Infection with *Chlamydia trachomatis* Alters the Tyrosine Phosphorylation and/or Localization of Several Host Cell Proteins Including Cortactin

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Infection of epithelial cells by two biovars of *Chlamydia trachomatis* results in the tyrosine phosphorylation of several host proteins. The most prominent change in host protein tyrosine phosphorylation involves a complex of proteins with molecular masses of 75 to 85 kDa (pp75/85) and 100 kDa (pp100). The *C. trachomatis*-induced tyrosine phosphorylation of pp75/85 and pp100 is observed in several cell lines, including epithelial cells, fibroblasts, and macrophages. Subcellular fractionation and detergent solubility properties of pp75/85 are consistent with its association with the cytoskeleton. Phosphoamino acid analysis demonstrates that the pp75/85 complex is phosphorylated on both tyrosine and serine residues. Immunofluorescence studies of chlamydia-infected cells by using fluorescein isothiocyanate-phalloidin and antibodies to phosphotyrosine and cortactin demonstrate that tyrosine-phosphorylated proteins, as well as cortactin, are localized to the chlamydial vacuole and that this process is facilitated by actin.

Chlamydia trachomatis, a gram-negative bacterium and an obligate intracellular parasite, is a major human pathogen worldwide. It is the leading cause of sexually transmitted disease in the Western world and the main cause of noncongenital blindness in developing nations (26). During its life cycle, *C. trachomatis* alternates between two distinct morphological forms: the replicative, intracellular reticulate body (RB) and the infectious but metabolically inactive elementary body (EB) (21, 33). EBs bind to an unknown receptor on the host cell, which, in the case of lymphogranuloma venereum (LGV) biovars, potentially involves an interaction with a bacterially derived heparan sulfate-like glycosaminoglycan (GAG) present on the chlamydial surface (36). Others have presented data that suggest that the chlamydial major outer membrane protein may function as the ligand (30). After binding, the bacteria are internalized, enveloped within membrane-bound compartments, and transported to a perinuclear location (15, 18). The *C. trachomatis* compartments fuse with each other to form one or a few large vacuoles. The mature bacterial vacuole appears to have little or no interaction with the endocytic pathway but receives ceramide in the form of sphingolipids from the *trans*-Golgi network (15, 16, 18, 27). Within the vacuole, EBs differentiate into RBs in 6 to 8 h; the RBs subsequently undergo binary fission to yield approximately 100 progeny. Between 18 and 72 h postinfection (hpi), the RBs redifferentiate into EBs and are released into the extracellular space to start a new round of infection.

The ability of chlamydiae to induce their uptake by epithelial cells and to remain sequestered in a compartment separate from lysosomes is integral to their successful replication and survival in the hostile intracellular environment of the host. By analogy to other pathogens that invade epithelial cells, including enteropathogenic *Escherichia coli* (25), *Salmonella* spp.

(23), *Shigella flexneri* (9), and *Yersinia* spp. (reviewed in reference 4), this process may involve interactions with host signal transduction pathways and is likely to start when the microorganism binds to its host cell receptor.

To dissect the interaction of chlamydiae with its host cell signal transduction pathways, we have investigated the changes in host protein tyrosine phosphorylation that occur upon infection with the LGV L2 serovar and the mouse pneumonitis (MoPn) biovar of *C. trachomatis*. While this work was in progress, Birkelund and coworkers demonstrated that upon infection of HeLa cells with the L2 serovar of *C. trachomatis*, the tyrosine phosphorylation of at least three host proteins was increased (3). We significantly extend these results.

MATERIALS AND METHODS

Cell culture. The human epithelial HeLa cell line (ATCC CCL2; American Type Culture Collection, Rockville, Md.) was grown in Dulbecco's modified essential medium H16 (DME-H16 medium) supplemented with 5% (vol/vol) fetal calf serum (FCS) (DME-5). The mouse fibroblast L929 cell line (ATCC CCL1) was grown in RPMI 1640 medium containing 25 mM HEPES and 10% (vol/vol) FCS. The murine macrophage cell lines RAW 264.7 (ATCC TIB 71) and J774A.1 (ATCC TIB 67) were cultured in DME-H21 medium supplemented with 5% (vol/vol) calf serum and 5% (vol/vol) FCS. All cell lines were grown at 37°C in 5% CO₂. Medium was obtained from the Cell Culture Facility, University of California, San Francisco. FCS was obtained from Gibco (Bethesda, Md.).

Growth and purification of chlamydiae. The MoPn biovar of *C. trachomatis* was routinely grown in HeLa cells for 48 h, after which it was purified as described previously (10). The LGV L2 serovar was obtained from Richard S. Stephens (University of California, Berkeley) and propagated in L929 cells. In some experiments, the MoPn biovar was purified by centrifugation on a discontinuous Renografin (Squibb Diagnostics, BMS Pharmaceutical Group, Irvine, Calif.) gradient (17). Infections were carried out at a multiplicity of infection (MOI) of 20 to 100 inclusion-forming units. Despite the high-MOI inocula used, no immediate cytotoxicity (prior to 18 h) was observed.

Chlamydial infection and protein extraction. Subconfluent monolayers of HeLa, L929, RAW, or J774 cells (2×10^5 cells) in 100-mm-diameter plates were infected for 1 h at 37°C with a purified preparation of MoPn or LGV in sucrose-phosphate-glutamine buffer (10) diluted in DME-5. The inoculum was removed and replaced by fresh medium, and the infection was allowed to proceed for different periods of time. For mock infections, the cells were incubated with a preparation of uninfected cells purified by the protocol used for chlamydiae. After the medium was removed, the cells were washed with and scraped into cold

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phosphate-buffered saline (PBS). The cells were then spun down in a microcentrifuge at $8,000 \times g$ and lysed in 200 μ l of Triton X-100 lysis buffer (20 mM Tris-HCl [pH 8.0], 137 mM NaCl, 1% [wt/vol] Triton X-100, 10% glycerol, 1 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM aprotinin) for 15 min at 4°C . The Triton X-100-soluble fraction of the lysate was separated from the insoluble fraction by centrifugation at $18,000 \times g$ for 5 min. The pellet was extracted in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 0.5% deoxycholate, 1 mM Na_3VO_4 , 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin). The soluble and insoluble fractions were mixed with $2\times$ and $5\times$ Laemmli buffer (19), respectively, to a $1\times$ final concentration and boiled prior to electrophoresis on SDS-polyacrylamide gels (SDS-PAGE) (22). Lysates from mock-infected cells were prepared identically. When used, cycloheximide, chloramphenicol, and rifampin were added at 100 $\mu\text{g}/\text{ml}$ each for 1 h prior to infection and were present throughout the infection. These concentrations of inhibitors have been previously demonstrated to inhibit eukaryotic protein or *C. trachomatis* protein or RNA synthesis (11).

For subcellular fractionation, L929 cells were infected with LGV or mock infected for 1 h at 37°C . The cells were washed three times with cold PBS and sonicated (Branson Sonifier 450; setting 7) for 10 1-s intervals in PBS containing 1 mM Na_3VO_4 , 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM aprotinin. The lysate was centrifuged at $2,500 \times g$, $10,000 \times g$, and $100,000 \times g$ for 15 min at 4°C . Pellets were extracted in Triton X-100 lysis buffer as described above.

Cytoskeletal extracts were prepared from monolayers of HeLa cells grown in 10-cm-diameter plates. Following infection with the MoPn biovar of *C. trachomatis* for 1 h at 37°C , the inoculum was removed, and the infected cells were washed with cold PBS. Cytoskeletal extracts were prepared as previously described (1). Equivalent amounts of all fractions were separated by gel electrophoresis, electroblotted to Immobilon (Amersham, Arlington, Ill.), and probed with 4G10 as described below. Successful fractionation was confirmed by Western blot analyses of the same samples with an antibody against human tubulin (a kind gift from Marc Kirschner, Harvard University, Cambridge, Mass.) used at a $1:1,000$ dilution in Tris-buffered saline-0.1% Tween 20 (TBST).

Western immunoblotting and immunoprecipitation. Western blot analyses were carried out according to the manufacturer's specifications, using a secondary antibody conjugated to alkaline phosphatase (Promega, Madison, Wis.) or to horseradish peroxidase (ECL kit; Amersham). The mouse monoclonal antiphosphotyrosine antibody 4G10 (Upstate Biotechnology Incorporated, Lake Placid, N.Y.) was used at a dilution of $1:1,000$ in TBST. The antipaxillin antibody (a kind gift of Christopher Turner, State University of New York Health Science Center, Syracuse) was used at a dilution of $1:10$, and the mouse monoclonal anticortactin antibody 4F11 (a kind gift of J. Thomas Parsons, University of Virginia Health Sciences Center, Charlottesville) was used at a dilution of $1:1,000$. Antibody against ezrin (a kind gift of Anthony Bretscher, Cornell University, Ithaca, N.Y.) was used at a dilution of $1:2,000$. Antibody against the 85-kDa subunit of phosphatidylinositol 3-kinase (a kind gift of Lewis Williams, Chiron Corporation, San Francisco, Calif.) was used at a dilution of $1:3$.

For immunoprecipitation experiments, 2×10^6 HeLa or RAW cells were infected with chlamydiae for 4 h at 37°C . The infected cells were lysed in 500 μ l of RIPA buffer for 15 min at 4°C . The lysates were boiled for 5 min and centrifuged at $18,000 \times g$ for 5 min. Supernatant fractions were incubated with protein A-Sepharose coupled to antiphosphotyrosine antibody 4G10 overnight at 4°C and washed with RIPA buffer; bound proteins were eluted by boiling in an equivalent volume of $2.5\times$ Laemmli buffer. Following gel electrophoresis, proteins were transferred to a nitrocellulose membrane and Western blot analyses were carried out as described above.

Immunofluorescence. HeLa or L929 cell monolayers were grown on 12-mm-diameter coverslips and infected with MoPn or L2. When indicated, cytochalasin D (1 $\mu\text{g}/\text{ml}$ in dimethyl sulfoxide; Sigma, St. Louis, Mo.) was added to the cells for 1 h prior to infection and was present throughout the infection. An equivalent amount of dimethyl sulfoxide was added to untreated control monolayers. The monolayers were fixed and stained by the pH-shift method (2), with minor modifications (32). Tyrosine-phosphorylated proteins were detected by using the mouse monoclonal antibody 4G10 at a $1:1,000$ dilution as the primary antibody. Cortactin was visualized by using the mouse monoclonal antibody 4F11 at a $1:500$ dilution. Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (Zymed, South San Francisco, Calif.) diluted $1:200$ was used as the secondary antibody. Vacuoles were visualized by staining with an antibody to the major outer membrane protein of *C. trachomatis* (L2-45 or MO-336; a kind gift of Harlan Caldwell, Rocky Mountain Laboratories, Hamilton, Mont.) that was directly conjugated to Texas red as instructed by the manufacturer (Molecular Bioprobes, Eugene, Oreg.). For the 4G10-4F11 double-immunofluorescence studies, antibody 4F11 was directly conjugated to Texas red. The monolayers were fixed and stained with antibody 4G10 as described above. They were then washed six times with PBS-fish skin gelatin-saponin and incubated with Texas red-conjugated antibody 4F11 for 1 h at 37°C . The monolayers were washed twice with PBS-fish skin gelatin-saponin, once with PBS, once with 0.1% Tween in PBS, and once with PBS, postfixed, and mounted as before. To visualize actin, FITC-conjugated phalloidin (Sigma) was used at 200 ng/ml.

All samples were examined by conventional and confocal fluorescence microscopy. For confocal microscopy, the samples were analyzed by using a krypton-

FIG. 1. Chlamydial infection of HeLa cells induces the tyrosine phosphorylation of five proteins. Lane 1, mock-infected HeLa cells; lane 2, HeLa cells infected with the MoPn biovar for 2 h; lane 3, HeLa cells exposed to heparin (1 mg/ml) for 1 h prior to and during a 2-h infection with the MoPn biovar. Lysates were subjected to SDS-PAGE (7% gel) and immunoblotted with the antiphosphotyrosine antibody 4G10. Tyrosine-phosphorylated proteins induced by the chlamydial infection are indicated by arrows on the left.

argon laser coupled with a Bio-Rad MRC600 confocal head, attached to an Optiphot II Nikon microscope with a Pan Apo $60\times$ 1.4-numerical-aperture objective lens. The samples were scanned simultaneously for FITC and Texas red emission, using the K1 and K2 filter blocks. Collection parameters were as follows: zoom of 1.0 0.5 s/scan, four frames/image, Kalman filter motor step size = 1 μm , and diaphragm set at $1/3$ open. The data were analyzed by using Cosmos software, and regions of colocalization were identified by using the merge side function. The images were converted to tagged-information-file format, and the contrast levels and colors of the images were adjusted with the Photoshop program (Adobe Co., Mountain View, Calif.) on a PowerPC 7100/80 (Apple Computer Inc., Cupertino, Calif.). The color images were imported into Pagemaker (Aldus Corporation, Seattle, Wash.) and printed.

Biotinylation of cell surface proteins. L929 cells were washed three times in cold PBS (pH 8.0) and then incubated in 0.5 mg of sulfo-succinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate (NHS-SS-biotin; Pierce, Rockford, Ill.) per ml for 30 min at room temperature. The cells were washed again, infected with LGV or mock infected for 1 h, and then lysed in Triton X-100 lysis buffer. Lysates (200 μg) were absorbed with streptavidin-agarose beads (50 μl) on a rotating wheel for 2 h at 4°C . The beads were washed three times in Triton X-100 lysis buffer and boiled in Laemmli buffer. Aliquots of total lysate, streptavidin-bound proteins, and unbound proteins were separated by SDS-PAGE. Western blots were probed with 4G10 and transferrin receptor antibody ($1:10,000$) (35) as a positive control.

In vivo labeling of pp75/85 and phosphoamino acid analysis. Subconfluent monolayers of HeLa cells (6×10^6) were labeled with 3 mCi of [^{32}P]orthophosphate (Amersham) for 3 h at 37°C in 1 ml of phosphate-free, serum-free DME-H16 medium supplemented with 2.5% bovine serum albumin and then infected with MoPn for 1 h at 37°C . The infected cells were washed three times with cold PBS and lysed in RIPA buffer as described above. Immunoprecipitation with antiphosphotyrosine antibody 4G10 was carried out as described above except that the lysates were incubated with the 4G10-coupled protein A-Sepharose beads for 3 h at 4°C . Following SDS-PAGE, the proteins were transferred to an Immobilon membrane and exposed to a Kodak Xomat X-ray film. The band of interest was cut out, and Cerenkov counts were determined. The Immobilon band was then washed with distilled H_2O and subjected to acid hydrolysis by boiling in HCl (Pierce) for 90 min. The acid hydrolysate was pelleted, washed with distilled H_2O , and dried. This was repeated four times. The pellet was then resuspended in buffer pH 1.9 (7.5% glacial acetic acid, 2.5% formic acid) to a final concentration of 1,000 cpm/ μl . An aliquot (1 μl) was spotted on a thin-layer chromatography plate and electrophoresed in buffer pH 1.9 at 1,500 V for 20 min followed by electrophoresis in the second dimension in buffer pH 3.5 (5% glacial acetic acid, 0.5% pyrimidine) at 1,300 V for 18 min. The thin-layer chromatography plate was dried and exposed to a Kodak Xomat X-ray film (7).

RESULTS

Induction of host protein tyrosine phosphorylation by infection with *C. trachomatis* occurs in several different cell types and with two different biovars. To study whether infection of epithelial cells by *C. trachomatis* would affect tyrosine phosphorylation of host proteins, lysates from both mock-infected and MoPn-infected HeLa cells were immunoblotted with the antiphosphotyrosine antibody 4G10 (Fig. 1). Increased tyrosine phosphorylation of at least five groups of proteins can be discerned (apparent molecular masses of 200 kDa [pp200], 160

FIG. 2. Tyrosine phosphorylation of pp75/85 occurs upon infection with two different biovars of *C. trachomatis*. Lane 1, uninfected HeLa cells; lane 2, HeLa cells infected with the L2 serovar of LGV for 2 h; lane 3, HeLa cells exposed to heparin (1 mg/ml) 1 h prior to and during a 2-h infection with L2; lane 4, HeLa cells exposed to chondroitin sulfate (1 mg/ml) 1 h prior to and during a 2-h infection with L2; lane 5, HeLa cells infected with the MoPn biovar of *C. trachomatis* for 2 h; lane 6, HeLa cells exposed to heparin (1 mg/ml) 1 h prior to and during a 2-h infection with MoPn; lane 7, HeLa cells exposed to chondroitin sulfate (1 mg/ml) 1 h prior to and during a 2-h infection with MoPn. Lysates were immunoprecipitated with 4G10, subjected to SDS-PAGE (7% gel), and immunoblotted with 4G10. Note that the two pp100 phosphoproteins seen upon infection with HeLa cells by L2 and MoPn comigrate, whereas the pp75/85 complex observed upon infection of HeLa cells by L2 migrates slightly faster than the pp75/85 complex observed upon infection of HeLa cells by MoPn. In this experiment, tyrosine-phosphorylated proteins of approximately 160 kDa are visible in the LGV-infected cells. As noted in Fig. 1 and in the text, the appearance of this protein was variable. The lower-molecular-weight bands present in all lanes represent immunoglobulin.

kDa [pp160], 140 kDa [pp140], 100 kDa [pp100], and 75 to 85 kDa [pp75/85]); however, in most experiments, increased phosphorylation of only the most prominent species, pp75/85 and pp100, can be routinely observed.

The increased tyrosine phosphorylation of all of these phosphoproteins is blocked if the cells are exposed to heparin (1 mg/ml) for 1 h prior to and during the addition of MoPn (Fig. 1, lane 3; Fig. 2, lane 6), a treatment that prevents attachment of MoPn to the host cell (12a). It is not inhibited by another highly negatively charged GAG, chondroitin sulfate (Fig. 2, lanes 4 and 7). While this GAG has a lower sulfation density than heparin, the results suggest that inhibition by heparin is not merely charge related. These data also demonstrate that free heparin is not sufficient to induce host protein tyrosine phosphorylation.

Birkelund and coworkers reported that infection of HeLa cells by L2 resulted in the increased tyrosine phosphorylation of three host proteins, a complex of proteins of 64, 66, and 68 kDa, a 100-kDa protein, and a 140-kDa protein (3). Figure 2 directly compares the induction of host tyrosine phosphorylation of pp75/85 and pp100 of HeLa cells infected with two different biovars of *C. trachomatis*, MoPn and L2. Infection with either biovar resulted in increased tyrosine phosphorylation of pp75/85 and pp100, although the MoPn-induced pp75/85 complex exhibited a slightly slower mobility. Based on these data, we believe that pp75/85 corresponds to the 64/66/68-kDa complex observed by Birkelund and coworkers. The finding that two different biovars of *C. trachomatis* which differ in their biological properties induce similar changes in host protein tyrosine phosphorylation upon entry suggests that these events likely play an important role in infection by *C. trachomatis*.

The patterns of host protein tyrosine phosphorylation of different host cells were compared. Increased phosphorylation of pp75/85 and pp100 was observed upon infection of L929 cells (Fig. 3), RAW cells, and J774.1 cells (data not shown). Thus, this phosphorylation is not cell type specific.

Phosphorylation of pp75/85 and pp100 occurs rapidly, is stable, and does not require de novo host or bacterial protein synthesis. The increased tyrosine phosphorylation of pp75/85 and pp100 occurs quickly upon addition of MoPn and LGV to

HeLa cells; the changes in tyrosine phosphorylation could be detected as early as 10 min postinfection and were detectable as late as 18 hpi, at which time the cells started to lyse (data not shown). The prolonged nature of the increased phosphorylation is not dependent on multiple rounds of chlamydial infection; it was observed even when subsequent rounds of infection were prevented by adding heparin (data not shown). Figure 3 demonstrates that the phosphorylation of pp75/85 and pp100 was not inhibited by the presence of chloramphenicol, rifampin, or cycloheximide, indicating that neither bacterial nor host de novo protein synthesis is required. Similar results were observed when these inhibitors were present for longer periods of time (up to 22 h [data not shown]). The persistence of the tyrosine-phosphorylated proteins in the absence of de novo host protein synthesis following a single round of infection indicates that the tyrosine-phosphorylated forms of pp75/85 and pp100 are quite stable.

The phosphorylated forms of pp75/85 and pp100 are found in both soluble and insoluble fractions in the host cell but are not membrane associated. The detergent solubility of pp75/85 and pp100 was assayed. Figure 4A illustrates that in LGV-infected L929 cells, pp75/85 and pp100 are found in both the Triton X-100-soluble and Triton X-100-insoluble fractions. Similar results were observed with MoPn-infected HeLa cells (data not shown). Furthermore, the relative amount of tyrosine-phosphorylated pp75/85 in either fraction does not change over the course of the infection (up to 18 hpi). The detergent-insoluble fraction of pp75/85 was resistant to solubilization by 5% Triton X-100 and 2 M KCl. This finding suggests that the insoluble portion of pp75/85 is not membrane bound but may be associated with the cytoskeleton. A fractionation protocol that enriches for intermediate filaments showed that pp75/85, although present in the intermediate filament preparation, is not enriched in that fraction (data not shown).

The localization of pp75/85 and pp100 was further investigated by subcellular fractionation and extraction of cellular lysates with detergent. Fractionation of LGV-infected L929 cells by differential centrifugation shows that pp75/85 and pp100 are enriched in the low- and intermediate-speed pellets and relatively diminished in the high-speed pellet and supernatant (Fig. 4B). Cell surface biotinylation of pp75/85 or pp100 was not detected in experiments where biotinylation of a

FIG. 3. Phosphorylation of pp75/85 and pp100 does not require de novo bacterial protein and RNA synthesis or host protein synthesis. Lane 1, mock-infected L929 cells; lane 2, L929 cells infected with LGV for 1 h in the presence of chloramphenicol (100 μ g/ml); lane 3, L929 cells infected with LGV for 1 h in the presence of cycloheximide (100 μ g/ml); lane 4, L929 cells infected with LGV for 1 h in the presence of rifampin (100 μ g/ml); lane 5, L929 cells infected with LGV for 1 h. Lysates (20 μ g) were subjected to SDS-PAGE (7.5% gel) and immunoblotted with 4G10. Positions of molecular weight markers (in kilodaltons) are indicated by arrows.

FIG. 4. The phosphorylated forms of pp75/85 and pp100 are found in both soluble and insoluble fractions in the host cell but are not membrane associated. L929 cells were infected with LGV (+) or mock infected (–) for 1 h; lysates (20 μ g) were subjected to SDS-PAGE (7.5% gel) and immunoblotted with 4G10. (A) Detergent extraction. Lane 1, mock-infected L929 cells, Triton X-100 (TX-100)-soluble fraction; lane 2, LGV-infected L929 cells, Triton X-100-soluble fraction; lane 3, mock-infected L929 cells, Triton X-100-insoluble fraction; lane 4, LGV-infected L929 cells, Triton X-100-insoluble fraction. (B) Subcellular fractionation. Lane 1, mock-infected L929 cells, low-speed pellet (LP); lane 2, LGV-infected L929 cells, low-speed pellet; lane 3, mock-infected L929 cells, medium-speed pellet (IP); lane 4, LGV-infected L929 cells, medium-speed pellet; lane 5, mock-infected L929 cells, high-speed pellet (HP); lane 6, LGV-infected L929 cells, high-speed pellet; lane 7, mock-infected L929 cells, high-speed supernatant (HS); lane 8, LGV-infected L929 cells, high-speed supernatant. Positions of molecular weight markers (in kilodaltons) are indicated by arrows.

known membrane-exposed protein, the transferrin receptor, was observed (data not shown). These results are consistent with the results of the detergent solubility experiments and indicate that pp75/85 and pp100 are not associated with the plasma membrane or microsomes.

pp75/85 is phosphorylated on serine and tyrosine residues upon infection with MoPn. Molecules involved in eukaryotic signal transduction are often phosphorylated on both tyrosine and serine or threonine residues. MoPn-infected HeLa cells were labeled in vivo with [32 P]orthophosphate, and the tyrosine-phosphorylated proteins were immunoprecipitated with 4G10. Following SDS-PAGE, the 75/85-kDa band was excised and subjected to phosphoamino acid analysis. In mock-infected cells treated similarly, no species corresponding to pp75/85 could be detected by autoradiography of 4G10 immunoprecipitates (Fig. 5A). Figure 5B demonstrates that pp75/85 is phosphorylated on serine residues in addition to tyrosine residues.

Cortactin and other eukaryotic tyrosine-phosphorylated proteins are associated with the chlamydial vacuole. To investigate whether infection of HeLa or L929 cells with either MoPn or L2 altered the distribution of tyrosine-phosphorylated proteins, we performed conventional and confocal epifluorescence microscopy. Figure 6A shows single-plane 1- μ m-thick confocal micrographs of vacuoles in HeLa cells infected with L2 for 4, 12, and 20 h followed by staining with 4G10. At 4 hpi, the vacuole stained intensely with 4G10. Individual chlamydia could also be observed to occasionally colocalize with the antiphosphotyrosine antibody. Similar results were observed as early as 1 hpi (data not shown). At 12 hpi, the entire vacuole stained, although less intensely. Two alternate patterns were seen at 20 hpi: either the vacuole stained diffusely, similar to the appearance at 12 hpi, or intense staining was observed between the vacuole and the nucleus (data not shown). The latter pattern was not observed by Birkelund and coworkers (3). No changes were discernible in the pattern of focal adhe-

sion staining at either time point (data not shown). Similar results were observed in HeLa cells infected with MoPn and in L929 cells infected with the L2 biovar (data not shown).

Cortactin is associated with the chlamydial vacuole. It has been previously suggested that actin participates in and colocalizes with the perinuclear aggregation of EBs that occurs early after infection (20). Cortactin, a 75- to 85-kDa tyrosine-phosphorylated actin binding protein, was found associated with the chlamydial vacuole at 4, 12, and 20 hpi in HeLa cells infected with LGV in a pattern similar to that observed with the antiphosphotyrosine antibody (Fig. 6B). Specifically, at 4 and 12 hpi, cortactin colocalized with the vacuole. Although cortactin staining could be detected throughout the cell at this plane, its colocalization with the vacuole is significant in this 1- μ m-thick cross-sectional image. In contrast, other cytoplasmic markers, such as FITC-conjugated dextran, transferrin, and transferrin receptor, are specifically excluded from the interior of the vacuole (32). However, at 20 hpi, intense staining of cortactin was primarily observed in the region between the vacuole and the nucleus of L2-infected HeLa cells, although some residual staining within the vacuole could be seen. Similar results were observed with MoPn (data not shown). The localization of both tyrosine-phosphorylated proteins and cortactin with the *C. trachomatis* vacuole suggested that this pool of cortactin was tyrosine phosphorylated or was closely associated with one or more tyrosine-phosphorylated proteins.

To further investigate this observation, double-staining experiments using 4G10 (visualized by incubation with goat anti-mouse immunoglobulin G conjugated to FITC) and the cortactin antibody 4F11 (directly conjugated to Texas red) were performed on L2-infected L929 cells. In Fig. 7A, it can be seen that the cortactin that colocalizes to the vacuole colocalizes with tyrosine-phosphorylated proteins. As expected, in other regions of the cell, cortactin and tyrosine-phosphorylated proteins did not always colocalize. Notably, there appears to be more staining of the vacuole by the antiphosphotyrosine antibody than the cortactin antibody, suggesting that other tyrosine-phosphorylated proteins associate with the vacuole, although differences in the affinity of these two antibodies could also explain this observation.

The apparent colocalization of tyrosine-phosphorylated pro-

FIG. 5. pp75/85 is phosphorylated on both serine and tyrosine residues in HeLa cells infected with MoPn. (A) HeLa cells were labeled in vivo with [32 P]orthophosphate for 3 h, after which they were infected for 1 h with MoPn. Immunoprecipitation with 4G10 was carried out on both mock-infected (–) and infected (+) HeLa cell lysates followed by separation on an SDS–7% polyacrylamide gel. Immunoprecipitated labeled proteins were transferred to an Immobilon membrane followed by exposure to Kodak XAR film. Molecular is indicated in kilodaltons. (B) Phosphoamino acid analysis of 32 P-labeled pp75/85 cut out from the Immobilon membrane (from lane + in panel A). (C) Migration of the amino acid standards serine (Ser), threonine (Thr), and tyrosine (Tyr) in this two-dimensional thin-layer chromatography separation.

FIG. 6. Time course of the association of tyrosine-phosphorylated proteins and cortactin with the chlamydial vacuole. HeLa cells were infected with LGV for 4, 12, or 20 h and incubated with antibody 4G10 (A) or the cortactin antibody (B) followed by FITC-conjugated goat anti-mouse secondary antibody. The *C. trachomatis* vacuole is visualized by staining with an antibody against the major outer membrane protein that had been directly conjugated to Texas red. Each panel is a representative 1- μ m-thick cross section visualized by confocal microscopy. The merge column displays the results of merging of the green and red channels to demonstrate the extent to which the antibody staining overlapped. +CD, cells preincubated with cytochalasin D for 1 h prior to and throughout the infection.

FIG. 7. Colocalization of cortactin and tyrosine-phosphorylated proteins, cortactin and actin, and chlamydiae and actin. (A) HeLa cells were infected with LGV for 12 h and incubated with antibody 4G10 followed by FITC-conjugated goat anti-mouse secondary antibody (green) and with cortactin antibody directly conjugated to Texas red (red). (B) HeLa cells were infected with LGV for 4 h and incubated with FITC-conjugated phalloidin to stain actin filaments (green) and with cortactin antibody directly conjugated to Texas red (red). (C) HeLa cells were infected with LGV for 4 h. The actin visualized by staining with FITC-phalloidin (green), and the vacuoles were visualized by staining with an antibody to the major outer membrane protein followed by incubation with rhodamine-conjugated goat anti-mouse secondary antibody (red). Each panel is a representative 1-μm-thick cross section visualized by confocal microscopy. The right-hand column displays the results of merging of the red and green channels. C, chlamydial vacuole; N, nucleus.

teins and cortactin combined with the finding that cortactin comigrates on Western blots with the pp85 component of pp75/85 (data not shown) suggested that cortactin was indeed tyrosine phosphorylated. To rigorously test this, lysates from *C. trachomatis*-infected HeLa cells were either immunoprecipitated with 4G10 followed by immunoblotting with the cortactin antibody or immunoprecipitated with the cortactin antibody followed by immunoblotting with 4G10. In neither case were we able to demonstrate increased tyrosine phosphorylation of cortactin compared to uninfected cells (data not shown). From this, we conclude that upon infection of epithelial cells with *C. trachomatis*, a portion of the cellular pool of cortactin is re-localized to the chlamydial vacuole and this pool of cortactin may be associated with other tyrosine-phosphorylated proteins.

Cortactin can be found associated with actin early in infection but not late in infection. To further investigate the relationship between cortactin, actin, and vacuoles, HeLa cells were infected with LGV and fixed after 4, 12, or 20 hpi, and double-label immunofluorescence for cortactin and actin or chlamydiae and actin was carried out. The vacuole was located

by phase microscopy. At 4 hpi, actin colocalized with vacuoles approximately 50% of the time (compare Fig. 7B and C). In contrast, cortactin was always observed to localize with the vacuole at 4 hpi. At 20 hpi, actin was never observed to localize with the vacuole (data not shown), despite the fact that cortactin was still associated with the chlamydial vacuole. The association of cortactin with actin at the periphery of the cell and in conjunction with focal adhesions did not appreciably change in chlamydia-infected HeLa cells compared to uninfected HeLa cells (data not shown). No change in the amount of stress fibers observed in infected cells compared to uninfected cells was evident (data not shown).

Cytochalasin D alters the association between actin, cortactin, and chlamydiae. HeLa cells were pretreated with cytochalasin D for 1 h and then infected with LGV for 4, 12, or 20 h, and the distribution of tyrosine-phosphorylated proteins, cortactin, and chlamydiae was observed by double-label immunofluorescence. Large aggregates of actin (data not shown), tyrosine-phosphorylated proteins (Fig. 6A), and cortactin (Fig. 6B) were present at the periphery of the cell. Especially at the

earlier times postinfection, the chlamydiae were located directly adjacent to these aggregates of actin, cortactin, and tyrosine-phosphorylated proteins and appeared to be extracellular. At later times postinfection, aberrant-appearing vacuoles that were associated with tyrosine-phosphorylated proteins and cortactin were formed. Many more chlamydiae remained at the periphery of the cell at these later time points in cytochalasin D-treated cells than in the untreated controls. These findings are consistent with data from others demonstrating that cytochalasin D has some inhibitory effect on infection of epithelial cells by *C. trachomatis* (24, 34). Taken together, these data indicate that tyrosine-phosphorylated proteins, including cortactin, are intimately associated with the chlamydial vacuole and that actin facilitates this process.

DISCUSSION

Many pathogenic bacteria induce their uptake into non-phagocytic eukaryotic cells. Often, this process of cell invasion involves triggering host cell signal transduction pathways. In some cases, the end result of this process is the rearrangement of the cytoskeleton, which facilitates bacterial uptake, as with enteropathogenic *E. coli* (25) and *Salmonella typhimurium* (13, 23). There is also evidence that demonstrates that secretion of the *Yersinia* protein serine threonine kinase YpkA (14) and the tyrosine phosphatase YopH (5) into host cells is necessary for virulence in vivo.

In this report, we demonstrate that infection of epithelial cells with the MoPn biovar of *C. trachomatis* results in the de novo or increased tyrosine phosphorylation of two to five host proteins. At least two of these host proteins are tyrosine phosphorylated upon infection with a second biovar (L2) of *C. trachomatis* that differs significantly from MoPn in its natural host and its biology. Reproducible detection of these potential signaling events required the use of high-MOI inocula. However, at these high MOIs, immediate cytotoxicity was not observed, suggesting that the tyrosine phosphorylation events reflect events that may occur during physiologic infection.

While this work was in progress, Birkelund and colleagues reported that infection of epithelial cells by L2 stimulated the tyrosine phosphorylation of a similar spectrum of host proteins (3). There are several differences between their observations and ours. First, the most prominent species that they observed migrated at 64 to 68 kDa; we infer that this corresponds to the pp75/85 species that we observed. The discrepancy in apparent molecular masses could be explained by a difference in electrophoretic or other experimental conditions. Second, we reproducibly observe that the pp75/85 and pp100 species are found in both detergent-soluble and detergent-insoluble fractions of the cell. In contrast, Birkelund et al. could detect it only in the detergent-insoluble fraction. One possible explanation is that if proteolysis of pp75/85 occurred under their extraction conditions, the solubility of the cleaved species may differ from that of the full-length protein.

In other work, we have shown that almost identical host protein phosphorylation events are observed when heparin-coated beads are endocytosed by epithelial cells (29). These results demonstrate unequivocally that the tyrosine-phosphorylated proteins are eukaryotic in origin. The observation that entry of *C. trachomatis* and heparin-coated beads result in the increased tyrosine phosphorylation of similar sets of proteins lends support for the hypothesis that at least the LGV biovars of *C. trachomatis* enter cells through a eukaryotic pathway involving the uptake of heparin-containing GAGs and/or proteoglycans. It is interesting to note the recent report that attachment of serovar E, an oculogenital strain, is not inhibited by heparin (8).

How these tyrosine-phosphorylated proteins are transported across the vacuolar membrane and what function they serve remain to be elucidated. Experiments with inhibitors of tyrosine kinases have been unrevealing. Like Birkelund and coworkers, we were unable to inhibit the tyrosine phosphorylation events with genistein, tyrphostin, lavendustin A, herbimycin, or staurosporine (12a). While orthovanadate, an inhibitor of tyrosine phosphatases, increased the amount of pp75/85 and pp100 tyrosine phosphorylation, it had an inhibitory effect on infection of cells by *C. trachomatis* (18a).

Several pieces of data suggest that these changes in tyrosine phosphorylation reflect early binding or entry events rather than inhibition of phagolysosomal fusion. First, the changes in tyrosine phosphorylation occur rapidly, being detectable as early as 10 min postinfection. At this time, the chlamydiae have not relocated to the peri-Golgi region. Second, the tyrosine phosphorylation changes are not dependent on bacterial protein synthesis, whereas the inhibition of phagolysosomal fusion is (28). Finally, similar events are observed upon internalization of heparin-coated beads, yet the beads are trafficked to lysosomes (29).

We provide evidence that these phosphorylation events coincide with the relocation of actin, cortactin, and other tyrosine-phosphorylated proteins to the vacuole. Multiple lines of evidence are consistent with the notion that actin facilitates this process, though it may not be absolutely required. First, approximately half of the time, actin is found associated with the vacuole in a manner similar to cortactin at 4 hpi, but at later times, it does not localize with the vacuole. Second, cytochalasin D inhibits the formation of vacuoles and the association of cortactin and other tyrosine-phosphorylated proteins with the vacuole in a similar manner. We speculate that the actin cytoskeleton provides the scaffolding for the association of these tyrosine-phosphorylated proteins, including cortactin, with the incoming chlamydiae.

The redistribution and tyrosine phosphorylation of cortactin that occurs upon infection of cells by *C. trachomatis* may be related to the intimate association of cortactin with focal adhesions. Focal adhesions are thought to function as signal transduction centers that permit communication between the extracellular matrix and the intracellular cytoskeleton. It is possible that since heparan sulfate-like GAGs are components of the extracellular matrix, and focal adhesions are formed where the cell contacts the extracellular matrix, then attachment of chlamydiae or heparin-coated beads mimics binding of the extracellular matrix to the cell surface. This may result in changes in the tyrosine phosphorylation of components of focal adhesions, such as cortactin, and their distribution to the chlamydial vacuole. Notably, Dehio and coworkers have demonstrated that entry of *S. flexneri* into epithelial cells stimulates the phosphorylation of cortactin through a pp60^{c-Src}-mediated pathway and is intimately involved in the recruitment of host cell actin to the invading bacteria (9). We have attempted to define a role for pp60^{c-Src} in *C. trachomatis* infection (32a). We have found that infection of NIH 3T3 cells that overexpress chicken pp60^{c-Src} is enhanced; however, pp60^{c-Src}-deficient cell lines are not defective in infection. We have not been able to detect a change in pp60^{c-Src} activity, nor have we been able to detect pp60^{c-Src} on the *C. trachomatis* vacuole by indirect immunofluorescence.

Identification of the tyrosine-phosphorylated proteins will yield further insights into their function. The phosphorylation of pp75/85 is unlikely to be the consequence of a chlamydia-induced degradation of a larger phosphoprotein, since we fail to detect the disappearance of a tyrosine-phosphorylated protein or ³²P-labeled protein upon chlamydial infection. We have tested by immunoprecipitation combined with immunoblotting and by fluorescence microscopy whether paxillin (31), ezrin (6), or the 85-

kDa subunit of phosphatidylinositol 3-kinase (12) is a component of pp75/85. These antibodies failed to recognize any component of pp75/85, nor were they found associated with the vacuole (12b). We have attempted to determine if differential glycosylation contributes to the multiple species. Treatment of pp75/85 immunoprecipitated from chlamydia-infected cells with endoglycosidase F failed to alter the mobility of the protein or reduce the number of bands visible by Western blot analysis with 4G10 (12a).

In summary, we have demonstrated that infection with *C. trachomatis* results in the increased tyrosine phosphorylation of several host proteins. This may reflect a novel signal transduction pathway that involves the recruitment of tyrosine-phosphorylated proteins, including cortactin, to the vacuole. Our data are consistent with a model wherein these signal transduction events reflect processes that occur after the binding of chlamydiae to a receptor but before their trafficking to their final intracellular destination. The study of these events during chlamydial infection may yield unique insights into host cell signal transduction. Moreover, the identification and function of these host phosphoproteins may allow novel therapeutic approaches to the treatment and prevention of chlamydial infections.

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