**Chlamydia trachomatis** uses host cell dynein to traffic to the microtubule-organizing center in a p50 dynamitin-independent process

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**Summary**

Chlamydiae are pathogenic obligate intracellular bacteria with a biphasic developmental cycle that involves cell types adapted for extracellular survival (elementary bodies, EBs) and intracellular multiplication (reticulate bodies, RBs). The intracellular development of chlamydiae occurs entirely within a membrane-bound vacuole termed an inclusion. Within 2 hours after entry into host cells, *Chlamydia trachomatis* EBs are trafficked to the perinuclear region of the host cell and remain in close proximity to the Golgi apparatus, where they begin to fuse with a subset of host vesicles containing sphingomyelin. Here, we provide evidence that chlamydial migration from the cell periphery to the peri-Golgi region resembles host cell vesicular trafficking. Chlamydiae move towards the minus end of microtubules and aggregate at the microtubule-organizing center (MTOC). In mammalian cells the most important minus-end-directed microtubule motor is cytoplasmic dynein. Microinjection of antibodies to a subunit of cytoplasmic dynein inhibited movement of chlamydiae to the MTOC, whereas microinjection of antibodies to the plus-directed microtubule motor, kinesin, had no effect. Surprisingly, overexpression of the protein p50 dynamitin, a subunit of the dynactin complex that links vesicular cargo to the dynein motor in minus directed vesicle trafficking, did not abrogate chlamydial migration even though host vesicle transport was inhibited. Nascent chlamydial inclusions did, however, colocalize with the p150(Glued) dynactin subunit, which suggests that p150(Glued) may be required for dynein activation or processivity but that the cargo-binding activity of dynactin, supplied by p50 dynamitin subunits and possibly other subunits, is not. Because chlamydial transcription and translation were required for this intracellular trafficking, chlamydial proteins modifying the cytoplasmic face of the inclusion membrane are probable candidates for proteins fulfilling this function.

Key words: *Chlamydia*, Cytoskeleton, Microtubules, Dynein, Dynactin

**Introduction**

*Chlamydia trachomatis* is the most common cause of sexually transmitted disease (STD) in developed countries and the most frequent cause of preventable blindness worldwide (Schachter, 1999). *Chlamydia* spp. are obligate intracellular bacteria with a unique developmental cycle that takes place entirely within a membrane-bound parasitophorous vacuole termed an inclusion (Friis, 1972; Moulder, 1991). Two morphologically distinct forms characterize the chlamydial developmental cycle: elementary bodies (EBs) are the extracellular, infectious form, and reticulate bodies (RBs) are the intracellular replicative form (Friis, 1972; Moulder, 1991). The EB-to-RB transition is initiated within the first few hours after internalization. Vesicles containing endocytosed EBs quickly cease interaction with the endocytic pathway (Scidmore et al., 2003), instead intersecting an exocytic pathway as evidenced by fusion with Golgi-derived sphingomyelin-containing vesicles (Hackstadt et al., 1995; Hackstadt et al., 1996).

Within the first few hours postinfection, endocytosed EBs are trafficked to the peri-Golgi region of the host cell where the inclusions remain for the duration of chlamydial intracellular development (Hackstadt, 1999). Recruitment of the nascent inclusion to the peri-Golgi region appears to require an active process on the part of the chlamydiae, given that inhibition of chlamydial transcription or translation blocks their translocation (Scidmore et al., 1996). *C. trachomatis* inclusions aggregate at a perinuclear or peri-Golgi location that corresponds to the microtubule-organizing center (MTOC) within 6 hours postinfection (Clausen et al., 1997; Hackstadt et al., 1996). The minus-end-directed microtubule motor, dynein, has been implicated in this process as dynein colocalizes with chlamydial early inclusions and Na3VO4, a general inhibitor of tyrosine kinases that also inhibits dynein, detrimentally affects chlamydial development when cells are treated during the course of infection (Clausen et al., 1997). Thus, like many cellular organelles, the chlamydial inclusion appears to be trafficked intracellularly via interactions with the microtubular network.

Microtubules are polarized structures with a minus end anchored at the MTOC and a plus end directed towards the periphery of the cell. Microtubules serve as a scaffold for the transport and sorting of various cellular cargoes and are
involved in such diverse cellular functions as chromosome segregation, organelle transport, and regulation of anterograde and retrograde trafficking through the Golgi complex. Transport along microtubules is mediated by ATP-dependent, microtubule-associated motor proteins. Kinesin superfamily proteins comprise the major plus-end-directed motors, whereas dynein superfamily proteins are the minus-end-directed motors. An accessory protein complex called dynactin is thought to be required for cargo binding to the dynein motor complex. Dynactin is a large multisubunit complex consisting of p150(Glued), p62, p50 dynamin, actin-related protein 1 (Arp1) and actin-capping protein (Hirokawa, 1998; Waterman-Storer et al., 1997). Overexpression of p50 dynamin inhibits the ability of dynein to interact with its cargo and results in the disruption of minus-end movement along microtubules (Burkhardt et al., 1997; Valetti et al., 1999).

Many bacterial and viral pathogens exploit the microtubular network to access specific sites within the host cell (Alonso et al., 2001; Kim et al., 2001; Ploubidou et al., 2000; Ye et al., 2000). In this study, we show that C. trachomatis modifies the inclusion membrane to recruit dynein and selected components of the dynactin complex for migration along microtubules in a fashion similar to host vesicular trafficking. However, chlamydial recruitment of dynein appears to be mechanistically distinct as migration to the MTOC is not disrupted by overexpression of the p50 dynactin subunit of dynamitin. These findings suggest that chlamydial circumvent the necessity for an intact dynein-dynactin motor complex in a unique manner in which a chlamydial protein supplants a requirement for at least the dynamitin component of dynactin.

Materials and Methods

Organisms, cell culture and infections

C. trachomatis serovars L2 (LGV-434) and D (UW3-Cx) were grown in HeLa or Cos-7 cells as previously described (Caldwell et al., 1981). EBs used for infections were purified by Renografin (E. R. Squibb and Sons, Princeton, NJ) density gradient centrifugation (Caldwell et al., 1981).

For live imaging, C. trachomatis L2 EBs were intrinsically labeled with the fluorescent dye 5-(and -6)-((4-chloromethyl)benzoyl)-amino)tetramethylrhodamine (CMTMR) as previously described (Boletti et al., 2000; Carabeo et al., 2002). Briefly, HeLa cells were infected with serovar L2 EBs at a multiplicity of infection (MOI) of 1 and incubated for an additional 24 hours with 25 μg of CMTMR cell tracker/ml added to the culture medium at 12 hours postinfection. EBs were then harvested by Renografin density gradient centrifugation. The infectivity of CMTMR-treated EBs was evaluated by inclusion-forming assay and was found to be unaffected by the labeling procedure (Carabeo et al., 2002).

For microscopy studies Cos-7 cells were seeded on 25 mm #1 borosilicate coverslips at a density of 3x10^5 cells per coverslip and cultivated overnight at 37°C in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 10 μg/ml gentamicin (Invitrogen, Carlsbad, CA) under 5% CO2.

All infections were carried out similarly unless otherwise noted. Cells were incubated with C. trachomatis EBs at an MOI of ~50 in Hanks Balanced Salts Solution (HBSS) (Invitrogen, Carlsbad, CA) for 10 minutes, after which the inoculum was removed and the coverslips were washed twice with HBSS with 100 μg/ml heparin (Pharmacia, Peapack, NJ), and once with HBSS without heparin. The HBSS was then replaced with RPMI media containing 10% FBS and 10 μg/ml gentamicin. Infections were allowed to proceed for appropriate times.

Transfections and microinjections

Cos-7 cells were seeded on 12 mm glass coverslips in 24-well plates to obtain a monolayer of approximately 50% confluency. Transfections of plasmid constructs were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s directions. The transfection mixture was prepared as follows: 0.8 μg of DNA was diluted in 50 μl of Optimem serum-free media (Invitrogen) and added to a solution of 50 μl of Optimem with 3 μl lipofectamine 2000. After a 20 minute incubation at room temperature, the complexes were added to one well of a 24-well plate containing 500 μl of RPMI with 10% FBS. Expression vectors used were enhanced green fluorescent protein (EGFP)-C1, EYFP-Golg (BD Biosciences, San Jose, CA) and GFP-dynamitin (Valetti et al., 1999). Expression from the transfected vectors was allowed to proceed for 24 to 30 hours before experimentation.

Microinjection of antibodies was performed using an automated microinjection system as described previously (Heinzen and Hackstadt, 1997). Microinjected antibodies used in these experiments were: mouse mAb to dynein intermediate chain dic74.1 (Covance); mouse mAb to dynein intermediate chain dic70.1 (Abcam, Cambridge, UK); mouse mAb to kinesin (Covance); and mouse mAb to Rickettsia rickettsii (Heinzen et al., 1999). All antibodies were purified using protein G sepharose columns (Pharmacia, Peapack, NJ) according to the manufacturers’ directions, followed by buffer exchange and concentration using microspin concentrators (Millipore, Bedford, MA). The antibodies were concentrated to 10 μg/ml in microinjection buffer (48 mM K2HPO4, 14 mMNaH2PO4, 4.5 mM KH2PO4, pH 7.2). Before the injections, cells on coverslips were removed from 24-well plates and placed in 100 cm diameter Petri dishes containing 10 ml of fresh RPMI plus 10% FBS. Microinjection of the cytoplasm of cells was done with a Micromanipulator 5171 and a Transjector 5426 plus (Eppendorf, Hamburg, Germany). Femtotips (Eppendorf) were backfilled with 3 μl samples by using Microloaders (Eppendorf). Injections were monitored by epifluorescence illumination on a Nikon TE 300 inverted microscope equipped with a Polychrome I polychromatic illumination system (Applied Scientific Instrumentation, Eugene, OR.). Oregon green dextran was used for monitoring injections (Molecular Probes, Eugene, OR) and was added to the antibodies at a final concentration of 0.5 mg/ml. All injections were conducted at room temperature and the delivery pressure and injection duration for most experiments was 1.4 lb/in2 and 0.5 seconds, respectively. This resulted in an estimated delivery volume of approximately 0.1 pl (Minaschek et al., 1989). Following injection, cells were washed once with RPMI plus 10% FBS, and fresh medium was added. Ten to fifteen minutes after injection, the coverslips were infected with C. trachomatis L2 or used in endocytic trafficking experiments. The injected antibodies were detected using AlexaFluor 488-conjugated goat anti-mouse IgG secondary antibodies.

Endocytic markers

For endocytosis assays, Cos-7 cells transfected with appropriate constructs or microinjected with various antibodies were incubated with 25 μg/ml AlexaFluor 594-transferrin (Tf) or 5 μg/ml AlexaFluor 594-cholera toxin B (Molecular Probes) in Optiemp serum-free media (Invitrogen) for 5 hours at 37°C. Cells were quickly rinsed in HBSS before fixing with freshly prepared 4% paraformaldehyde in PBS for 10 minutes at room temperature.

Immunofluorescence staining

For fluorescent antibody staining, cells were fixed with cold methanol for 10 minutes. The cells were washed three times with PBS. Antibodies used in these experiments were mouse mAb to dynein intermediate chain dic70.1 diluted 1:100 (Abcam), mouse mAb to p150(Glued) 2.5 μg/ml (BD Biosciences, San Jose, CA) and mouse mAb to β-tubulin diluted 1:300 (Sigma). Primary antibodies were...
incubated on cells for 3 hours followed by three washes with PBS. To visualize the primary antibodies the cells were incubated with AlexaFluor 488-conjugated goat anti-mouse IgG 4 μg/ml. To simultaneously visualize chlamydiae, cells were also stained using a rabbit polyclonal Ab to C. trachomatis serovar L2 at a dilution of 1:1000. This antibody was detected using AlexaFluor 594-conjugated goat anti-rabbit IgG secondary antibody 4 μg/ml. For cytochalasin D experiments, cells were fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 0.05% triton-X 100 and stained using FITC-phalloidin 10 U/ml (Molecular Probes). All fluorescence images were obtained with a Zeiss Axiovert LSM 510 confocal microscope. Projections were constructed using the ImageJ image software (written by Wayne Rasband at the U.S. National Institutes of Health and available by anonymous FTP from zippy.nimh.nih.gov).

Quantification of fluorescent microscopy
Quantification of peri-Golgi fluorescence was conducted using ImageJ software. Regions of interest were chosen that represented the C. trachomatis localization in individual cells from the micrographs. The fluorescence intensity of this region was represented as a percentage of the total fluorescence signal of the entire cell.

Live-cell confocal microscopy
For live-cell imaging, cells were subcultured into 35 mm glass bottom Petri dishes (Mat Tek Corp, Ashland, MA). The dishes were placed in a temperature-controlled chamber and maintained at 37°C throughout the experiment. To observe normal trafficking of host cell vesicles, cells were labeled with fluorescent N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)-6-aminocaproyl-D-erythro-sphingosine (C6-NBD-Cer) (Molecular Probes). C6-NBD-Cer was complexed with 0.034% defatted bovine serum albumin (dfBSA) in HBSS, as described previously (Pagano and Martin, 1988), to yield complexes equal to a concentration of 5 μM in both dfBSA and C6-NBD-Cer. Cells were incubated with the dfBSA/NBD-Cer complex at 37°C for 15 minutes, washed with HBSS and incubated for 2 hours in HBSS with 0.34% dfBSA to back-exchange excess probe from the plasma membrane. These cells were simultaneously infected with CMTMR-labeled L2 EBs. The infection was allowed to proceed for 2 hours before viewing. Images were acquired on a Zeiss Axiovert LSM 510 confocal microscope and a 64×, 1.4 NA lens with a computer-controlled 488 nm argon laser to excite NBD or a 564 nm krypton laser to excite CMTMR. Images were collected at 15-second intervals using the Zeiss LSM 510 software.

Results
C. trachomatis nascent inclusions aggregate at a perinuclear site corresponding to the MTOC of infected cells and are dependent on host microtubules
Within the first 2-4 hours postinfection, nascent C. trachomatis inclusions are translocated to a perinuclear site of the infected cell. Simultaneous visualization of both the microtubule network by indirect immunofluorescence staining of β-tubulin and C. trachomatis L2 inclusions in Cos-7 cells revealed that the chlamydiae aggregate at the MTOC (Fig. 1). Interestingly, chlamydiae aggregate at both spindle poles in mitotic cells. This localization is not specific to infections at a high multiplicity of infection (MOI) as individual inclusions also localize to the MTOC of cells infected at an MOI <1 (data not shown). Cells infected with C. trachomatis serovar D showed an identical phenotype to L2-infected cells. Thus, this migration and aggregation to the MTOC appears to be common to both lymphogranuloma venereum (LGV) and non-LGV strains of C. trachomatis.

Chlamydial migration is delayed due to a requirement for chlamydial protein synthesis
Approximately 4-5 hours is required for most inclusions to aggregate at the MTOC. The speed of host vesicle movement along microtubules is about ~0.5 μm/second (Garcia-Mata et al., 1999; Schroer, 2000; Toomre et al., 1999); thus, the time it takes for perinuclear delivery of chlamydiae could be due to either slow movement along microtubules or a delay in the initiation of migration. Chlamydial modification of the inclusion membrane occurs very soon after infection (Hackstadt, 1999). A requirement for chlamydial protein synthesis suggests that modification of the nascent inclusion by chlamydiae is necessary to initiate migration along microtubules and could explain this observed lag in perinuclear...
To confirm a role for chlamydial protein synthesis in Cos-7 cells, the cells were incubated with nocardazole for 4 hours before fixing and staining for *C. trachomatis* and tubulin. In these cells the chlamydial early inclusions did not aggregate at a single perinuclear site but remained widely dispersed throughout the cytoplasm. The cells were fixed and stained for chlamydiae and observed simultaneously with GFP signal. The nascent inclusions aggregated normally even though the Golgi was dispersed, as can be seen by the dispersed GFP-Golgi signal. Disruption of the actin cytoskeleton with cytochalasin D also does not inhibit chlamydial aggregation. FITC-phalloidin staining of the F-actin cytoskeleton shows that the actin cytoskeleton has been disrupted but the nascent inclusions are still aggregated at a single site within the cell.

Rapid movement along microtubules

Although the necessity for chlamydial protein synthesis accounts for the observed lag in perinuclear delivery it does not indicate if *Chlamydia* are capable of rapid travel on microtubules. To address this question, we synchronized chlamydial migration using nocardazole to inhibit migration and allowed the chlamydiae time to modify the inclusion. This was followed by a washout of nocardazole to re-initiate migration. Cos-7 cells were infected with L2 for 30 minutes at 4°C before the addition of nocardazole. Infected cells were incubated in the presence of nocardazole for 4 hours, after which time the medium was replaced with fresh media without drug. The cells were subsequently fixed and stained for both microtubules and *C. trachomatis* after either 15 minutes or 1 hour additional incubation. By 15 minutes the microtubule network was partially restored and many cells had obvious MTOCs and some microtubules. In these cells, many of the inclusions were already aggregated at the reorganizing MTOC. By 1 hour after nocardazole washout, the microtubules were more organized and the majority of the chlamydiae were
Chlamydia microtubule interactions

Chlamydial microtubule interactions

Chlamydiae are capable of rapid migration in host cells. Chlamydial migration was inhibited after entry by treating infected cells with nocodazole for 4 hours. This allowed time for chlamydial protein synthesis and modification of the inclusion membrane. Nocodazole was replaced with complete media and cultures were further incubated for 15 minutes or 1 hour. Simultaneous visualization of cells stained with antibodies to tubulin and L2 EBs revealed that after 15 minutes of nocodazole washout a few microtubules had reformed and there was an obvious MTOC (arrowheads). The merged image shows many of the nascent inclusions are aggregated at this site. After one hour the microtubule network is nearly restored. The tubulin staining shows two MTOC in this cell (arrowheads). In the merged image the majority of the chlamydial staining localizes to one or the other MTOC. Bar, 10 μM.

Chlamydia inclusions colocalize with dynein

Dynein has previously been shown to be associated with early chlamydial inclusions (Clausen et al., 1997). To confirm this in Cos-7 cells, cultures were infected with C. trachomatis L2 and fixed and stained with the dic70.1 monoclonal antibody at 5 hours and 24 hours postinfection. Confocal micrographs revealed that dynein localized along microtubules and to the nascent inclusions in the perinuclear region (Fig. 6). When the migration of the chlamydial nascent inclusions was inhibited by nocodazole treatment, dynein was still recruited to the chlamydial inclusion (Fig. 6). This recruitment could be inhibited by treatment with chloramphenicol, indicating that chlamydial protein synthesis was necessary for this interaction (data not shown). Colocalization of dynein with the chlamydial inclusion was not restricted to early time-points as dynein staining of the mature chlamydial inclusions remained apparent at 24 hours postinfection (Fig. 6). At this time the multiple individual inclusions had fused and the inclusion had taken on its characteristic spherical structure with a spacious, fluid-filled center rimmed by chlamydial RBs.

Chlamydia microtubule migration is independent of the dynactin complex

To investigate chlamydial interactions with the dynein-dynactin system, we
transiently transfected Cos-7 cells with a plasmid encoding GFP-dynamitin. Overexpression of dynamitin causes dissociation of the dynactin complex, thus decoupling dynein-binding and cargo-anchoring functions and effectively acting as a dominant negative for dynactin activity (Echeverri et al., 1996; Wittmann and Hyman, 1999). Cells overexpressing dynamitin were infected with *C. trachomatis* L2 for 5 or 24 hours in the presence or absence of nocodazole. Cells were stained for simultaneous visualization with mAb DIC 70.1 to visualize the dynein intermediate chain and a polyclonal antibody to L2 EBs to visualize the nascent inclusions. By 5 hours postinfection, the dynein antibody revealed staining consistent with it being localized along microtubules, as well as associated with the aggregated chlamydiae. In cells treated with nocodazole, dynein staining of the microtubules is absent and much of the staining is evident around the nuclear envelope. The nascent inclusions show dramatic dynein recruitment even in the presence of nocodazole. Dynein remains associated with the mature chlamydial inclusion even after infection for 24 hours. Bars, 10 μM.

To verify that the GFP-dynamitin construct was indeed acting as a dominant negative for the dynactin-dynein pathway, we tested the effect of overexpression on trafficking of two model proteins, transferrin (Tf) and cholera toxin subunit B (CTX), trafficked by microtubule-dependent pathways. Tf binds to the Tf receptor on the cell surface and is delivered to late endosomes in the peri-Golgi region. This trafficking is inhibited by overexpression of dynamitin (Valetti et al., 1999). Alexa-594-conjugated Tf was incubated with either GFP or GFP-dynamitin-transfected cells for 5 hours. The cells were

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**Fig. 6.** Dynein colocalizes with nascent inclusions by 5 hours postinfection and remains associated with the inclusion as late as 24 hours postinfection. Cos-7 cells were infected with *C. trachomatis* for 5 or 24 hours in the presence or absence of nocodazole. Cells were stained for simultaneous visualization with mAb DIC 70.1 to visualize the dynein intermediate chain and a polyclonal antibody to L2 EBs to visualize the nascent inclusions. By 5 hours postinfection, the dynein antibody revealed staining consistent with it being localized along microtubules, as well as associated with the aggregated chlamydiae. In cells treated with nocodazole, dynein staining of the microtubules is absent and much of the staining is evident around the nuclear envelope. The nascent inclusions show dramatic dynein recruitment even in the presence of nocodazole. Dynein remains associated with the mature chlamydial inclusion even after infection for 24 hours. Bars, 10 μM.

**Fig. 7.** Chlamydial migration is not inhibited by disruption of the dynactin complex due to overexpression of p50 dynamitin. Cos-7 cells were transiently transfected with GFP-dynamitin before infection with *C. trachomatis* or labeling with Alexa-Tf or Alexa-CTX. Cells expressing GFP-dynamitin (green in merged image) show no defects in chlamydial migration. The nascent inclusions stained with an antibody to L2 EBs (red in merged image) aggregate at a single perinuclear site in GFP-positive cells (arrowhead). Transiently transfected cells incubated with Alexa-Tf (red in merged image) show that Tf accumulated normally at a peri-Golgi region (arrowheads) in untransfected cells (no green signal in merged image) but Tf trafficking was inhibited in a neighboring cell expressing GFP-dynamitin (green cell in merged image). Similar results were observed for Alexa-CTX trafficking. CTX (red in merged image) was delivered to the Golgi (arrowhead) in the untransfected cell (no green signal in merged image) but delivery was abolished in GFP-dynamitin-expressing cells (green in merged image). Bars, 10 μM.
then fixed and observed by confocal microscopy. Cells expressing GFP-dynamitin had little to no Tf signal in the perinuclear region, and most of the signal was distributed throughout the periphery. This is in contrast to untransfected cells, which had significant signal in the perinuclear region (Fig. 7). The Tf signal in cells expressing GFP only were indistinguishable from untransfected cells (data not shown).

The trafficking of CTX to the Golgi involves binding to the lipid GM1, a ganglioside on the cell surface, followed by internalization and delivery by vesicle trafficking directly to the Golgi without interacting with transferrin-containing late endosomes (Nichols et al., 2001; Nichols, 2002). This pathway is distinct from that of transferrin but dynein-dynactin dependent. We investigated the effect of dynamitin overexpression on the delivery of CTX to the Golgi. Cos-7 cells were transfected with plasmids expressing either GFP or GFP-dynamitin for 24 hours before incubating the cells for 5 hours with Alexa 594 CTX. Cells expressing GFP-dynamitin did not accumulate CTX in the perinuclear region, whereas untransfected cells on the same coverslip had substantial CTX signal in a distinct perinuclear pattern (Fig. 7). The localization of CTX in GFP-expressing cells was identical to neighboring untransfected cells (data not shown).

The differential effect of GFP-dynamitin overexpression on the intracellular trafficking of Tf and CTX compared with C. trachomatis L2 was quantified by measuring the percent fluorescent signal in the perinuclear region compared with the total signal in the cell. Cells transfected with GFP-dynamitin were subsequently incubated with Alexa 594-Tf or Alexa 594-CTX, or infected with C. trachomatis L2 for 5 hours. Cells incubated with Tf or CTX were fixed and observed directly and cells infected with C. trachomatis were fixed and the bacteria were visualized by indirect immunofluorescence. Dual color micrographs were taken of both transfected cells (bright GFP signal) and untransfected cells (no GFP signal) on the same coverslips. In untransfected cells, about 65% of the fluorescence signal for Tf, CTX and chlamydial nascent inclusions was perinuclear by 5 hours after treatment (68%±3, n=14; 62%±3, n=16; and 67%±5, n=34, respectively). By contrast, cells that expressed GFP-dynamitin had very limited amounts of Tf and CTX signal in the perinuclear region (10%±2, n=15 for Tf and 7%±2; n=13 for CTX). However, chlamydial inclusions localized no differently in transfected versus untransfected cells (71%±3, n=31 and 67%±5, n=34, respectively). These data suggest that chlamydial perinuclear migration may be mechanistically distinct from currently described systems.

Chlamydial perinuclear aggregation is dependent on dynein

Because chlamydial aggregation was unaffected by dynamitin overexpression, we asked whether chlamydiae require dynein for their minus-end-directed microtubule motor activity. For these experiments we microinjected monoclonal antibodies against the dynein intermediate chain to determine any effect on chlamydial intracellular trafficking. The monoclonal antibody dic74.1 was microinjected into Cos-7 cells followed by infection with C. trachomatis L2. The cells were incubated for 5 hours to allow the chlamydiae to initiate development and translocate before fixing and staining for chlamydiae and the microinjected antibody. Cells that had been microinjected showed a chlamydial aggregation phenotype that was indistinguishable from nocodazole-treated cells, in that there was no perinuclear aggregation, and the nascent inclusions were scattered throughout the cytoplasm (Fig. 8). This was, to some extent, dependent on antibody concentration as cells that stained weakly for microinjected antibody showed an intermediate phenotype with many aggregated and unaggregated nascent inclusions. We confirmed the requirement for dynein using a second antibody dic70.1 that also recognizes the dynein intermediate chain. Microinjection of the dic70.1 antibody showed an identical phenotype (data not shown). To confirm the specificity of the antibody, two irrelevant antibodies, against either R. rickettsii or the major plus-end-directed microtubule motor protein, kinesin, had no effect on chlamydial movement as chlamydiae aggregated normally (arrowheads) at the MTOC of both injected (green cells) and noninjected cells (no green signal in merged image). Bars, 10 μM.
these data show that dynein is recruited to the chlamydial inclusion and is necessary for migration and that this recruitment is dependent on expression of a chlamydial protein or proteins.

The p150\(^{(Glued)}\) subunit of dynactin colocalizes with the chlamydial nascent inclusions

Because overexpression of the p50 dynamitin subunit does not affect chlamydial intracellular aggregation but dynein is recruited and required for this movement, we asked whether p150\(^{(Glued)}\), the largest subunit of the dynactin complex, colocalized to the chlamydial inclusion. Indirect immunofluorescence showed that the p150\(^{(Glued)}\) subunit of dynactin is recruited to the chlamydial inclusion (Fig. 9). Like dynein, p150\(^{(Glued)}\) also colocalized with nascent inclusions in nocodazole-treated cells (Fig. 9). This recruitment is also dependent on chlamydial protein synthesis as recruitment is inhibited by chloramphenicol (data not shown).

Overexpression of the p50 dynamitin subunit of dynactin disrupts the interaction between the motor complex, dynein, and the activating and cargo-binding complex, dynactin (Kamal and Goldstein, 2002; Karcher et al., 2002). Because migration of the nascent inclusion was unaffected by overexpression of GFP-dynamitin, we asked whether this overexpression was able to disrupt the ability of the chlamydial inclusion to recruit p150\(^{(Glued)}\). Cos-7 cells were transfected with GFP-dynamitin for 24 hours before infection with C. trachomatis. The infection was allowed to proceed for 5 hours before fixation and staining. The cells were observed by three-color confocal microscopy for GFP signal, as well as immunofluorescence staining for chlamydiae and p150\(^{(Glued)}\). The p150\(^{(Glued)}\) colocalized with the chlamydial inclusions in the cells that expressed GFP-dynamitin in the same fashion as untransfected cells (Fig. 9). The overexpression of GFP-dynamitin did not remove p150\(^{(Glued)}\) from the inclusion, suggesting that this interaction is independent of p50 dynamitin.

Discussion

Nascent chlamydial inclusions are translocated to a perinuclear/peri-Golgi region of the host cell relatively quickly following endocytosis (Higashi, 1965). This perinuclear aggregate is centered at the MTOC [this manuscript and (Clausen et al., 1997)] and dependent on the host cell microtubule network. The process of translocation is dependent on the host minus-end-directed microtubule motor complex dynein as migration is inhibited by microinjection of antibodies to the dynein intermediate chain. This is in agreement with studies that have previously localized dynein to the developing inclusions and shown that Na\textsubscript{3}VO\textsubscript{4}, a general inhibitor of tyrosine phosphatases that inhibits dynein, detrimentally affects chlamydial development when cells are treated concurrently with infection (Clausen et al., 1997). In addition, optimal growth of C. psittaci appears to be dependent upon a functional microtubule network (Escalante-Ochoa et al., 2000). Although chlamydial migration requires dynein and appears to have many similarities to host endocytic trafficking, it differs significantly in that an intact dynactin complex is apparently not necessary. Modification of the inclusion membrane by chlamydiae is required, however, suggesting that a chlamydial protein may supersede the requirement for the dynactin complex subunit, p50 dynamitin. The identity of the required chlamydial protein or proteins responsible is currently unknown, but chlamydiae are known to modify the inclusion within the first few hours of infection with several integral inclusion membrane proteins, collectively termed Incs, many of which are exposed to the cytoplasm (Hackstadt et al., 1999; Rockey et al., 1995; Rockey et al., 1997; Scidmore and Hackstadt, 2001; Scidmore-Carlson et al., 1999).

The exploitation of the host cell
Chlamydia microtubule interactions

The microtubule network is not unique to chlamydiae. Several large DNA viruses including adenovirus, African swine fever virus, vacinia virus and herpes viruses use the dynein-dynactin machinery for intracellular migration (Alonso et al., 2001; Dohner et al., 2002; Leopold et al., 2000; Ploubidou et al., 2000). At least one other intracellular bacterium, Orientia tsutsugamushi, also uses this system for intracellular migration (Kim et al., 2001). However, each of these examples differ from chlamydiae in that their migration is disrupted by the overexpression of the dynactin subunit p50 dynamitin. Indeed, overexpression of this dynactin subunit inhibits all reported dynein-dynactin-dependent processes in host cells. We confirmed that overexpression of p50 dynamitin inhibits the perinuclear accumulation of transferrin as well as the delivery of cholera toxin subunit B to the Golgi apparatus. The overexpression of p50 dynamitin has been reported to inhibit many diverse host functions that rely on minus-directed motor activity such as: endocytic vesicle trafficking, the organization of the Golgi complex, the formation of perinuclear aggresomes, reorientation of the MTOC after injury to fibroblasts, axonal transport in motor neurons, and the assembly and organization of the intermediate filament network (Echeverri et al., 1996; Garcia-Mata et al., 1999; Helfand et al., 2002; LaMonte et al., 2002; Palazzo et al., 2001; Valetti et al., 1999).

An intact dynactin complex is believed to be a necessary component in dynein-mediated minus-end-directed microtubule-mediated transport. Overexpression of the dynactin subunit p50 dynamitin dissociates dynactin into two subcomplexes: a dynein-binding subunit containing p150(Glued) and a cargo-binding subunit that contains Arp1 (Burkhardt et al., 1997). Unlike most intracellular pathogens, interactions of chlamydial inclusion with microtubules appear unique in that dynamitin overexpression fails to disrupt trafficking of endocytosed EBs to the MTOC. Although transport of the nascent chlamydial inclusion is not sensitive to the overexpression of p50 dynamitin, the dynactin subunit p150(Glued) is recruited to the inclusion, suggesting that the dynactin complex may have multiple and distinguishable functions. Our results suggest that p150(Glued) may be required for dynein activation or processivity but that the dynamitin subunits and possibly other subunits required for cargo binding are dispensable for interaction with C. trachomatis inclusions. In the model by Hirokawa (Hirokawa, 1998), the chlamydial inclusion may supply the cargo binding activity in the form of chlamydial proteins (Fig. 10). This model is supported by the report that Arp1 but not p150(Glued) is removed from microtubules by p50 dynamitin overexpression (Vaughan et al., 1999), suggesting that the functional interaction of dynein and p150(Glued) may not require the entire dynactin complex. An alternative model might be that chlamydial proteins may stabilize the interactions of the cargo-binding and dynein-binding subunits of dynactin to the extent that it is no longer susceptible to dissociation by overexpression of dynamitin. A detailed characterization of the components of dynein-dynactin complex associated with chlamydial inclusions and the chlamydial proteins involved will be required to distinguish between these models. In either case, the novel interactions of the chlamydial inclusion with the dynactin complex may reveal some interesting biology of the early events in chlamydial development but may also help elucidate mechanisms by which the dynactin complex and dynein interact.

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References

Alonso, C., Miskin, J., Hernaez, B., Fernandez-Zapatero, P., Soto, L., Canto, C., Rodriguez-Cresco, I., Dixon, L. and Escrivan, J. M. (2001). African swine fever virus protein p54 interacts with the microtubular motor complex through direct binding to light-chain dynamin. J. Virol. 75, 9819-9827.

Boleti, H., Ojcius, D. M. and Dautry-Varsat, A. (2000). Fluorescent labelling of intracellular bacteria in living host cells. J. Microbiol. Methods 40, 265-274.

Burkhardt, J. K., Echeverri, C. J., Nilsson, T. and Valee, R. B. (1997). Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. J. Cell Biol. 139, 469-484.

Caldwell, H. D., Kromhout, J. and Schachter, J. (1981). Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis. Infect. Immun. 31, 1161-1176.

Carabeo, R. A., Grieshaber, S. S., Fischer, E. and Hackstadt, T. (2002). Chlamydia trachomatis induces remodeling of the actin cytoskeleton during attachment and entry into HeLa cells. Infect. Immun. 70, 3793-3803.

Clausen, J. D., Christiansen, G., Holst, H. U. and Birkelund, S. (1997).
Chlamydia trachomatis utilizes the host cell microtubule network during early events of infection. Mol. Microbiol. 25, 441-449.

Dohner, K., Wolfstein, A., Prank, U., Echeverri, C., Dujardin, D., Vallee, R. and Sodeik, B. (2002). Function of Dynexin and dynactin in herpes simplex virus capsid transport. Mol. Biol. Cell 13, 2795-2809.

Echeverri, C. J., Paschal, B. M., Vaughan, K. T. and Vallee, R. B. (1996). Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. J. Cell Biol. 132, 617-633.

Escalante-Ochoa, C., Ducattele, R. and Haesebroeck, F. (2000). Optimal development of Chlamydiophila psittaci in L929 fibroblast and BGM epithelial cells requires the participation of microfilaments and microtubule-motor proteins. Microb. Pathog. 28, 321-333.

Friis, R. R. (1972). Interaction of L cells and Chlamydia psittaci: entry of the parasite and host responses to its development. J. Bacteriol. 110, 706-721.

García-Mata, R., Bebok, Z., Sorscher, E. J. and Sztul, E. S. Friis, R. R. Heinzen, R. A., Grieshaber, S. S., Van Kirk, L. S. and Devin, C. J. Hackstadt, T., Rockey, D. D., Heinzen, R. A. and Scidmore, M. A. Hackstadt, T., Scidmore, M. A. and Rockey, D. D. Higashi, N. Helfand, B. T., Mikami, A., Vallee, R. B. and Goldman, R. D. Leopold, P. L., Kreitzer, G., Miyazawa, N., Rempel, S., Pfister, K. K., Tokito, M., Van, W. T., Howland, D. S. and Holzbaur, E. L. Tokito, M., Van, W. T., Howland, D. S. and Holzbaur, E. L. Waterman-Storer, C. M., Karki, S. B., Kuznetsov, S. A., Tabb, J. S., Weiss, M. S., Moreau, V., Ashman, K., Reckmann, I., Gonzalez, C. and Way, M. (2000). Vaccinia virus infection disrupts microtubule organization and centrosome function. EMBO J. 19, 3932-3944.

Palazzo, A. F., Joseph, H. L., Chen, Y. J., Dujardin, D. L., Alberts, A. S., Pfister, K. K., Vallee, R. B. and Gundersen, G. G. (2001). Cdc42, dynexin, and dynactin regulate MTOC reorientation independent of Rho-regulated microtubule stabilization. Curr. Biol. 11, 1536-1541.

Plouidou, A., Moreau, V., Ashman, K., Reckmann, I., Gonzalez, C. and Way, M. (2000). Vaccinia virus infection disrupts microtubule organization and centrosome function. EMBO J. 19, 3932-3944.

Rockey, D. D., Greenbach, D., Hruby, D. E., Peacock, M. G., Heinzen, R. A. and Hackstadt, T. (1997). Chlamydia psittaci IncA is phosphorylated by the host cell and is exposed on the cytoplasmic face of the developing inclusion. Microbiol. 24, 217-228.

Schachter, J. (1999). Infection and disease epidemiology. In Chlamydia, Intracellular Biology, Pathogenesis, and Immunity (ed. Stephens, R. S.), pp. 101-138. Washington, DC: ASM Press.

Schroer, T. A. (2000). Motors, clutches and brakes for membrane traffic: a commemorative review in honor of Thomas Kreis. Traffic. 1, 3-10.

Scidmore, M. A. and Hackstadt, T. (2001). Mammalian 14-3-3beta associates with the Chlamydia trachomatis inclusion membrane via its interaction with IncG. Mol. Microbiol. 39, 1638-1650.

Scidmore, M. A., Rockey, D. D., Fischer, E. R., Heinzen, R. A. and Hackstadt, T. (1996). Vesicular interactions of the Chlamydia trachomatis inclusion are determined by Chlamydial early protein synthesis rather than route of entry. Infect. Immun. 64, 5366-5372.

Scidmore, M. A., Fischer, E. R. and Hackstadt, T. (2003). Restricted fusion of Chlamydia trachomatis vesicles with endocytic compartments during the initial stages of infection. Infect. Immun. 71, 973-984.

Scidmore-Carlson, M. A., Shaw, E. I., Dooley, C. A., Fischer, E. R. and Hackstadt, T. (1999). Identification and characterization of a Chlamydia trachomatis early operon encoding four novel inclusion membrane proteins. Mol. Microbiol. 33, 73-80.

Toomre, D., Keller, P., White, J., Olivo, J. C. and Simons, K. (1999). Dual-color visualization of trans-Golgi network to plasma membrane traffic along microtubules in living cells. J. Cell Sci. 112, 21-33.

Valetti, C., Wetzel, D. M., Schrader, M., Hasbani, M. J., Gill, S. R., Kreis, T. E. and Schroer, T. A. (1999). Role of dynactin in endocytic traffic: effects of dynamin overexpression and colocalization with CLIP-170. Mol. Biol. Cell 10, 4107-4120.

Vaughn, K. T., Tynan, S. H., Faulkner, N. E., Echeverri, C. J. and Vallee, R. B. (1999). Colocalization of cytoplasmic dynein with dynactin and CLIP-170 at microtubule distal ends. J. Cell Sci. 112, 1437-1447.

Waterman-Storer, C. M., Karki, S. B., Kuznetsov, S. A., Tabb, J. S., Weiss, D. G., Langford, G. M. and Holzbaur, E. L. (1997). The interaction between cytoplasmic dynein and dynactin is required for fast axonal transport. Proc. Natl. Acad. Sci. 112, 12180-12185.

Wittmann, T. and Hyman, T. (1999). Recombinant p50/dynamitin as a tool to examine the role of dynactin in intracellular processes. Methods Cell Biol. 61, 137-143.

Ye, G. J., Vaughan, K. T., Vallee, R. B. and Rizkman, B. (2000). The herpes simplex virus 1 UL34 protein interacts with a cytoplasmic dynein intermediate chain and targets nuclear membrane. J. Virol. 74, 1355-1363.