Chlamydia infection of epithelial cells expressing dynamin and Eps15 mutants: clathrin-independent entry into cells and dynamin-dependent productive growth

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SUMMARY

Chlamydiae enter epithelial cells via a mechanism that still remains to be fully elucidated. In this study we investigated the pathway of entry of C. psittaci GPIC and C. trachomatis LGV/L2 into HeLa cells and demonstrated that it does not depend on clathrin coated vesicle formation. We used mutant cell lines defective in clathrin-mediated endocytosis due to overexpression of dominant negative mutants of either dynamin I or Eps15 proteins. When clathrin-dependent endocytosis was inhibited by overexpression of the dynK44A mutant of dynamin I (defective in GTPase activity), Chlamydia entry was not affected. However, in these cells there was a dramatic inhibition in the proliferation of Chlamydia and the growth of the chlamydia vacuole (inclusion). When clathrin-dependent endocytosis was inhibited by overexpression of an Eps15 dominant negative mutant, the entry and growth of Chlamydia was unaltered. These results indicate that the effect on the growth of Chlamydia in the dynK44A cells was not simply due to a deprivation of nutrients taken up by endocytosis. Instead, the dominant-negative mutant of dynamin most likely affects the vesicular traffic between the Chlamydia inclusion and intracellular membrane compartments. In addition, cytochalasin D inhibited Chlamydia entry by more than 90%, indicating that chlamydiae enter epithelial cells by an actin-dependent mechanism resembling phagocytosis. Finally, dynamin is apparently not involved in the formation of phagocytic vesicles containing Chlamydia.

Key words: Chlamydia, Dynamin, Eps15, Endocytosis, Vesicular traffic, Phagocytosis

INTRODUCTION

Chlamydia are obligate intracellular eubacteria that are major human pathogens world wide and have been implicated in a wide spectrum of diseases in humans, other mammals, and birds (for recent reviews see Stephens, 1993; Bavoil et al., 1996). In humans, they are the leading cause of sexually transmitted bacterial diseases in the western world and they are the main cause of noncongenital blindness in developing countries (Schachter and Dawson, 1990). The pathogen exists in two distinct morphological forms: the replicative, intracellular reticulate body (RB, 1 μm in diameter), and the infectious but metabolically inactive elementary body (EB, 0.3 μm in diameter). Infection is initiated by adherence of EBs to the host cells through an unknown receptor that probably binds with a bacterially derived heparan sulfate-like glycosaminoglycan present on the chlamydial surface (Kuo et al., 1973; Zhang and Stephens, 1992; Gutiérrez-Martin et al., 1997). Several other ligands for Chlamydia binding have also been proposed, including hsp70 and omp2 (Joseph and Bose, 1991; Swanson and Kuo, 1994; Ting et al., 1995; Su et al., 1996; Raulston et al., 1998). After binding, the Chlamydia are internalized, enveloped within membrane-bound compartments that are subjected to bacteria induced modifications both in their luminal environment and in their membrane composition, and transported to a perinuclear location. These modified Chlamydia-containing membrane compartments are termed inclusions. Although the mechanism of internalization remains controversial (reviewed by Moulder, 1991; Bavoil et al., 1996) the bacteria appear to survive within the inclusion in host epithelial cells through their ability to inhibit fusion between the entry inclusions and lysosomes (Eissenberg et al., 1983; Scidmore et al., 1996b). Within the inclusions, the EBs differentiate into RBs which proliferate to give rise within 24 hours to large inclusions often reaching a size larger than that of the nucleus. Between 24 and 72 hours post-infection the RBs redifferentiate into EBs and are released into the extracellular space to start a new round of infection.

It is not clear whether Chlamydia enter host cells by means of actin-dependent phagocytosis or clathrin-dependent endocytosis or indeed whether both pathways operate (Bavoil et al., 1996). Part of the confusion may be due to the
possibility that Chlamydia may use either of these mechanisms of entry in different host cells, or different chlamydial strains may use different modes of entry (reviewed by Moulder, 1991; Raulston, 1995; Bavoil et al., 1996). Moreover, contradictory results may be due to limitations of techniques used in the past.

Several molecules have been recently identified to play essential roles in endocytic processes amongst which is the large GTPase dynamin. It is a member of a structurally related, functionally heterogeneous family of GTPases that exhibits a diverse array of functional properties in vitro (reviewed by Damke, 1996; Urrutia et al., 1997). Studies using either the temperature sensitive mutant of fruit fly shibire, either the temperature sensitive mutant of fruit fly using either the temperature sensitive mutant of fruit fly (reviewed by Damke, 1996; Urrutia et al., 1997). Studies using either the temperature sensitive mutant of fruit fly shibire (Chen et al., 1991; van der Bieec and Meyerowitz, 1991), which possesses a point mutation near the GTP-binding domain of dynamin, or overexpression of the dynK44A mutant (Damke et al., 1994) in mammalian epithelial cells, have shown that dynamin participates in a clathrin-based endocytic process by severing clathrin-coated invaginations from the plasma membrane. Recently, dynamin was also localized to the Golgi complex of mammalian cells by biochemical, immunological and morphological techniques (Henley and McNiven, 1996; Maier et al., 1996) and was shown to participate in vesicle trafficking to and from the Golgi apparatus (Jones et al., 1998; Llorente et al., 1998) and in the liberation of caveolae (Henley et al., 1998; Oh et al., 1998). Finally, localization studies of the various dynamin molecules and their spliced variants further support the view that different dynamins function at different cellular sites (Cao et al., 1998).

The Eps15 protein is a newly identified constituent of plasma membrane clathrin-coated pits that is ubiquitously and constitutively associated with AP-2 clathrin adaptor protein complex within coated pits (Benmerah et al., 1995; Tebar et al., 1996). Inhibition of the AP2/Eps15 interaction inhibits endocytosis both in vivo and in vitro, showing that Eps15 is required for the early steps of clathrin-mediated endocytosis (Benmerah et al., 1998; A. Benmerah et al., unpublished). Eps15 is a highly conserved protein organized in three distinct structural domains (Fazioli et al., 1993; Wong et al., 1994). Its N-terminal domain (DI) is composed of three imperfect repeats, the Eps15 homology (EH) domains; a central domain (DII) which is involved in the oligomerization of Eps15 (Cupers et al., 1997; Tebar et al., 1997); and a C-terminal domain (DIII) which contains the AP-2 binding site (Benmerah et al., 1996; Iannolo et al., 1997). Overexpression of a dominant negative mutant of Eps15 consisting of its entire C-terminal domain fused to the Green Fluorescent Protein (GFP) strongly inhibited endocytosis of transferrin (Benmerah et al., 1998), demonstrating that interaction between Eps15 and AP-2 is required for efficient receptor-mediated clathrin-dependent endocytosis.

We studied the entry and growth of Chlamydia in a stable cell line in which a dynamin molecule defective in GTP binding and hydrolysis (dynK44A) (Damke et al., 1994) was overexpressed. This molecule inhibits clathrin-mediated endocytosis, but is probably also involved in other intracellular vesicular traffic events. We extended these studies in transiently transfected HeLa cells that overexpress the GFP-EA 95/295 Eps15 mutant (Benmerah et al., 1999), which inhibits specifically clathrin-dependent endocytosis. Our results demonstrate that the entry of C. psittaci GPIC or C. trachomatis LGV/L2 into epithelial cells is not a clathrin-dependent process. We also demonstrate that dynamin plays a role at a later step of Chlamydia intracellular growth. We conclude that the entry of Chlamydia into cervical epithelial cells is an actin-dependent mechanism resembling phagocytosis.

MATERIALS AND METHODS

Antibodies and other reagents

The FITC-labeled anti-Chlamydia mAb was purchased from Argene, BIOSOFT; the mAb against the human transferrin receptor (TIR) Okt9 (IgGI) was from ATCC; the mAb against the influenza hemagglutinin tag (HA) 12CA5 was from R. A. Lerner’s laboratory and was a kind gift from Dr S. Schmid (The Scripps Research Institute, La Jolla, CA). Texas Red-conjugated goat anti-mouse and Texas Red-conjugated goat anti-rabbit Abs were from Molecular Probes (Interchim, France); FITC rabbit anti-mouse Ab was from DAKO (A/S Denmark); and the phycoerythrin-conjugated goat anti-mouse Ab was from Immunotech (Marseille, France). Mowiol was from Calbiochem (La Jolla, CA, USA). Texas Red Tf was prepared according to the protocol recommended by the manufacturer (Molecular Probes) while FITC Tf was prepared as described previously (Moya et al., 1985). Cytochalasin D was obtained from Sigma (St Louis, MO).

Generation of polyclonal antisera against C. psittaci IncA protein

To generate polyclonal antisera, the open reading frame of the incA gene was amplified by PCR from C. psittaci genomic DNA. Chlamydia genomic DNA was prepared from purified bacteria using the RapidPrep Micro genomic DNA isolation Kit (Pharmacia Biotech). The oligonucleotides used in the PCR reaction for cloning of the IncA cDNA were the following: 5’ EcoRI IncA: CCGGAA TTCA TGACAGTA TCCACAGACAACAC and 3’SpeI IncA: CTCTCGTCGACTTAACTA TCTTTA TGCTCACC.

The IncA cDNA was then subcloned into the pMalC2 and pGEX-4T-1 vectors, allowing the expression of the IncA protein as a fusion protein with the maltose binding protein or glutathione transferase, respectively. The maltose binding fusion protein was purified and injected into rabbits for generation of polyclonal antisera against C. psittaci IncA and the glutathione transferase fusion protein was used to affinity-purify the anti-IncA antisera.

Cells and Chlamydia strains

The HeLa cells were from ATCC. The guinea pig inclusion conjunctivitis (GPIC) serovar of C. psittaci was obtained from Roger Rank (University of Arkansas) while the C. trachomatis strain of Lymphogranuloma venereum (LGV)/L2 strain was obtained from ATCC. Chlamydiae were prepared from infected cells by a modification of a method described earlier (Gutiérrez-Martin et al., 1997). Briefly, the Chlamydiae were propagated in HeLa cells grown in DMEM-G (DMEM, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 4.5 g/l glucose) for 48 hours. For routine preparation of bacteria, about thirty (10 cm diameter) tissue culture dishes were infected and the cells were harvested at 48 hours post-infection. The supernatant and the cells were collected and centrifuged (12,000 rpm, 60 minutes, in a Sorval type GSA rotor). The pellet containing the bacteria and the cells was resuspended in 10 ml ice-cold SPG (218 mM sucrose, 3.76 mM KH2PO4, 7.1 mM K2HPO4, 4.9 mM glutamate, pH 7.4) (Bovarnick et al., 1950). The cells were broken by passing the pellet through a 22 G needle and the resulting suspension was centrifuged (10 minutes, 2000 rpm, Sorval SS34 rotor) to remove unbroken cells and nuclei. The new supernatant was
centrifuged in the same rotor (15,000 rpm, 30 minutes, 4°C) to collect the bacteria. The bacteria pellet was subsequently homogenized, resuspended in ice-cold SPG, aliquoted and frozen at −80°C. Depending on the preparation, between 25 and 50% of the bacteria were infectious.

Infections of HeLa cells by Chlamydia

Cells were incubated with C. psittaci GPIC or C. trachomatis LGV/L2 diluted in SPG/DMEM-G (1:1) at a density resulting in 30-80% infected cells. After a 1.5 hour adhesion step, the bacteria were removed from the supernatant, the cells were washed once with DMEM-G, fresh medium was added, and incubations continued for 24 up to 48 hours post-infection at 37°C.

The effect of cytochalasin D (1 µg/ml) was studied at various times post-infection. The drug was dissolved in the culture medium and was added to the cultures during the adhesion step of the bacteria (time ‘0’ study) and for the whole length of the infection period or after the adhesion step at the indicated time points. When the effect of the drug was studied at time ‘0’ of the infection, the cells were pretreated with the drug (30 minutes, 37°C) before adding the bacteria.

Infection of dynamin mutant cells.

The HeLa cell line, tTA-HeLa (Gossen and Bujard, 1992) stably transformed with the cDNAs for dyn WT and dyn K44A dynamin (Damke et al., 1994), was maintained in DMEM-G medium supplemented with antibiotics (400 µg/ml G418, 4 µg/ml tetracycline, 200 µg/ml puromycin and 20 µg/ml gentamycin). Cultures were only used for up to two months after which they were replaced by freshly thawed cells. For infection with Chlamydia, dyn WT and dyn K44A cells were cultured for two days in the presence of 0.4 µg/ml tetracycline, and then for 48 hours in the complete absence of tetracycline. For the experiments, the cultures were maintained at less than 80% confluency. Infection was performed 48 hours post-induction and the infected cells were analysed either at 5 hours post-infection to assess internalization or at 24 hours post-infection to assess productive infection.

Transfection of cells with the GFP-Eps15 constructs

The plasmids used for the expression of GFP or the GFP-EA 95/295 Eps15 mutant were described previously (Benmerah et al., 1998, 1999). Transfections of subconfluent HeLa cells were performed using the CalPhos Maximizer Transfection Kit from CLONTECH (Palo Alto, CA). For transfections, the cells were plated in 6-well (3.5 cm diameter) dishes on 10 mm coverslips. Infection of transfected cells with Chlamydia was done 24 hours post-transfection. The expression of GFP or GFP-EA 95/295 Eps15 was assessed either by fluorescence microscopy using the FITC filter or by cytofluorimetry.

Measurements of endocytosis

Endocytosis of transferrin (Tf) was assessed as follows: cells growing on coverslips were incubated in DMEM supplemented with 20 mM Hepes, pH 7.2 at 37°C for 30 minutes, to chase receptor-bound Tf. Subsequently, they were incubated (15 minutes, 37°C) in the same medium (100 µl/coverslip) containing 1 mg/ml BSA and 0.1 µM FITC- or Texas Red-conjugated Tf. Endocytosis was stopped by cooling on ice and washing with the same medium at 0°C. Cells were washed further with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (30 minutes, 4°C) and processed for immunofluorescence.

When Tf endocytosis was analysed by cytofluorimetry, the cells were detached from the tissue culture dishes by PBS-EDTA (5 minutes, 37°C), centrifuged (1200 rpm, 5 minutes) and resuspended in 200 µl DMEM, 20 mM Hepes pH 7.2, 1 mg/ml BSA, containing FITC-conjugated Tf (0.1 µM). As above, TF was internalised for 15 minutes (37°C) and endocytosis was stopped by cooling on ice. The cells were subsequently washed with the same medium and PBS at 0°C. They were subsequently fixed and labelled for plasma membrane or intracellular antigens.

**Immunofluorescence microscopy**

For immunofluorescence analysis, cells were fixed with 4% paraformaldehyde, incubated with Abs and mounted as previously described (Ojcius et al., 1996). For labelling of plasma membrane markers the antibodies were diluted in PBS containing 2 mg/ml BSA while for labelling of internal antigens the antibodies were diluted in permeabilization buffer (PBS, 2 mg/ml BSA, 0.05% saponin). The DNA was visualized by staining with the Hoechst dye (5 µg/ml, 5 minutes, at room temperature). When intracellular antigens were followed the cells were washed with PBS containing 0.05% saponin. The samples were examined under an epifluorescence microscope (Axioquant, Zeiss, Germany) attached to a cooled CCD-camera (Photometrics, Tucson, AZ), using ×63 Neofluar or ×63 Apochromat lenses. Images were acquired using the IPLab software and analysed by the NIH or Adobe Photoshop software. For analysis of the size of Chlamydia inclusions the area of each inclusion was measured as square pixels and square pixels were converted to µm by measuring the area of images of fluorescent latex beads of 0.5 µm diameter (Latex Fluospheres, Molecular Probes).

For quantitative analysis of infection, bacteria or inclusions were counted in each field. Data were combined for each experiment and the results were presented as an average from the different experiments ± the standard deviations.

**Cytotr fluorimetry**

Cells were harvested by detaching with PBS/EDTA, washed once with PBS and fixed with 4% paraformaldehyde (30 minutes, room temperature). Excess paraformaldehyde was neutralised with 50 mM NH4Cl (10 minutes, room temperature), the cells were washed with PBS and subsequently stained with antibodies directed against either plasma membrane or intracellular antigens. For labelling of internal antigens, cells were permeabilized with saponin (0.05% in PBS). When cells were double labelled for a surface marker and an internal antigen, labelling of the plasma membrane marker was performed first in non-permeabilized cells and subsequently the internal antigen was visualised after permeabilization. Analysis of the fluorescence was performed with a Becton Dickinson FACScan instrument. At least 10,000 cells were analysed for each sample.

**RESULTS**

**Overexpression of dyn K44A dynamin mutant inhibits infection of HeLa cells by C. psittaci**

The pathway of entry of Chlamydia into host cells still remains controversial. Several early studies implicated clathrin in the entry or the intracellular redistribution of C. psittaci GPIC and C. trachomatis LGV into their host cells (Prain and Pearce, 1989; Wyrick et al., 1989; Reynolds and Pearce, 1990), while others found no role for clathrin (Byrne and Moulder, 1978; Ward and Murray, 1984; Prain and Pearce, 1989). Part of the disagreement may be due to the methods used (Moulder, 1991; Bavoil et al., 1996). Besides the pharmacological methods and morphological ultrastructure studies applied thus far, new tools have been developed to approach these questions. We took an alternative approach to understand the mechanism of Chlamydia entry into cells using a stable cell line where the overexpression of dyn K44A mutant dynamin (a dynamin molecule defective in GTPase activity) (Damke et al., 1994) strongly inhibits clathrin-mediated endocytosis. As the dyn K44A dynamin is not homogeneously

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membrane expression. Dyn K44A and dyn WT HeLa cells were
induced for 48 hours for the overexpression of the dominant negative dynamin mutant, the infection by C. psittaci GPIC was inhibited by about 45% (Fig. 2C). Infection was assessed at 22-24 hours post-infection, a time point at which wild-type cells had formed large Chlamydia inclusions. Infection in a given cell was determined by immunofluorescence with antibodies against Chlamydia surface antigens, and a cell was considered infected when the inclusion area was equal to or larger than approximately 1 µm² (size of smallest inclusions found in dynWT infected cells). When the infection was assessed in individual cells with high levels of staining for plasma membrane TfR, it was found to be inhibited by about 60% with respect to the infection observed in the dynWT cells (Fig. 2C). As the % of infected cells varied from experiment to experiment and with respect to the bacterial preparation, the data were normalized with respect to the control, i.e. the % of the infected dynWT cells. Similar data were obtained when the infected cells were analyzed by cytofluorimetry (data not shown). Similarly, when the level of infection was correlated to the level of TfR staining on the plasma membrane by cytofluorimetry, we found that the population of the cells infected was reduced to about 30% (data not shown). In other words, cells expressing the highest levels of the dynK44A mutant were the least likely to be productively infected by C. psittaci GPIC.

**Overexpression of dynK44A dynamin mutant does not affect C. psittaci internalization**

Since dynamin is involved in the fission of clathrin-coated vesicles from the plasma membrane, the decrease in Chlamydia infection could be due to reduced entry of the bacteria. We therefore analyzed the internalization of C. psittaci GPIC at 5 hours post-infection in dynK44A cells as compared to dynWT cells as described earlier (Ojcius et al., 1998). HeLa cells were incubated with bacteria at a multiplicity of infection of 0.15. Unbound bacteria were washed away and the host cells were fixed. The bacteria that were bound to the cells but not internalized were revealed by incubating the cells with an FITC-labelled anti-Chlamydia mAb without permeabilization, followed by incubation with a Texas Red-conjugated secondary Ab. Internalized bacteria were subsequently revealed by permeabilizing the cells and incubating them with the FITC-labelled anti-Chlamydia mAb. Thus, internalized bacteria appeared green while the external bacteria appeared yellow (green and red). Fig. 3 shows the % of Chlamydia internalized by the dynWT and the dynK44A cells 5 hours post-infection. No significant difference was observed in the internalization efficiency of C. psittaci GPIC by both cell lines. As a control, when the adherence step was performed at 0°C, no bacteria were detected inside the cells. The above results suggested that overexpression of the dynamin dominant

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**Fig. 1.** Effects of dynK44A mutant overexpression on TfR plasma membrane expression. DynK44A and dynWT HeLa cells were examined by cytofluorimetry for the accumulation of TfR on their plasma membrane by staining fixed, non-permeabilized cells with the anti-TfR monoclonal antibody OKT-9 followed by a secondary anti-mouse antibody conjugated to phycoerythrin (as described in Materials and Methods). (A) Representative histogram from cytofluorimetry of dynK44A induced cells (bold line) and dynK44A non-induced cells (normal line). P1, the population of dynK44A cells with high levels of TfR on their plasma membrane representing the population of dynK44A cells overexpressing the dynamin mutant. The same population P1 was plotted in B. (B) NI, non-induced cells; IN, induced cells. Results from five separate experiments. Error bars represent standard deviations.

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expressed within the population of these cells (Damke et al., 1995) we first assessed the clathrin-dependent endocytic activity by analyzing the levels of transferrin receptor (TfR) at the plasma membrane by immunofluorescence with antibodies against TfR. When the dynK44A mutant is expressed at high levels, endocytosis of TfR is inhibited (Damke et al., 1994) and thus its accumulation at the plasma membrane can be used as a criterion to assess the level of inhibition of clathrin-mediated endocytosis. The levels of TfR on the plasma membrane were quantitated by cytofluorimetry. About 55-70% of the dynK44A cells expressing the mutant dynamin show an accumulation of TfR on their plasma membrane (Fig. 1A,B). The levels of surface labelling of TfR for the induced dynWT cells were similar to those in the non-induced cultures growing in tetracycline (Fig. 1B).
negative mutant did not inhibit entry of *C. psittaci* GPIC into HeLa cells.

**Overexpression of dynK44A dynamin mutant inhibited the growth of the *C. trachomatis* inclusion**

Infection of the dynK44A cells expressing the dominant negative dynamin mutant with the *C. trachomatis* LGV/L2 strain did not show major differences in terms of the % of cells which were found infected by immunofluorescence. Fig. 4 shows that the infected dynK44A cells were infected only 25±5% less efficiently than dyn WT cells (Fig. 4A). However, a more careful analysis of the immunofluorescence images (Fig. 4C) of the infected cells clearly showed that the *C. trachomatis* LGV/L2 inclusions were significantly smaller than the inclusions observed in the dyn WT cells (Fig. 4B). Thus, the dynamin dominant negative mutant dynK44A has a clear effect on the growth of *C. trachomatis* LGV although this effect is not as dramatic as for the *C. psittaci* GPIC strain. However, we cannot exclude the possibility that the entry of *C. trachomatis* LGV may be inhibited in the dynamin mutant cells, and that the smaller size of the vacuole could therefore be due to delayed entry.

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**Fig. 2.** Overexpression of dynK44A dynamin mutant inhibits infection of HeLa cells by *C. psittaci*. DynK44A and dyn WT HeLa cells were induced to overexpress the mutant or wild-type dynamin for 48 hours, then infected with *C. psittaci* GPIC (MOI of 0.3-0.8), stained 24 hours post-infection with the FITC-conjugated monoclonal antibody against *Chlamydia*, and analysed for the presence of inclusions by immunofluorescence. The TIR on the plasma membrane was stained with the OKT-9 anti-TIR antibody followed by a Texas Red-conjugated anti-mouse antibody and the % of dynK44A cells overexpressing the mutant dynamin was evaluated by counting the cells with strong plasma membrane staining for TIR. (A,B). Immunofluorescence images of dynK44A cells infected for 24 hours with *C. psittaci* GPIC. (A) A cell with high levels of TIR (green pseudocolor, strong staining) on the plasma membrane. (B) A cell with low levels of TIR (green pseudocolor, weak staining). Red pseudocolor, the *C. psittaci* GPIC staining. (C) Quantitation of infection efficiency by immunofluorescence. The results for the infection of the dynK44A cells were normalized to the infection of the dyn WT cells. Black bar, dyn WT infected cells; white bar, dynK44A infected cells; hatched bar, the population of dynK44A infected cells with high levels of TIR on their plasma membrane. More than 160 cells were counted for each determination. For the total dynK44A cell population (white bar), data from six different experiments were compiled, while for the dynK44A cell population with high levels of TIR on the plasma membrane (hatched bar), data from three different experiments were compiled. Error bars represent standard deviations.

**Fig. 3.** Expression of dynK44A dynamin mutant has no effect on the internalization of *C. psittaci* by HeLa cells. Internalization of *Chlamydia* by Hela dynK44A (white bar) and dyn WT (black bar) cells was assessed by incubating the cells with *C. psittaci* GPIC (approximately 6-9 bound bacteria/cell) for 1.5 hours at room temperature, washing the unbound bacteria and incubating the cells at 37°C for another 3.5 hours (total length of infection, 5 hours). The population of non-internalized bacteria was differentiated from the internalized bacteria as described in Materials and Methods. The hatched bar represents the % of bacteria internalized by HeLa dynK44A or dyn WT cells when the adherence step was performed on ice and the cells were subsequently washed, fixed and analyzed for surface bound or internalized bacteria (results from 3 separate determinations in which a total number of 354 cells were analyzed). The results for internalization at 37°C are from two separate experiments where a total number of 360 cells were analyzed. Error bars represent standard deviations.
Overexpression of an Eps15 dominant negative mutant had no effect on *Chlamydia* intracellular growth

Recent studies have demonstrated that iron restriction causes a significant reduction in infectivity of *C. trachomatis* in an in vitro model of human genital infection using the intracellular iron-chelating agent desferoxamine mesylate (Desferal) (Raulston, 1997). To investigate the possibility that the reduced infectivity of *Chlamydia* in the HeLa DynK44A cells was not due to reduced iron or nutrient uptake or even inhibition of lipid recycling due to the reduced endocytic activity in these cells, we studied the infection of *C. psittaci* in HeLa cells transiently transected with a construct encoding as a GFP fusion protein the Eps15 deletion mutant (EΔ95/295) lacking the second and third EH domains. This dominant negative mutant of Eps15 inhibits clathrin-dependent endocytosis (A. Benmerah et al., 1999). As expected, almost all cells expressing the GFP-EΔ95/295 Eps15 protein accumulated TfR on their surface because its endocytosis was inhibited (data not shown). In addition, about 80% of cells expressing the GFP-EΔ95/295 mutant showed reduced Tf endocytosis while for the mock-transfected cells with constructs encoding GFP alone, this population was about 20% (Fig. 5D). We observed no significant differences in the infection efficiency of *C. psittaci* in transiently transected HeLa cells overexpressing the Eps15 deleted mutant as compared to the infection in cells transiently mock-transfected with constructs encoding the GFP protein (Fig. 5C). Large size *Chlamydia* inclusions were observed in the infected cells expressing the GFP-EΔ95/295 Eps15 mutant, similar to inclusions observed in control (mock transfected) cells (Fig. 5A,B). These results confirm that *Chlamydia* do not enter cells via clathrin-coated pits and further suggest that the inhibition of *Chlamydia* productive infection in the HeLa dynK44A cells was not due to iron or nutrient deficiency.

**C. psittaci** enter HeLa cells by an actin-dependent process

As clathrin-mediated endocytosis was not involved in *Chlamydia* entry we evaluated whether entry of *C. psittaci* GPIC and *C. trachomatis* LGV/L2 could be actin-dependent. HeLa cells were treated with cytochalasin D (1 μg/ml) 30 minutes before infection (37°C) and the drug was left in the incubation medium during the infection time (or added every hour for 4 hours). Infection was performed at a multiplicity of approximately 0.5 and the % of infected cells was assessed 24 hours post-infection by looking for the presence of *Chlamydia* inclusions in drug treated and untreated cells. When cells were treated with cytochalasin D, the population of infected cells, assessed as cells containing inclusions 24 hours post-infection, was reduced by 90% with respect to untreated cells. Similar results were observed when the drug was added at 1 hour post-infection, right after the bacteria adhesion step. However, the drug had a diminished effect when it was added later, indicating that it acts at the internalization step (Fig. 6). The internalization of *Chlamydia* was quantitated as described in
DISCUSSION

We readdressed the issue of whether *Chlamydia* uses clathrin-coated pits to enter cells by using HeLa cells that can be induced to express a mutant form (dynK44A) or the wild-type (dynWT) of dynamin. The expression of dominant-negative dynamin mutants defective in GTPase activity (dynK44A) inhibits the formation of clathrin-coated vesicles (Damke et al., 1996; Taraska et al., 1996) and suggests that chlamydiae do not use clathrin-coated vesicles to enter these cells. Nonetheless, the overexpression of the dynK44A mutant had a strong effect on the productive growth of *C. psittaci* and a partial effect on the productive growth of *C. trachomatis*. The fact that *C. psittaci* could grow in cells expressing the Eps15 mutant indicates that the inhibitory effect observed in the dynK44A cells is not due to a deprivation of nutrients (i.e. iron, lipids) resulting from the reduced endocytic capacity of these cells, but that the dynK44A mutant acts at a later step of *Chlamydia* intracellular life.

The events following the entry of *Chlamydia* into host cells are not very well understood and the origin and composition of the *Chlamydia* inclusion membrane still needs to be fully defined. Work from the Engel laboratory (Van Ooij et al., 1997) suggests that the chlamydial vacuole interacts with the endocytic pathway of the host but is a unique and dynamic organelle that shares several characteristics with recycling endosomes (Mukherjee et al., 1997), which would therefore provide a source of membrane or nutrients for the replicating organisms. Work from other teams has failed to localise markers of the endocytic compartment in the inclusion (Heinzen et al., 1996; Taraska et al., 1996) and suggests that for both *C. psittaci* and *C. trachomatis*, the late inclusion (Taraska et al., 1996) interrupts an exocytic pathway from the trans-Golgi to the plasma membrane (Hackstadt et al., 1995, 1996; Scidmore et al., 1996a). It is clear, though, that soon after their entry into the host cell, chlamydiae express factors which modify the inclusion membrane (Rockey et al., 1995;...
cells (drug-free cultures) analysed in parallel in each experiment. The efficiency of infection was normalized to the infection of control experiment. The error bars represent standard deviations separate experiments in which 50 to 600 cells were counted per antibody as described in Materials and Methods. Results from three bacteria were visualized by the FITC-conjugated anti-

hours post-infection as described in previous figures. Internalized addition apparently represents differences in the metabolism of structures characteristic of clathrin coated pits in chlamydia

Fig. 6 Effect of cytochalasin D on the infection of HeLa cells by C. psittaci GPIC. HeLa cells were infected with C. psittaci at an MOI of approximately 0.3-0.8, in the presence or absence of the drug. At the end of the binding step (see Materials and Methods) the bacteria in suspension were removed, the cells were washed and further incubations were carried out at 37°C. To assess the effect of cytochalasin D at time zero of infection, cells were incubated with (1 µg/ml) cytochalasin D for 30 minutes at 37°C prior to the addition of C. psittaci. For the rest of the conditions tested, the drug was added at the indicated time of infection and was left until the end of the incubation. Cells were analyzed by immunofluorescence at 24 hours post-infection as described in previous figures. Internalized bacteria were visualized by the FITC-conjugated anti-Chlamydia antibody as described in Materials and Methods. Results from three separate experiments in which 50 to 600 cells were counted per experiment. The error bars represent standard deviations. The efficiency of infection was normalized to the infection of control cells (drug-free cultures) analysed in parallel in each experiment.

Scidmore et al., 1996b) and inhibit fusion of the early inclusion vesicle with host cell lysosomes. Recent studies have consistently demonstrated that the inclusion obtains sphingolipids from the Golgi apparatus. Trafficking of Golgi-derived sphingolipids to the chlamydial inclusion has been demonstrated (Hackstadt et al., 1995, 1996) and in addition Chlamydia receives host cell derived glycerophospholipids (PE, PG, PS and cholesterol) (Wylie et al., 1997), suggesting that the inclusion interacts with other intracellular compartments besides the Golgi.

Brefeldin A, an inhibitor of anterograde vesicular traffic from the Golgi apparatus (Misumi et al., 1986; Klausner et al., 1992), inhibits transport of the fluorescent (ceramide) lipid probe to the inclusion (Hackstadt et al., 1995) and influences the morphology of the inclusion. Chlamydia inclusions in the presence of brefeldin A were smaller in size and appeared more densely packed (Hackstadt et al., 1996). This result is consistent with the hypothesis that Golgi derived lipids contribute to the growth of the inclusion membrane.

Consistent with the results above, we believe that the inhibitory role of dynamin on Chlamydia productive growth is due to an inhibition of the vesicular traffic between the Golgi compartment and the Chlamydia inclusion. The effect of dynK44A on the growth of C. trachomatis resembles the effect of brefeldin A, as the inclusions are smaller and more compact. The larger effect on the productive growth of the C. psittaci inclusion apparently represents differences in the metabolism of the two Chlamydia species and dependence of the two pathogens on the supply of host cell lipids.

Although dynamin was originally thought to be involved only in clathrin-mediated endocytosis (Damke, 1996), it has recently been implicated in several other unique functions, including endosome to Golgi transport (Llorente et al., 1998) and formation of nascent secretory vesicles from the trans-Golgi network (Jones et al., 1998). Several alternatively spliced forms of the three dynamin genes have been identified, and they were localized to several distinct (membrane or cytoplasmic) compartments (Cao et al., 1998) where they may participate in various membrane trafficking events. Dynamins have a high homology and they function as oligomers (Damke, 1996; Urrutia et al., 1997), which explains the dominant negative effect of dynK44A mutant of dynamin I, which is not normally expressed in HeLa cells (Damke et al., 1994). The dynK44A mutant thus may interact with an isoform of dynamin II in HeLa cells acting on the vesicular traffic between the Golgi and the Chlamydia inclusion. To further characterize the mechanism of Chlamydia entry into HeLa cells, we examined the role of actin. Cytochalasin D dramatically inhibited Chlamydia infection (C. psittaci by 90% and C. trachomatis completely); cytochalasin D prevented the entry of Chlamydia but not its growth. Addition of the drug 4 hours post-infection had a significantly smaller inhibitory effect, while addition after 10 hours had almost no effect. In parallel, we found that no bacteria had been internalized in cells pre-treated with cytochalasin D (data not shown). The effect of cytochalasin D on Chlamydia entry thus confirms earlier studies (Ward and Murray, 1984; Majeed et al., 1991, 1993; Schramm and Wyrick, 1995; Ojcius et al., 1998) but its effect at later stages of infection had not been evaluated. Our results indicate that in HeLa cells the entry of C. psittaci GPIC and C. trachomatis LGV/L2 is actin-dependent, suggesting that Chlamydiae enter host cells through a process resembling phagocytosis.

Early morphological studies suggested a possible role for clathrin in Chlamydia entry. In one study (Reynolds and Pearce, 1990), electron dense material resembling clathrin patches were found on the Chlamydia early inclusion. An elegant earlier study by high resolution electron microscopy with professional phagocytes, macrophages taking up latex beads (Aggeler and Werb, 1982), had shown that up to one half of the phagosomes observed after a short (2-5 minutes) phagocytic pulse had areas of clathrin basketwork associated with them, suggesting that there may be an early transient association of clathrin with phagosomes in macrophages. It is possible that this may be the case for phagosomes in non-professional phagocytes as well. On the other hand, polarized epithelial cells were used in another study (Wyrick et al., 1989) suggesting a role for clathrin in the entry of C. trachomatis, because of the presence of Chlamydia in membrane pits and vesicles coated with electron dense material resembling clathrin coat. The possibility still exists that the mechanism of entry of Chlamydia may be different in polarized epithelial cells compared to monolayer cells. However, ultrastructural studies that identify structures characteristic of certain entry pathways (e.g. coated pits) have important drawbacks with regards to statistical significance or operator bias. Additionally, the studies mentioned above which suggested the implication of structures characteristic of clathrin coated pits in chlamydia...
entry did not establish the presence of clathrin by immunocytchemistry. Recent functional in vivo studies and ultrastructural and biochemical analyses have shown that dynamin, besides mediating clathrin-dependent endocytosis, is involved in the internalization of caveolae in mammalian cells (Henley et al., 1998; Oh et al., 1998). Our data suggest that dynamin is not involved in the formation of Chlamydia containing phagocytic vesicles.

The mutant cell lines defective in endocytosis have allowed us to establish that C. trachomatis and C. psittaci enter HeLa cells via a mechanism that is clathrin-independent and actin-dependent. Since binding of Chlamydia also takes place at 4°C (Gutiérrez-Martin et al., 1997) and is receptor mediated (Su et al., 1996; Gutiérrez-Martin et al., 1997) we conclude that Chlamydia entry is a receptor-mediated bacteria-induced process resembling phagocytosis.

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