Chlamydia trachomatis Induces Remodeling of the Actin Cytoskeleton during Attachment and Entry into HeLa Cells

Reynaldo A. Carabeo,1 Scott S. Grieshaber,1 Elizabeth Fischer,2 and Ted Hackstadt1*

Host-Parasite Interactions Section, Laboratory of Intracellular Parasites,1 and Rocky Mountain Microscopy Branch,2 Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana 59840

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To elucidate the host cell machinery utilized by Chlamydia trachomatis to invade epithelial cells, we examined the role of the actin cytoskeleton in the internalization of chlamydial elementary bodies (EBs). Treatment of HeLa cells with cytochalasin D markedly inhibited the internalization of C. trachomatis serovar L2 and D EBs. Association of EBs with HeLa cells induced localized actin polymerization at the site of attachment, as visualized by either phalloidin staining of fixed cells or the active recruitment of GFP-actin in viable infected cells. The recruitment of actin to the specific site of attachment was accompanied by dramatic changes in the morphology of cell surface microvilli. Ultrastructural studies revealed a transient microvillar hypertrophy that was dependent upon C. trachomatis attachment, mediated by structural components on the EBs, and cytochalasin D sensitive. In addition, a mutant CHO cell line that does not support entry of C. trachomatis serovar L2 did not display such microvillar hypertrophy following exposure to L2 EBs, which is in contrast to infection with serovar D, to which it is susceptible. We propose that C. trachomatis entry is facilitated by an active actin remodeling process that is induced by the attachment of this pathogen, resulting in distinct microvillar reorganization throughout the cell surface and the formation of a pedestal-like structure at the immediate site of attachment and entry.

Chlamydia trachomatis is a gram-negative bacterium that absolutely requires an intracellular niche for its replication (42–44). Because of their obligate intracellular nature, chlamydiae have evolved very efficient means of entering host eukaryotic cells, a process which has been described as parasite-directed entry (11, 12). Chlamydiae have a biphasic developmental cycle consisting of infectious and replicative forms. Infection of eukaryotic host cells is initiated by elementary bodies (EBs). EBs can superficially be considered spore-like, in that they are metabolically inactive and relatively stable in the extracellular environment so as to promote their survival for sufficient time to encounter a susceptible host cell. Through largely unknown mechanisms, EBs attach to and induce their internalization by host cells. Once internalized, EBs transform into a larger and more pleomorphic form called the reticulate body (RB) within the first few hours postinfection. RBs are metabolically active, and they replicate; however, they are non-infectious. Eighteen hours following infection with C. trachomatis L2, increasing proportions of the dividing RBs revert to EBs until the cell lyses at 40 to 44 h postinfection. Non-lymphogranuloma venereum (LGV) strains (serovars A to K) typically have a somewhat longer developmental cycle.

The precise molecular mechanisms of chlamydial attachment and entry have not been defined. However, chlamydiae, like other pathogens such as Toxoplasma gondii (15) and varicella-zoster virus (62), attach to host cells via a relatively weak and reversible electrostatic interaction with heparan sulfate proteoglycans (53, 59) and a stronger, more specific binding to an as yet unknown secondary receptor (14). Once attached, a majority of the EBs are internalized.

Actin is a critical component of receptor-mediated endocytosis and phagocytosis in a variety of cell types (3, 31, 47), and a number of studies have demonstrated that the cytoskeleton can be manipulated by microbial pathogens to facilitate productive infection (6, 19, 20, 24). For example, enteropathogenic Escherichia coli has the ability to aggregate actin to form its pedestal structures, a trademark of attaching and effacing lesions (4, 26), and Salmonella induces membrane ruffles for internalization (24, 27, 30).

The role of actin-dependent mechanisms in chlamydial internalization has long been debated. Early studies using cytochalasin B as an inhibitor of microfilament function found no effect on chlamydial internalization (32, 51). Subsequent studies using the more efficient agent cytochalasin D have generally observed a significant reduction in internalization (8, 46, 48, 56), although the degree of inhibition was dependent upon C. trachomatis serovar (48) and means of inoculation (46). A consensus exists that chlamydiae likely have the ability to utilize multiple means of entry (43).

In this report, we confirm a requirement for actin during C. trachomatis serovar L2 and D entry. In addition, we demonstrate that the actin reorganization induced by EB attachment leads to the subsequent formation of pedestal-like structures and hypertrophic microvilli on the host cell surface. Inhibition of the formation of these cell surface projections by cytochalasin D correlated with a significant reduction of chlamydial internalization. Thus, we conclude that the sensitivity of C. trachomatis entry to cytochalasin D is manifested in the ability of this compound to inhibit actin polymerization at the site of infection.

* Corresponding author. Mailing address: Host-Parasite Interactions Section, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT 59840. Mailing address: Phone: (406) 363-9308. Fax: (406) 363-9253. E-mail: Ted_Hackstadt @NIH.gov.
attachment for the formation of phagocytosis-competent ped- estal and microvillus structures.

MATERIALS AND METHODS

Organisms and cells. Chlamydia trachomatis serovars L2 and D were grown in HeLa 229 cells as previously described (13). A protocol for the preparation of 5-(and-6)-(4-chloromethyl)benzoylamine)tetramethylrhodamine (CMTMR)-labeled C. psittaci was adapted to C. trachomatis L2 EBs (9). Briefly, HeLa cells were inoculated with serovar L2 EBs at a multiplicity of infection (MOI) of 1 and incubated for an additional 24 h with 1 µg of CMTMR cell tracker/ml added to the culture medium at 12 h postinfection. EBs were then harvested by Renografin density gradient centrifugation as previously described (13). The infectivity of CMTMR-treated EBs was evaluated by inclusion-forming assay (28) and was found to be unaffected by the labeling procedure (data not shown). CHO-K1 and GAG-deficient pgk-1/45 (21) were generously provided by J. Eso (University of California, San Diego). The D4.1-3 cell line was also previously described (14).

Internalization assay. Cells grown on 12-mm glass coverslips were washed three times with ice-cold Hanks’ balanced salt solution (HBSS) (Invitrogen, Frederick, Md.) prior to infection. C. trachomatis L2 EBs, suspended in cold HBSS at the desired MOI, were inoculated onto the cell monolayers and sub- sequently incubated at 4°C for 1 h to allow for chlamydial attachment. The inoculum was removed, and the cells were washed three times with cold HBSS to remove unbound EBs. The cultures were then overlaid with prewarmed medium and incubated for 30 min at 37°C. When required, cytochalasin D (Sigma, St. Louis, Mo.) (2.5 µg/ml) was present for 30 min prior to and during infection. The medium was removed, and the cells were washed three times with cold HBSS and once with cold phosphate-buffered saline (PBS) (150 mM NaCl, 50 mM NaPO4, pH 7.4) and fixed on ice with freshly prepared 4% formaldehyde for 30 min. This fixation protocol maintained the integrity of the plasma membrane, as demonstrated by the inaccessible internalization of EBs to exogenously added anti-L2 EB antibody. After fixation, the cells were incubated in 3% bovine serum albumin in PBS for 30 min at 4°C and washed at least five times with cold PBS. The mouse monoclonal antibody (Mab) L2 I-45 against L2 major outer membrane protein was added to the cells and allowed to incubate at room temperature (RT) for a maximum of 2 h. The antibody was removed, and the cells were rinsed thoroughly with PBS. Cells were then subjected to a second fixation with methanol (MeOH) for 15 min. This fixation permeabilized the cells and allowed detection of EBs that remained extracellular as well as of those that had been internalized. The MeOH-fixed cells were rinsed thoroughly with PBS, incubated with a rabbit polyclonal anti-C. trachomatis L2 EB antiserum at RT for 3 to 6 h, and washed thoroughly with PBS. Secondary antibodies, Cascade Blue (CB)-conjugated goat anti-mouse immunoglobulin G (IgG) (Molecular Probes, Eugene, Ore.) and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Zymed, South San Francisco, Calif.) in PBS, were allowed to bind overnight at RT. When required, human convalescent-phase serum was substituted for the L2 I-45 antibodies along with its appropriate secondary antibody. The cells were washed thoroughly and fixed for observation using a Nikon FXA photomicroscope with a Nikon 60X Planachromat objective. Images were recorded digitally using a Dage-MTI CCD 72 camera attached to a DSP2000 image processor (Dage-MTI, Inc., Michigan City, Ind.) and processed using Adobe Photoshop v. 6.0 software. For quantification of internalization, images obtained from the CB and TRITC channels were the total (internalized plus extracellular) organisms. EBs were enumerated, and efficiency of internalization was calculated using the formula (TRITC EBs – CB EBs)/TRITC EBs) × 100. A minimum of 65 cells each from three separate coverslips were analyzed, and the data are presented as means ± standard deviations (SD).

To characterize uptake of EBs by Fc-mediated endocytosis, C. trachomatis L2 EBs were opsonized with the MAb L2 I-45 against serovar L2 major outer membrane protein. EBs in HBSS were mixed with MAb L2 I-45 to a final antibody concentration of 150 µg/ml and incubated at RT for 1 h. Infection of HeLa cells on coverslips was performed at RT for 1 h in the presence of 100 µg of heparin (Amersham Pharmacia Biotech, Piscataway, N.J.)/ml to promote EB attachment to and internalization by FcγRII receptor only. After rinsing unbound serovar L2 EBs with cold HBSS plus 100 µg of heparin/ml, internalization of serovar L2 EBs was initiated by incubation at 37°C for 1 h in the presence of heparin (100 µg/ml). Efficiency of internalization was determined as described above. Inhibition of chlamydial entry by wortmannin (40 nM) involved pretreat- ment of HeLa cells for 1 h and the continuous presence of the drug during attachment at RT and entry at 37°C.

Confocal microscopy. HeLa cells grown on 12-mm glass coverslips were infected with 1000 L2 EBs (MOI = 50) for 3 h at 4°C and 30 min at 37°C to allow for attachment and internalization, respectively. Cells were fixed with freshly prepared 4% formaldehyde in PBS for 30 min at RT. The fixed cells were permeabilized with 1% Triton X-100 in PBS for 4 min at RT. The permeabilization buffer was removed, and the cells were washed three times with PBS. TRITC-conjugated phallolidin (Molecular Probes) was added to the permeabilized cells for 40 min at RT. Cells were then incubated with a 1:1,000 dilution of anti-L2 EB rabbit polyclonal antibody for 3 h. After thorough washing with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. All fluorescence images were obtained with a Zeiss Axiosvert Zoom LSM 510 confocal microscope using excitation wavelengths of 488 nm for green fluorescent protein (GFP) or fluorescein and 543 nm for CMTMR.

Fluorescence recovery after photobleaching (FRAP). HeLa cells grown on coverslips were transfected with 800 ng of GFP-actin expression vector (36) with the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, Calif.) in accordance with the manufacturer’s instructions. The transfected HeLa cells were inoculated with CMTMR-labeled L2 EBs (MOI = 50) for 1 h at 4°C. Unbound EBs were rinsed away with cold HBSS. Areas to be photobleached were determined, and their fluorescence levels were monitored before and at 0 and 15 min after photo- bleaching. Photobleaching and subsequent fluorescence recovery were con- densed at 37°C on the microscope stage using a ×63, 1.4 numerical aperture objective. Selective photobleaching was performed using an excitation wave- length of 488 nm with the laser at full power, and recovery was then monitored by time-lapse imaging at low-intensity illumination. Images were analyzed using ImageJ software (written by Wayne Rasband at the U.S. National Institutes of Health and available by anonymous file transfer protocol from http://rsb.info- ni.nih.gov/ij/).

Electron microscopy. Cells for scanning and transmission electron microscopy studies were grown on Thermofax coverslips to 90% confluence. EBs were added to an MOI of 200, and the infected cells were incubated at 4°C for 1 h to allow for attachment. The cells were rinsed with cold HBSS three times to remove unattached EBs and were shifted to 37°C. At various times, the coverslips were fixed with 2.5% glutaraldehyde–4% paraformaldehyde in 0.1 M sodium cacodylate–0.1 M sucrose buffer. Samples for scanning electron microscopy (SEM) were postfixed with 1% osmium tetroxide and dehydrated in a graded ethanol series. The samples were critical-point dried under CO2 in a Bal-Tec model cpd 030 dryer (Balzers, Liechtenstein), mounted on aluminum studs, and sputter coated with 150 A of iridium in a model IB5/TM2000 ion beam sputterer (VCR Group, South San Francisco, Calif.). Samples were viewed at 5 kV in a Hitachi S-4500 field emission SEM (Hitachi, Tokyo, Japan), and the images were processed using the Imagepro Interface program (GW Electronics, Norcross, Ga.). Samples for transmission electron microscopy were postfixed with 0.5% OsO4–0.8% K2Fe(CN)6, followed by 1% tannic acid and stained overnight on bloc in 1% uranyl acetate. Samples were dehydrated in a graded ethanol series and embed- ded in Spurr’s resin. Thin sections were cut with an RMC MT-7000 ultramic- rotome (Ventana, Tucson, Ariz.), stained with 1% uranyl acetate and Reynolds’ lead citrate, and observed on a Phillips CM100 transmission electron microscope (FEI, Hillsboro, Oreg.). Images were acquired with an Amount digital camera (Advanced Microscopy Techniques, Chazy, N.Y.) and processed using Adobe Photoshop v. 6.0 software (Adobe Systems, Mountain View, Calif.).

RESULTS

Inhibition of chlamydial entry by cytochalasin D. The actin machinery has been implicated in the colonization and/or inva- sion of host cells by a number of microbial pathogens (7, 18, 23, 29, 54). Because there are conflicting data (8, 32, 46, 48, 51, 56) regarding the role of actin in chlamydial entry, we examined the involvement of actin in the infection of HeLa cells by C. trachomatis serovars L2 and D by using an assay that quantitatively determines the level of internalization at times very early in infection. HeLa cells were treated with cytochalasin D, a compound that disrupts the actin-based cytoskeleton, prior to and during infection by C. trachomatis serovars L2 and D. The percentage of EBs internalized was evaluated by differential staining of intracellular versus extracellular EBs and quanti- tation with fluorescence microscopy. Representative images of HeLa cells infected with serovar L2 EBs in the presence or

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absence of cytochalasin D (2.5 μg/ml) are shown in Fig. 1. Treatment with cytochalasin D resulted in 41- and 13-fold reductions in internalization for L2 and D EBs, respectively. Because the concentration of cytochalasin D used was sufficient to disrupt actin structures (data not shown), the results indicate that actin is required for entry by serovar L2 and D EBs and that the two serovars share similar actin-dependent mechanisms of entering HeLa cells.

Recruitment of F-actin to sites of chlamydial attachment. Internalization of Salmonella requires actin polymerization, which is manifested by membrane ruffling and inhibited by cytochalasin D (24, 27, 30). Therefore, we investigated actin polymerization during chlamydial infection by using TRITC-phalloidin and confocal microscopy. Polymerized actin colocalized with C. trachomatis L2 EBs attached to the surface of HeLa cells (Fig. 2A). Background actin filaments observed in the periphery of the cells correspond to focal adhesions and stress fibers. The actin aggregation appears to be restricted to the area immediately surrounding the EBs (Fig. 2A and B). In contrast, Salmonella-induced membrane ruffling typically involves a much larger area of the plasma membrane that extends beyond the immediate vicinity of the organism. Interestingly, a view of the x-z plane showed an upward extension (relative to the coverslip) of these actin-rich structures. EBs were localized at or near the apex (Fig. 2B), reminiscent of the pedestal structure induced by enteropathogenic E. coli (4, 26). Pedestal-like structures could be observed more clearly in transmission electron micrographs (TEM) of infected HeLa cells, with L2 or D EBs localized at the apical surface of these projections (Fig. 2C, D, and E). Scanning electron micrographs also showed raised structures immediately underneath L2 or D EBs, with surrounding extended microvilli (Fig. 2F, G, and H).

In combination, these findings demonstrate cytoskeletal remodeling during C. trachomatis infection.

Active recruitment of GFP-actin to sites of attachment and entry. To determine if actin monomers were actively recruited to sites of chlamydial attachment, GFP-actin was utilized in conjunction with FRAP to observe localized actin recruitment in real time. HeLa cells transiently transfected with a GFP-actin expressing vector (34) were infected with live or heat-killed serovar L2 EBs fluorescently labeled with CMTMR cell tracker. Regions with EBs were selected for photobleaching, and recovery of fluorescence was monitored and quantitated. A progressive recovery of fluorescence contributed by GFP-actin at sites of chlamydial attachment indicated that GFP-actin was recruited to those sites (Fig. 3, top left, top right, and bottom left panels). Compared with regions of the cell lacking EBs, fluorescence recovery (i.e., recruitment of actin) of areas that contained EBs demonstrated threefold greater fluorescence. The areas of aggregation perfectly

FIG. 1. EBs of serovars L2 and D were allowed to attach to HeLa cells that were untreated or pretreated with 2.5 μg of cytochalasin D/ml at 4°C and shifted to 37°C for 30 min to allow chlamydial entry. Cytochalasin D, when required, was present at all times during the 37°C incubation. Internalized and uninternalized EBs were detected as described in Materials and Methods. Total EBs (red), uninternalized EBs (green), merged (yellow). (A to C) Serovar L2, untreated. (D to F) Serovar L2, cytochalasin D treated.
correlated with the presence of L2 EBs and could easily be distinguished from areas of the cell devoid of EBs. In contrast, heat-killed EBs were unable to recruit GFP-actin to their sites of attachment (data not shown).

Microvillar hypertrophy during chlamydial infection. The pedestal-like structures shown in Fig. 2 indicate morphological changes occurring on the cell surface. Specifically, the phalloidin staining of the cell surface projections indicated F-actin-
To directly observe possible morphological changes in the cell microvilli during infection, we utilized SEM to monitor the cell surfaces of infected and mock-infected cells. This technique allowed the visualization of any structural changes to the cell surface associated with chlamydial infection. Infected and mock-infected samples for SEM were fixed at different time points (0 to 60 min) during _C. trachomatis_ L2 infection, and representative images are shown in Fig. 4. Note the uniform length and distribution of cell surface microvilli in the mock-infected sample after incubation at 37°C for 30 min (Fig. 4A). The infected cells, however, exhibited a distinct cell surface morphology (Fig. 4B to H). There was a progressive elongation and clustering of microvilli in the infected cells which was visible at the 0 min time point and became more dramatic by 20 to 40 min. At 20 min postinfection, other nonmicrovillar cell surface projections in the proximity of EBs and microvilli became apparent. These structures may be related to those shown in the TEM images in Fig. 3. Interestingly, beginning at 50 min postinfection, microvillar hypertrophy became less dramatic and normal microvillar morphology was restored with regards to length and distribution. These data demonstrate induction of active actin remodeling and microvillar hypertrophy during interaction of EBs with host cells. These unique microvillar structures may correspond to the cell surface projections that were intensely stained by TRITC-phalloidin. Similar morphological changes were observed during infection of HeLa cells by serovar D EBs (Fig. 4I and J). Induction was less dramatic, which may have been due to the less efficient interaction of this serovar with HeLa cells. These microvillar structures were unique to _C. trachomatis_-infected cells, in that HeLa cells exposed to zymosan failed to produce the same result (Fig. 4K and L).

To elucidate the interaction of _C. trachomatis_ with host cells, we monitored the attachment and infection of a serovar L2 EB with a GFP-actin-expressing HeLa cell by using time-lapse confocal microscopy (Fig. 5). Over a period of 35 min, we visualized the microvillus-mediated retraction of an EB to the cell surface and its subsequent internalization.

Microvillar hypertrophy is cytochalasin D sensitive. The dramatic changes in cell surface morphology associated with _C. trachomatis_ infection appeared to be dependent on the formation of filamentous actin and may be critical in chlamydial entry. Thus, we investigated the effects of cytochalasin D, which we have shown to potently inhibit chlamydial entry, on the formation of these structures. HeLa cells growing on Thermannox coverslips were either left untreated, treated with cytochalasin D before and during infection with _C. trachomatis_ L2, or allowed to recover after cytochalasin D removal. Cytochalasin D-treated infected HeLa cells, which we found refractory to chlamydial invasion, showed a significant reduction in the number and length of microvilli (Fig. 6). In general, HeLa cells treated with cytochalasin D were devoid of cell surface microvilli. In these cells, EBs could be observed attached to a featureless plasma membrane (Fig. 6B). When treated cells were allowed to recover after cytochalasin D treatment, microvilli began to appear, first at the site of EB attachment (Fig. 6C). Therefore, chlamydiae appear to trigger localized microvillus formation and the cytochalasin D inhibition of _C. trachomatis_ entry may be due to the ability of the drug to inhibit the formation of these structures.

A structural component(s) of EBs is required for induction of microvillar hypertrophy. Because _C. trachomatis_ EBs are able to induce gross rearrangements of cell surface microvilli, it is possible that factors responsible for actin remodeling and induction of morphological changes in the microvilli may be structural components of the EBs. To test this hypothesis, L2 EBs in sucrose-phosphate-glutamate buffer (13) were heated to 90°C or were treated with 10 mM dithiothreitol (DTT)–10 mM iodoacetamide (IAA) or both or were fixed with 2.5% glutaraldehyde–4% formaldehyde. Treated EBs were subsequently assayed for the ability to attach and form stable secondary attachments to and enter cells (Table 1). Viability of the treated EBs was assayed by inclusion formation.

The treated EBs were then tested for their ability to induce microvillar hypertrophy on HeLa cells. Interestingly, infection of HeLa cells with EBs that were heat killed or fixed did not result in the formation of such structures (Fig. 7). In contrast, treatment of EBs with 10 mM DTT, 10 mM IAA, or both did not affect the ability of EBs to induce such structures. In addition, treated EBs, with the exception of those heat killed or fixed, retained their ability to bind heparan sulfate at approximately the same level as that of the untreated EBs. Stable post-heparan sulfate binding to the secondary receptor (14) was only achieved by the DTT- and/or IAA-treated EB group along with the untreated group, in correlation with the ability to enter HeLa cells (Table 1). The ability of differently treated EBs to induce microvillar hypertrophy correlated precisely with their ability to achieve both types of binding. The data implicate binding to the putative secondary receptor of EBs as an essential step for inducing actin remodeling and microvillar hypertrophy, which are prerequisites to internalization of chlamydial EBs.

Induction of microvilli structures requires primary and secondary binding steps of chlamydial attachment to host cells. It is likely that chlamydial attachment initiates a signal transduction cascade, leading to elongation and subsequent clustering of cell surface microvilli. Indeed, treatments of L2 EBs that eliminated their ability to achieve the post-heparan sulfate-binding step also eliminated their ability to induce microvillar hypertrophy. To confirm this observation, we utilized a mutant CHO cell line, D4.1-3, that has been recently described to support heparan sulfate-dependent binding but not the more stable secondary binding step required for entry by members of the LGV biovar only (14). Figure 8A shows the microvillar hypertrophy induced by serovar L2 EB attachment in the wild-type CHO-K1 cells. However, L2 EBs did not induce the same effect on microvilli morphology in the D4.1-3 cells (Fig. 8B and E). Uninfected cells did not show qualitative differences in the size or number of microvilli on the cell surface, further supporting the hypothesis of a chlamydia-induced microvilli hypertrophy. Serovar D EBs, which efficiently infect both wild-type CHO-K1 and mutant D4.1-3 cells (14), were able to induce microvillar hypertrophy in both cell lines. Thus, the resistant and susceptible phenotypes of D4.1-3 cells to serovar L2 and D infection, respectively, correlated with induction or absence of microvillar hypertrophy. The heparan sulfate-deficient pgsA 745 cell line (21) failed to produce the microvillar structures upon infection by either serovar L2 or D EBs (data...
FIG. 4. SEM of HeLa cells infected with serovar L2 or D EBs. HeLa cells were infected at an MOI of 100 at 4°C for 1 h and shifted to 37°C. Fixation to inhibit infection was performed at the indicated time points, and samples were processed for SEM. (A) Uninfected, 30 min. (B) Serovar L2, 0 min. (C) Serovar L2, 10 min. (D) Serovar L2, 20 min. (E) Serovar L2, 30 min. (F) Serovar L2, 40 min. (G) Serovar L2, 50 min. (H) Serovar L2, 60 min. (I) Serovar D, 0 min. (J) Serovar D, 30 min. (K) Zymosan, 0 min. (L) Zymosan, 30 min. Note the phases of morphological changes in and distribution of microvilli. Scale bar, 0.8 μm.
not shown). These results indicate that the more stable binding step of chlamydia to the D4.1-3 protein, which occurs subsequent to the heparan sulfate-binding step, was required for inducing the changes in microvillus morphology.

Chlamydial entry is independent of PI-3 kinase activity. Phosphatidylinositol (PI)-3 kinase has been implicated in a number of processes that require rapid actin remodeling, including invasion of host mammalian cells by bacterial and protozoan pathogens (7, 18, 23, 29, 54). For example, infection of mammalian cells by Cryptosporidium parvum results in a PI-3 kinase-dependent microvillar hypertrophy (19, 20, 25). The involvement of PI-3 kinase in chlamydial entry was investigated using a specific inhibitor of PI-3 kinase, namely, wortmannin. HeLa cells were treated with 40 nM wortmannin prior to infection and tested for their ability to internalize C. trachomatis L2 EBs by using our internalization assay (Fig. 9). To control for effects of wortmannin, PI-3 kinase-mediated internalization of antibody-opsonized EBs by HeLa cells was assessed in parallel. Wortmannin inhibited Fc-receptor-mediated entry of opsonized serovar L2 EBs 7.5-fold. However, the same treatment appeared to be ineffective in inhibiting internalization of untreated EBs. These results indicate that PI-3 kinase is not required for chlamydial entry in HeLa cells.

DISCUSSION

By employing an assay that distinguishes chlamydial attachment from entry, we demonstrated that actin is required for the internalization of C. trachomatis LGV serovar L2 and trachoma serovar D by HeLa epithelial cells. This requirement for actin was shown by the inhibition of internalization of both serovars by cytochalasin D. In addition, the interactions of C. trachomatis with the cell surface resulted in a localized recruitment of actin to the sites of attachment that was accompanied by a dramatic reorganization of cell surface microvilli. The failure of heat-killed or fixed EBs to induce actin aggregation

FIG. 5. Interaction of a HeLa cell with a C. trachomatis serovar L2 EB. Time-lapse confocal images of HeLa cells transfected with a GFP-actin expression vector reveal cell surface projections interacting with a fluorescently tagged EB and leading to the retraction of the microvillus-like structure and subsequent internalization of the EB. The site of entry is shown as an x-z image in the inset panel.

FIG. 6. Inhibitory effects of cytochalasin D treatment on microvillar hypertrophy. HeLa cells were either treated with cytochalasin D or left untreated at 4°C during infection with serovar L2 EBs. When required, cytochalasin D was present during the 37°C incubation for 30 min. Samples were processed for SEM. (A) Untreated. (B) Cytochalasin D treated. (C) Cytochalasin D removal and recovery (10 min). Scale bar, 1.5 μm.
suggests a requirement for a conformation-dependent component on the EB surface. Two distinct morphological events were observed upon C. trachomatis attachment, (i) a very localized recruitment of actin to the site of EB attachment that resulted in an apparent modification of microvillar structure to form a pedestal-like configuration that is associated with internalization and (ii) a more generalized microvillar hypertrophy that was evidenced by increased number and length of microvilli with clustering at the center of the cell. Thus, our results support a role for actin polymerization in the entry of chlamydiae into epithelial cells and demonstrate a previously unrecognized microvillar remodeling of the host cell surface during the process of chlamydial internalization.

Chlamydiae attach to and enter eukaryotic host cells very efficiently, yet the nature of the mechanisms by which they accomplish these essential functions has been controversial. Several different ligands, host receptors, and mechanisms have been proposed to play a role in the entry process (reviewed in reference 33). A role for heparin-sulfate-like proteoglycans in an initial electrostatic interaction with the host cell surface is presently a favored model (53, 59), although this mechanism may not be operant for all serovars (17). Subsequent to this initial reversible interaction, an irreversible association of EBs with the host cell surface has recently been demonstrated and has been proposed to precede entry (14). Although neither the parasite ligand nor host receptor has yet been identified, a mutant CHO cell line that was defective in this irreversible attachment step for the LGV serovars of C. trachomatis was susceptible to infection by serovar D, B, or MoPn. The specific defect in LGV attachment suggests that distinct receptors may be involved for the uptake of trachoma versus LGV serovars. The actual entry mechanism, endocytosis versus phagocytosis,
is also not well characterized, since evidence for (8, 45, 46) and against (32, 45, 46, 48, 51) a role for the actin cytoskeleton has been presented. Some of these disparate results can be ascribed to different cell types or experimental conditions. Even within the same cell type, however, the conditions of infection, i.e., static incubation versus centrifugation of the inoculum, can alter the percentage of EBs entering by cytochalasin D-sensitive or -insensitive mechanisms (45, 46). It is likely that chlamydiae enter by multiple means. While we find that *C. trachomatis* L2 and D EBs enter HeLa cells predominantly by a cytochalasin D-sensitive mechanism, it is possible that in other cells or culture conditions, the proportion of EBs entering by cytochalasin D-sensitive versus -insensitive mechanisms may differ.

Ultrastructural analyses of events surrounding chlamydial uptake typically describe EBs associated with microvilli (36, 37, 49, 57, 58), from which they are believed to be internalized into tight endocytic vesicles. SEM of the cell surface at close intervals during the *C. trachomatis* entry process demonstrated a marked rearrangement of microvilli on the cell surface, which was evident by 10 min after shifting the temperature to 37°C to induce internalization of attached EBs. This change in cell surface architecture is characterized by elongation of microvilli as well as an increase in the number of microvilli. Whether this increased number of cell surface microvilli increases susceptibility to subsequent infection by additional EBs remains to be determined. Microvillar hypertrophy is accompanied by transient rearrangement of the actin cytoskeleton, with a localized concentration of actin at the site of EB attachment and the apparent development of a structure smaller than, but reminiscent of, the attaching and effacing lesion (or pedestal) of enteropathogenic *E. coli* (26). Interestingly, a mutant CHO cell line that is defective in internalization of serovar L2 EBs supports entry of serovar D (14). The inability of serovar L2 to invade the mutant cells correlated with failure to induce microvillar hypertrophy, which is clearly induced by serovar D. The CHO cell mutation has not been characterized, and thus it is not clear whether the lesion represents loss of a receptor or simply an allelic substitution (14). Reports of a lack of competition between LGV and trachoma biovars for attachment suggest there may be distinct receptors (17). If the receptors are indeed different, similar signals seem to be transduced to the host cell, since the same microvillar hypertrophy is observed with both L2 and D.

![Image of EBs entering cells](http://iai.asm.org/)

**FIG. 8.** Attachment to the secondary receptor is required for induction of microvillar hypertrophy. Wild-type CHO-K1 and D4.1-3 cells were examined for their ability to support microvillar hypertrophy during infection with serovar L2 EBs. (A-C) CHO-K1. (D-F) D4.1-3. (A and D) Uninfected. (B and E) Serovar L2 infected. (C and F) Serovar D infected. Scale bar, 5 μm.

**FIG. 9.** EBs enter via a PI-3 kinase-independent mechanism. Wortmannin was tested for its ability to inhibit entry of untreated or opsonized *C. trachomatis* L2 EBs via the normal or Fc receptor-mediated endocytic pathway. Internalization efficiencies in both experimental groups were determined as described in Materials and Methods. Data are from a minimum of 60 cells and expressed as means ± SD.
Rearrangement of the actin cytoskeleton at the sites of parasite adherence or entry is a common theme among pathogens. In addition to the pedestals induced at the sites of adherence of enteropathogenic E. coli (26), bacterial and protozoan intracellular parasites trigger cytoskeletal rearrangement to facilitate their uptake by eukaryotic cells (reviewed in references 7, 18, 23, 29, and 54). Salmonella enterica serovar Typhimurium (24, 30, 60, 61) and Shigella flexneri (1, 2, 16, 35, 41, 50, 55) both induce large-scale membrane ruffling, or macropinocytosis, which serves to take up the bacteria into vacuoles. This triggering mechanism is in contrast to that of bacteria that use a “zipper”-type mechanism that involves direct contact between the bacterium and host cell surface and results in a tight association between bacterial ligands and host receptors to effect envelopment of the parasite. An example of bacteria that enter by a zipper-type mechanism would be Listeria monocytogenes (40). Although the cell surface rearrangements are not as excessive as ruffling, localized actin cytoskeleton reorganization is required. Attachment of the protozoan parasite Cryptosporidium parvum also induces microvillar hypertrophy characterized by long branched microvilli clustered around the cryptosporidial vacuole (19, 20, 25).

The interaction of C. trachomatis EBs with the host cell surface elicited a highly localized recruitment of actin at the sites of attachment. This actin recruitment may have resulted from a signal transduction cascade initiated by the attachment of the EB to the cell surface receptor and transduced to the actin nucleating machinery of the host cell. The microvillar reorganization observed with C. parvum-infected cells appears to be dependent upon the activity of a PI-3 kinase and various actin-binding proteins such as members of the ezrin-radixin-moesin family, Wiskott-Aldrich syndrome protein, and the Arp2/3 complex (10, 19, 20, 25). Whereas the C. parvum invasion of host cells is inhibited by wortmannin (25), the chlamydial internalization was not, suggesting a PI-3 kinase-independent mechanism. A similar PI-3 kinase-independent mechanism of invasion has been demonstrated for Salmonella (38). Despite the apparent lack of involvement of PI-3 kinase in chlamydial internalization, it is possible that proteins mediating actin remodeling during C. parvum infection could also be involved in chlamydial infection via the recruitment and/or activation of these proteins by an alternate mechanism(s) independent of PI-3 kinase activity (39).

Actin remodeling induced by many gram-negative bacterial pathogens is dependent upon bacterially encoded factors secreted by a type III secretion system (27). This type III mechanism in mediating bacterial internalization involves the engagement of cell surface receptors with ligands on the EB surface. The adhesin-receptor interaction induces recruitment of actin with accompanying morphological changes to microvillar structure and ultimately leads to internalization. Our results are consistent with those involving receptor-mediated phagocytosis of C. trachomatis EBs by epithelial cells. A complete understanding of the chlamydial entry process, including identification of the chlamydial ligand(s), host receptor(s), signaling pathways, and actin-interacting proteins, would be a significant advance in knowledge of the biology of these complex pathogens.

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REFERENCES


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