Analysis of *Chlamydia caviae* entry sites and involvement of Cdc42 and Rac activity

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Summary

In epithelial cells, endocytic activity is mostly dedicated to nutrient and macromolecule uptake. To invade these cells, *Chlamydiaceae*, like other pathogens, have evolved strategies that utilise the existing endocytic machineries and signalling pathways, but little is known about the host cell molecules involved. In this report, we show that within five minutes of infection of HeLa cells by *Chlamydia caviae* GPIC strain several events take place in the immediate vicinity of invasive bacteria: GM1-containing microdomains cluster, tyrosine-phosphorylated proteins accumulate, and intense actin polymerization occurs. We show that actin polymerization is controlled by the small GTPases Cdc42 and Rac, which become activated upon infection. Expression of dominant negative forms of these GTPases inhibits *C. caviae* entry and leads to abnormal actin polymerization. In contrast, the small GTPase Rho does not seem essential for bacterial entry. Finally, phosphatidylinositol 3-kinase activity is also required for internalization of *C. caviae*, probably downstream of the other molecular events reported here. We present the first scheme of the events occurring at the sites of invasion of epithelial cells by a member of the *Chlamydiaceae* family.

Key words: *Chlamydiaceae*, Phagocytosis, Small GTPases, Actin polymerization, Lipid rafts, PI 3-kinase

Introduction

Pathogenic bacteria are able to utilise genuine cellular processes to survive within their host. For example, they have developed different ways to interact with the molecules involved in the internalization of extracellular components by eukaryotic cells, either to promote their own entry or to prevent it, depending on their ability to survive within cells. Because they are obligate intracellular parasites, *Chlamydiaceae* depend on their ability to be internalised by host cells. Their principal target are epithelial cells, in which they multiply within a membrane-bound compartment, called an inclusion. The developmental cycle takes 2 to 3 days, depending on the species, after which bacteria are released in the extracellular space from where a new infectious cycle can begin. The three species pathogenic to humans, *C. trachomatis*, *C. pneumoniae* and *C. psittaci* infect different epithelia and cause a number of diseases, including trachoma, pelvic inflammatory disease, pneumoniae and their sequelae (Gregory and Schaffner, 1997; Kuo et al., 1995; Stamm, 1999).

The first step in the infection by *Chlamydiaceae* is the attachment of the infectious form, the elementary body (EB), to a host cell. A number of surface molecules have been proposed to function as adhesins, including the major outer membrane protein (Su et al., 1996), heat shock protein 70 (Raulston et al., 1993), OmcB (Ting et al., 1995) and heparan sulfate-like glycosaminoglycans (Zhang and Stephens, 1992). The host surface molecules involved in adhesion have not been clearly identified either. We and others have shown that *Chlamydiaceae* bind preferentially to specific lipid microdomains on the host cell surface, suggesting that the host receptors are enriched in these structures (Jutras et al., 2003; Norkin et al., 2001; Stuart et al., 2003). These lipid domains, also called detergent-resistant lipid microdomains (DRMs) or rafts, are enriched in cholesterol and sphingolipids (Simons and Ehehalt, 2002). One difficulty in understanding the mechanism of attachment of *Chlamydiaceae* is that it may rely on different molecules depending on the strain, which may also interact with different receptors depending on the cell type.

Owing to the small size of EBs (0.3 μm in diameter), their entry has been essentially studied by electron microscopy (Friis, 1972; Hodinka et al., 1988; Kuo et al., 1988; Ward and Murray, 1984; Wyrick et al., 1989). As with most pathogens, the actin cytoskeleton is involved in the entry step, since actin depolymerizing drugs such as cytochalasin D inhibit the entry of most *Chlamydiaceae* species, including *C. caviae* (Boleti et al., 1999; Coombes and Mahony, 2002; Ward and Murray, 1984). Recent observations by confocal microscopy and scanning electron microscopy showed that *C. trachomatis* attachment on HeLa cells induced a transient actin recruitment and microvillar reorganization (Carabeo et al., 2002). Similarly, microvilli were observed on HEp2 cells infected with *C. pneumoniae*, and disappeared 2 hours after infection, concomitant with the end of the entry step (Coombes and Mahony, 2002).

Early microscopic observations had suggested that clathrin-coated pits may be involved in the entry of *Chlamydiaceae* (Hodinka et al., 1988; Wyrick et al., 1989). We have shown that the overexpression of dominant negative mutants of two

proteins, dynamin-1 and Eps15, which both inhibit clathrin-dependent endocytosis, did not affect *C. caviae* GPIC strain entry. Therefore, by specifically inhibiting clathrin-coated pit-dependent entry in epithelial cells, we have shown that *C. caviae* does not use this endocytic pathway (Boleti et al., 1999).

These three characteristics – the involvement of actin, the independence from clathrin-mediated endocytosis and from dynamin-1-mediated endocytosis – indicate that this process is related to phagocytosis although it occurs mostly in cells that are not 'professional' phagocytes (Tse et al., 2003). In macrophages, the molecular basis of phagocytosis has been intensively studied, and several of the players have been identified (Underhill and Ozinsky, 2002). Fc receptors (FcRs) for the constant region of immunoglobulins and receptors for complement (CR) mediate the clearance of pathogens opsonized by specific antibodies or complement respectively. Both FcR- and CR-mediated signalling pathways lead to the activation of specific small GTP-binding proteins belonging to the Rho family: Cdc42 and Rac for FcR signalling and Rho for CR signalling (Caron and Hall, 1998). Rho GTPases function as molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state. They interact with a diversity of downstream effectors which, by controlling actin filament assembly and organization, regulate phagocytosis (Bishop and Hall, 2000).

The molecular mechanisms involved in internalization of several micro-organisms have been investigated in the last decade. With the exception of several membrane receptors, most of the components of the phagocytic machinery are present in nonprofessional phagocytic cells such as epithelial cells. Intracellular pathogens use such existing machinery and signalling pathways to enter their target cells. However, each pathogen has developed different invasive strategies, and the signalling pathways involved vary (Mecsas et al., 1998). In the case of *Chlamydiaceae*, little has been determined. Tyrosine phosphorylation of host proteins was detected early after infection of HeLa cells by *C. trachomatis* L2 and of endothelial cells by *C. pneumoniae* (Birkelund et al., 1994; Fawaz et al., 1997; Krüll et al., 1999). Two signalling pathways were shown to be activated in HEp2 cells upon infection by *C. pneumoniae*: MEK-dependent phosphorylation and activation of ERK1/2, and PI 3-kinase-dependent phosphorylation and activation of Akt (Coombes and Mahony, 2002; Krüll et al., 1999). However, the molecules directly involved in the reorganization of the actin cytoskeleton and internalization process were not identified.

We have analysed *C. caviae* entry sites by fluorescence microscopy and we describe some of the very early events that take place upon infection. We also investigated the role of the GTPases of the Rho family in entry. We show that Cdc42 and Rac are activated and are necessary for this step.

Materials and Methods

Cells, bacteria, antibodies, plasmids and other reagents

The human cervical adenocarcinoma cell line, HeLa 229, was from the American Type Culture Collection and was grown in Dulbecco's modified Eagle's medium with Glutamax (Life Technologies) supplemented with 10% foetal calf serum (complete medium). The GPIC serovar of *C. caviae* was obtained from Roger Rank (University of Arkansas). *Chlamydiaceae* were prepared as described previously (Boleti et al., 1999). Mowiol, wortmannin and LY294002 were from Calbiochem (LaJolla, CA), saponin, fluorescein isothiocyanate (FITC), FITC-cholera toxin and DABCO (1,4-diazabicyclo-[2.2.2] octane) from Sigma and Alexa546- and Alexa488-phalloidin were from Molecular Probes. Epidermal differentiation inhibitor (EDIN) from *S. aureus* was generously provided by Dr Michel Robert Popoff (Institut Pasteur, Paris). The mouse anti-*Chlamydia* antibodies (unlabelled and FITC-labelled) were purchased from Argene, Biosoft (#12-114,Varilhes, France). Cy™-3- and Cy™-5-conjugated goat anti-mouse antibodies were from Amersham. Anti-myc antibodies (9E10) were a kind gift from Alain Israel (Institut Pasteur, Paris), anti-phosphotyrosine antibody 4G10 was from Upstate (Lake Placid, NY). Anti-Cdc 42 antibodies were a kind gift from Dr Philippe Chavrier (Institut Curie, Paris) and anti-Rac1 mAb was purchased from BD Transduction Laboratories. Plasmids coding for myc-tagged Cdc42N17, RacN17, RhoAN19, PAK GBD and WASP GBD in the pKR5 vector, and for GST-CRIB were a kind gift from Alan Hall (University College, London). Expression of GST-CRIB in *E. coli* was induced with 0.2 mM isopropyl β-D-thiogalactoside (IPTG) for 5 hours at 25°C. The fusion protein was purified by affinity chromatography on glutathione-Sepharose beads (Amersham Biosciences, Little Chalfont, UK) and stored in 20 mM Tris, pH 7.4, 2 mM EDTA, 100 mM NaCl, 10% glycerol and 2 mM β-mercaptoethanol.

FITC labelling of bacteria

All steps were performed at 4°C. A 0.4 ml aliquot of bacteria in SPG buffer (218 mM sucrose, 3.76 mM KH_2PO_4, 7.1 mM K_2HPO_4, 4.9 mM glutamate, pH 7.4) stored at –80°C was thawed on ice, 0.5 ml of phosphate-buffered saline (PBS) was added and the bacteria were centrifuged at 18,000 ***g*** for 5 minutes. Bacteria were resuspended in 200 μl of 0.5 mg/ml FITC in PBS, 10% DMSO, and incubated for 30 minutes in the dark with gentle agitation. SPG (0.5 ml) was added to the bacteria, which were centrifuged for 5 minutes at 18,000 ***g***, washed with 1 ml of SPG, centrifuged for 5 minutes at 18,000 ***g***, resuspended in 200 μl of SPG and finally centrifuged for 5 minutes at 700 ***g*** to eliminate large aggregates. The supernatant was aliquoted and stored at –80°C until use. As a control for the efficiency of coupling, an aliquot of bacteria was labelled with anti-*Chlamydia* antibody followed by Alexa546 anti-mouse antibody. Nine out of ten bacteria seen with the red filter were also seen with the green filter, showing that there was a 90% coupling efficiency.

Visualization of entry sites

HeLa cells grown in 24-well plates were washed twice in PBS and infected in 0.25 ml of culture medium and immediately centrifuged for 5 minutes at 770 ***g*** at room temperature. Plates were then incubated at 37°C for the indicated times, washed twice in PBS and fixed in 4% paraformaldehyde (PFA), 120 mM sucrose in PBS for 30 minutes at room temperature. The cells were washed in PBS, incubated for 10 minutes in 50 mM NH_4Cl in PBS at room temperature and permeabilized in 0.05% saponin, 1 mg/ml BSA in PBS. Actin was visualized using Alexa546-coupled phalloidin, and the ganglioside GM1 was labelled with 5 μg/ml FITC-coupled cholera toxin. The samples were examined with a Leica confocal microscope. To observe tyrosine-phosphorylated proteins the cells were first labelled with 4G10 antibody before being incubated with anti-mouse Cy™-5-antibody together with Alexa546-coupled phalloidin. In the series of experiment involving small GTPases, transfected cells were identified with an anti-myc antibody, before being incubated with anti-mouse Cy™-5 antibody together with Alexa546-coupled phalloidin. Coverslips were mounted in Mowiol with 100 mg/ml DABCO and examined under an epifluorescence microscope (Axiophot, Zeiss, Germany) attached to a cooled CDD camera (Photometrics, Tucson, AZ), using a ×63 Apochromat lens. Images were acquired in the

green, red and far-red channels using the IPlab Spectrum program (Signal Analytics Corporation), and were then superimposed.

Measure of *C. caviae* entry in cells expressing GTPases mutants

HeLa cells, which were less than 50% confluent, were transfected with the indicated plasmids using Fugene reagent (Roche Applied Science). The following day, the cells were washed twice in PBS and incubated in complete medium with bacteria for 1 hour 30 minutes at 37°C. The cells were then washed twice in PBS, complete medium was added and incubation continued for 2 hours 30 minutes before cell fixation and quenching as described above. Extracellular bacteria were labelled with anti-*Chlamydia* antibody followed by anti-mouse Cy™-5 antibody. The cells were then permeabilized in 0.05% saponin, 1 mg/ml BSA in PBS, and incubated with FITC-conjugated anti-*Chlamydia* antibody to label intracellular bacteria together with anti-myc antibody 9E10 to label transfected cells. Anti-myc antibody was revealed in a last step, by incubating the cells with anti-mouse Cy™-3 antibody and this colour was used to identify transfected cells. Images were acquired in the FITC, Cy™-3 and Cy™-5 channels and were then superimposed. The number of intracellular bacteria (green only) and surface-associated bacteria (far-red or yellow) was counted for each transfected and non-transfected cell in the fields (about 100 cells were counted per experiment). Results are expressed as the efficiency of entry in transfected versus non-transfected cells (i.e. internal/total cell-associated in transfected cells × total cell-associated/internal in non-transfected cells × 100). On average, 5-20 bacteria were associated with control cells, and the efficiency of entry ranged between 25 and 50%.

Measure of *C. caviae* entry after cell treatment with EDIN or PI 3-kinase inhibitors

Toxin EDIN

HeLa cells were washed twice in PBS and preincubated for 2 hours in complete medium containing 10^{-7} M EDIN, before diluting bacteria directly into this medium. After 1 hour 30 minutes at 37°C, the cells were washed twice in PBS, complete medium was added and incubation continued for 2 hours 30 minutes before fixation. No further addition of toxin was needed as the effects of the toxin is not reversible.

PI 3-kinase inhibitors

HeLa cells were washed twice in PBS and preincubated for 30 minutes in complete medium containing 100 nM wortmannin or 50 μM LY294002, before diluting bacteria directly into this medium. After 1 hour 30 minutes at 37°C, the cells were washed twice in PBS, complete medium with PI 3-kinase inhibitors was added and incubation continued for 2 hours 30 minutes before fixation. Differential labelling of surface and intracellular bacteria was performed as described above. For visualization of actin reorganization upon infection, cells were preincubated for 30 minutes at 37°C with or without 50 μM LY294002, before centrifugation with FITC-coupled bacteria and incubation for 5 minutes at 37°C, as described above. Actin was observed using Alexa546-coupled phalloidin.

Determination of Cdc42 and Rac activation by pull-down assay

HeLa cells (5×10^6 per time point) grown overnight at subconfluence in serum-free conditions were detached with PBS-EDTA and resuspended in DMEM medium. Cells were mixed with the bacterial inoculum in 0.1 ml of DMEM, centrifuged for 20 seconds at 16,000 ***g*** and transferred to 37°C (*t*=0 minutes). After incubation for different times, the cells were lysed on ice for 15 minutes in lysis buffer (150 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris pH 7.5). Cell lysates were centrifuged at 16,000 ***g*** at 4°C for 10 minutes. Equal volumes of supernatant for each time point were incubated for 90 minutes with 50 μg GST-CRIB in the presence of 0.5% BSA and glutathione-Sepharose. The resin was washed extensively with washing buffer (150 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 50 mM Tris pH 7.5), boiled in SDS-PAGE sample buffer, and bound proteins were resolved on SDS gels followed by western blot analysis using anti-Cdc42 and anti-Rac antibodies.

Results

Infection by *C. caviae* induces rapid and transient actin polymerization

Prior to infection, bacteria were coupled to FITC as described in the Materials and Methods section. We checked that FITC-labelled bacteria were still infectious by measuring their ability to form inclusions 24 hours after infection, which was identical to that of non-labelled bacteria (data not shown). One difficulty in the observation of bacterial entry is in the asynchronicity of attachment of bacteria to host cells and therefore of downstream events. To circumvent this problem, the bacterial inoculum was laid on the cells in 24-well plates at 4°C, centrifuged for 5 minutes at 770 ***g*** at room temperature and incubated at 37°C (time zero) to allow internalization to proceed. The cells were fixed at different times post-infection (p.i.) and filamentous actin was labelled using phalloidin coupled to a red fluorochrome. Local actin polymerization was observed as early as 5 minutes p.i., and each actin patch contained a bacterium (Fig. 1). Actin polymerization around invasive bacteria was a transient phenomenon since it was still observed 15 minutes p.i. but not 45 minutes p.i. In addition to the local actin rearrangement around bacteria, infection also induced a general actin cytoskeleton remodelling with the appearance of abundant actin spikes all over the cell surface, which were still visible 45 minutes p.i. (Fig. 1).

Observation of cholesterol-rich domain clustering upon infection

We have previously shown that after binding to HeLa cells, *C. trachomatis* LGV-L2 and *C. caviae* GPIC are associated with DRMs, which can be isolated by fractionation of infected cells and flotation on a sucrose gradient. Moreover, extraction of plasma cholesterol inhibited infection, indicating that these domains participate in the entry process (Jutras et al., 2003). To visualize the distribution of DRMs during infection, we used FITC-coupled cholera toxin, which binds to the ganglioside GM1, a widely used marker for DRMs (Streuli et al., 1981). As shown in Fig. 2, clustering of GM1 was observed 5 minutes p.i., at the spots of actin polymerization. In control cells (time 0 minute p.i.), GM1 was evenly distributed on the cell surface. This experiment, together with our previous observations, indicates that, upon attachment of bacteria to cholesterol-rich membrane domains, these microdomains cluster and actin polymerization is rapidly initiated at these sites.

Rapid appearance of phosphorylated proteins at the sites of entry

One of the early events that was observed upon *Chlamydiaceae* infection is the phosphorylation of host proteins, as measured

mostly by western blotting using anti-phosphotyrosine antibodies (Birkelund et al., 1994; Coombes and Mahony, 2002; Fawaz et al., 1997; Krüll et al., 1999). To gain information on the distribution of phosphorylated proteins during the entry process, they were observed early post-infection (Fig. 3). In non-infected cells, tyrosine-phosphorylated proteins were enriched at the periphery of the cell, in a punctate pattern corresponding to the distribution of focal adhesion complexes (Maher et al., 1985). Five minutes p.i., the distribution was largely changed: the highest concentration of tyrosine-phosphorylated proteins was found associated with the actin patches. This result is in agreement with the protein phosphorylation observed 5 minutes p.i. during *C. pneumoniae* infection (Coombes and Mahony, 2002). One of the phosphorylation substrates identified in this study was the focal adhesion kinase, which is tyrosine-phosphorylated and localized in focal adhesion in resting cells (Hanks et al., 1992). Therefore, recruitment of the phosphorylated focal adhesion kinase at the site of bacterial entry may contribute to the general redistribution of tyrosine-phosphorylated proteins observed at early times of infection. Since there is also a general increase in tyrosine-phosphorylated proteins during infection, it is probable that the accumulation of phosphorylated proteins around invasive bacteria corresponds to local phosphorylation events at the sites of entry.

C. caviae entry requires Cdc42 and Rac, but not Rho, activities

The Rho family of small GTPases is involved in the reorganization of filamentous actin structures in response to extracellular stimuli. To examine whether the activity of these proteins is involved in *C. caviae* entry, we analysed the ability of cells expressing dominant negative forms of Cdc42, Rac and RhoA, the three main players of the family, to internalize *C. caviae*. Cells transfected for 18 hours with one of the dominant negative constructs were infected and fixed 4 hours p.i. Transfected cells and bacteria were identified with anti-myc and anti-*Chlamydia* antibodies, respectively (Fig. 4A). The protocol used allowed us to distinguish between internal bacteria and surface-bound bacteria as described in the Materials and Methods section, and entry was quantified in transfected and non-transfected cells (Fig. 4B). Overexpression

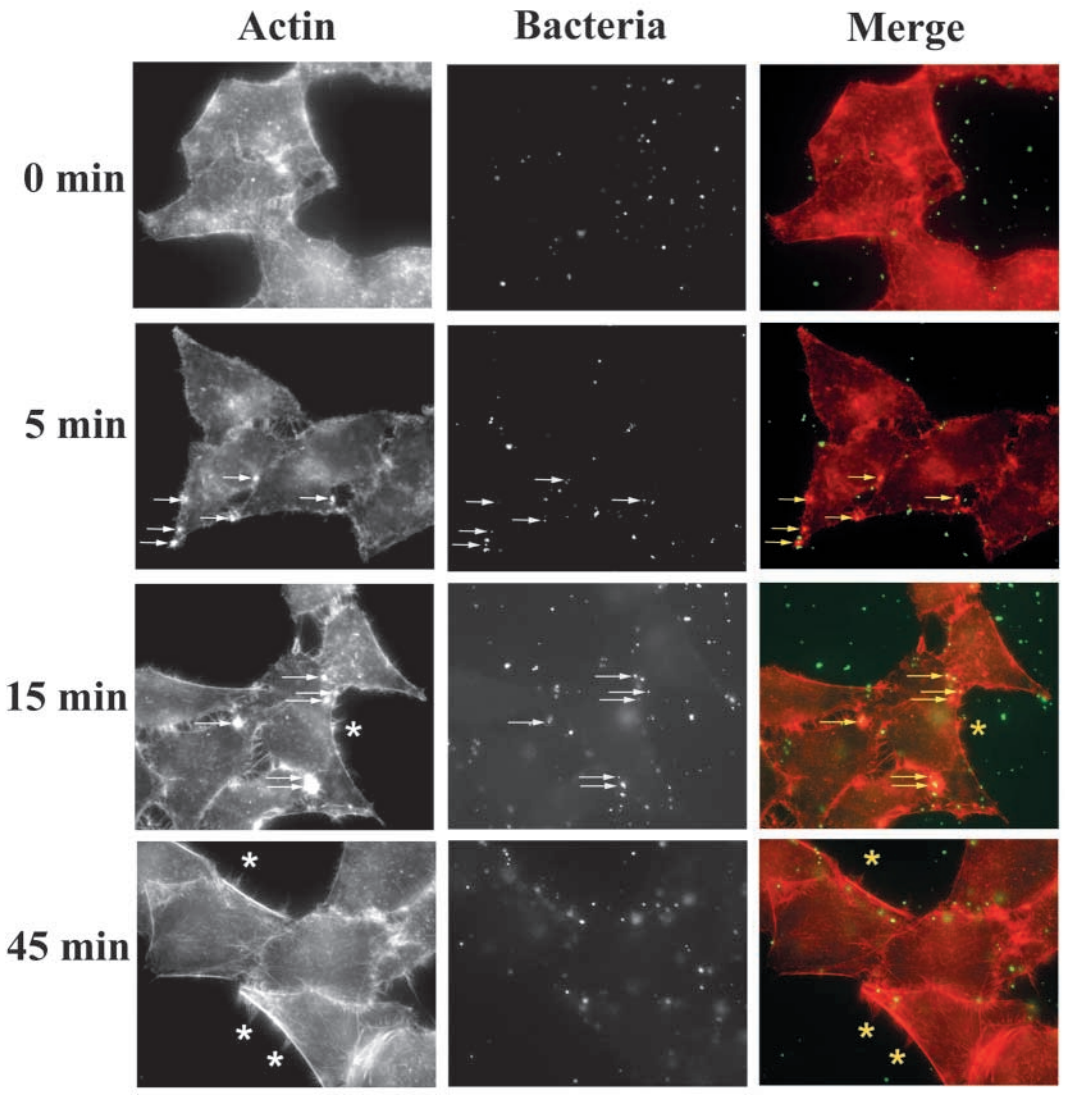

Fig. 1. Actin rearrangements upon bacteria entry. HeLa cells were infected with FITC-coupled bacteria for the indicated times before fixation and actin labelling using Alexa546-coupled phalloidin. Notice that a bacterium is visible in each of the actin patches, which transiently appeared (5 minutes and 15 minutes, arrows). At later times, infection also induced a general actin remodelling (15 minutes and 45 minutes, asterisks).

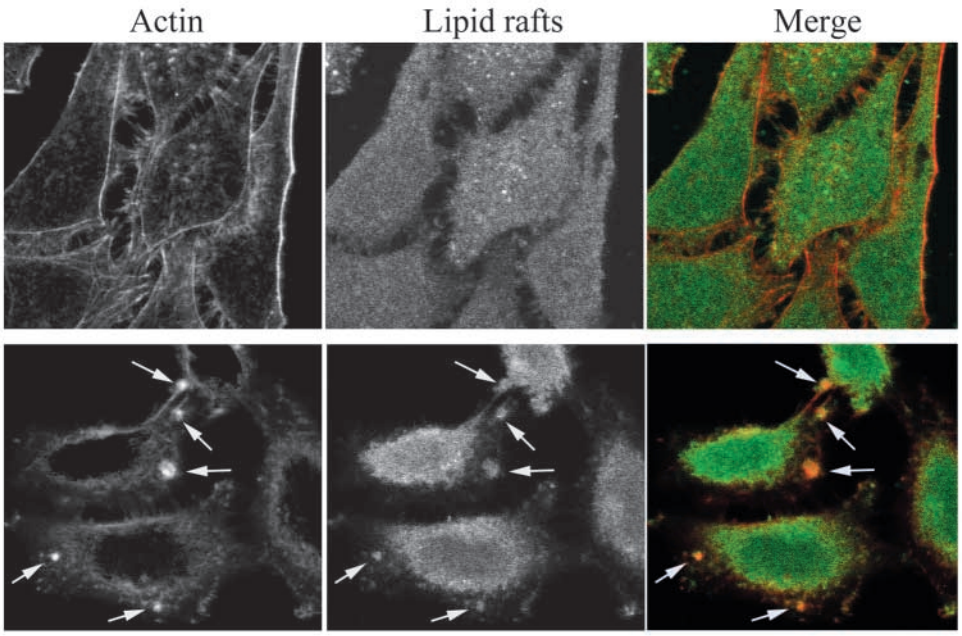

Fig. 2. Distribution of DRMs during bacterial entry. HeLa cells were infected with *C. caviae* GPIC for 0 minute (top) or 5 minutes (bottom) before fixation. Actin was visualized with Alexa546-coupled phalloidin (left) and GM1 gangliosides were stained using FITC-cholera toxin (middle). Superimposition of the two stainings is shown (right), clusters of GM1 are indicated (arrows).

of dominant negative Cdc42 and Rac induced a decrease in the entry efficiency of 45 and 64% respectively. In contrast, overexpression of dominant negative Rho did not significantly affect bacterial entry.

As an alternative method to inhibit Cdc42 and Rac activity, we overexpressed domains of proteins that interact with the GTP-bound forms of these GTPases, thereby inhibiting their action. The GTPase binding domain of the serine/threonine kinase PAK (p21 activated kinase) binds preferentially to activated Rac, and, to a smaller extent, to activated Cdc42 (Manser et al., 1994). Its expression in HeLa cells inhibited *C. caviae* entry efficiency by 62% (Fig. 4). Similarly, overexpression of the GTPase binding domain of WASP, an effector of Cdc42 (Nagata et al., 1998), inhibited bacterial entry by 52%.

To further assess a putative role for Rho in bacterial entry we used the *Staphyloccocus aureus* epidermal differentiation inhibitor (EDIN). This toxin has an ADP-ribosylation activity similar to that of *Clostridium botulinum* C3 transferase and specifically inhibits Rho activity without affecting Cdc42 nor Rac (Barbieri et al., 2002). After 2 hours of incubation with 10^{-7} M EDIN, the morphology of the actin cytoskeleton was markedly changed (data not shown). The cells rounded up and not surprisingly, upon infection, the number of attached bacteria was reduced compared to control cells. However, this treatment had only a minor effect on *C. caviae* GPIC entry, as the percentage of internalized bacteria relative to total cell-associated bacteria was reduced only by 20% (Fig. 4B).

Altogether, these experiments show that both Cdc42 and Rac activities are necessary for the entry of *C. caviae* in HeLa cells. In contrast, Rho activity does not seem to be necessary for this step.

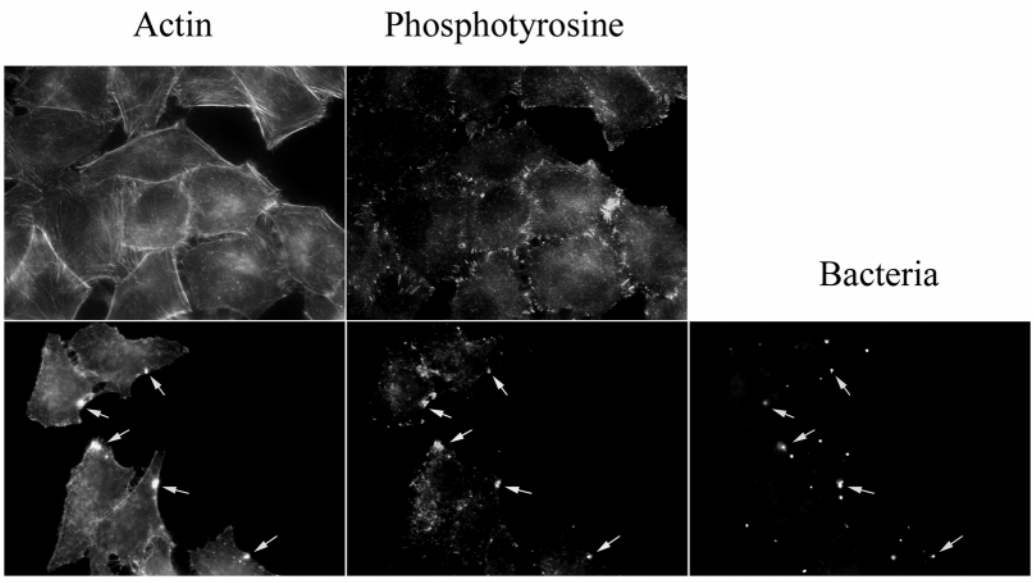

Fig. 3. Distribution of phosphorylated proteins during bacterial entry. Non-infected cells (top) and cells infected for 5 minutes with FITC-coupled *C. caviae* GPIC (bottom) were labelled with anti-phosphotyrosine antibody (middle) while actin was visualized using Alexa546-coupled phalloidin (left). Upon infection, phosphorylated proteins were concentrated at the sites of intense actin polymerization, where bacteria are found (arrows).

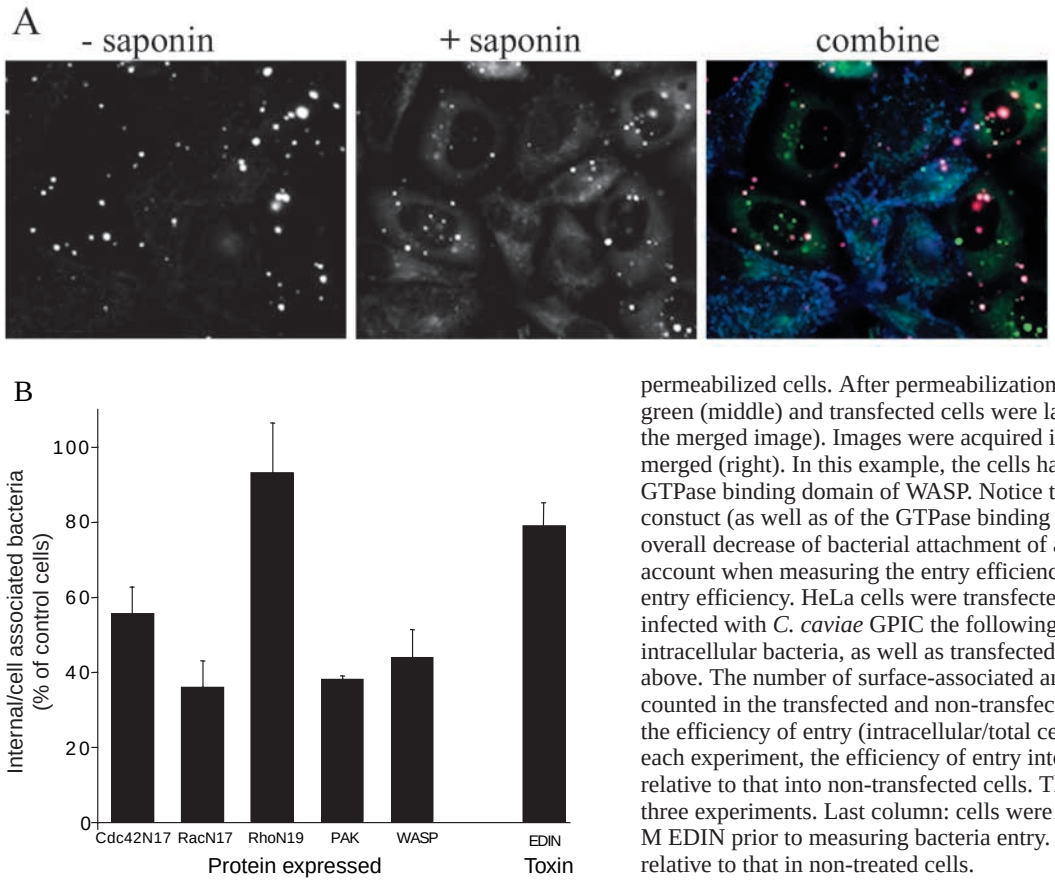

Fig. 4. Effect of the inhibition of small GTPases of the Rho family on *Chlamydia* entry. (A) Illustration of the method used for the quantification of entry efficiency. Transiently transfected HeLa cells were infected with *C. caviae* for 4 hours as described in the Materials and Methods section. Extracellular bacteria were labelled in far-red (Cy^{TM}-5, left) in non-permeabilized cells. After permeabilization, total bacteria were labelled in green (middle) and transfected cells were labelled in red (they appear blue in the merged image). Images were acquired in the three channels and were merged (right). In this example, the cells had been transfected with the GTPase binding domain of WASP. Notice that, overexpression of this constuct (as well as of the GTPase binding domain of PAK) induced an overall decrease of bacterial attachment of about 50%, which was taken into account when measuring the entry efficiency. (B) Quantification of *C. caviae* entry efficiency. HeLa cells were transfected with the indicated plasmids and infected with *C. caviae* GPIC the following day. Extracellular and intracellular bacteria, as well as transfected cells, were labelled as described above. The number of surface-associated and intracellular bacteria were counted in the transfected and non-transfected population (n>25 cells) and the efficiency of entry (intracellular/total cell-associated) was calculated. For each experiment, the efficiency of entry into transfected cells is expressed relative to that into non-transfected cells. The data shown are the average of three experiments. Last column: cells were pretreated for 2 hours with 10^{-7} M EDIN prior to measuring bacteria entry. Efficiency of entry is expressed relative to that in non-treated cells.

Rac and Cdc42 are activated upon infection

The active GTP-bound forms of small GTPases have a much higher affinity for downstream partners than the inactive GDP-bound forms. This property has been used to develop pull-down assays to measure in vitro activation of these molecules. We monitored the activation levels of endogenous Cdc42 and Rac during *C. caviae* infection using the GST-fused Rac interactive binding domain of PAK (GST-CRIB) that specifically binds the GTP-bound forms of Rac and, when the quantity of GST-CRIB is not limiting, of Cdc42 (Burbelo et al., 1995). To that end, cells were infected for various times at 37°C, lysed on ice, and activated Rac and Cdc42 were pulled-down using 50 μg of GST-CRIB at each time point. The amount of precipitated GTPases was quantified by western blotting using specific anti-Cdc42 and anti-Rac antibodies (Fig. 5). Rac and Cdc42 had similar kinetics of activation, which peaked 5 minutes p.i. and returned to a basal level within 45 minutes.

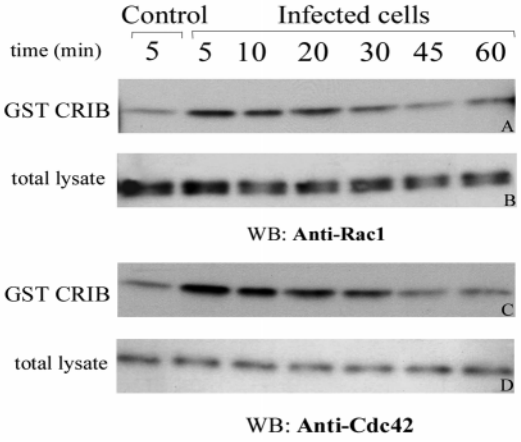

Fig. 5. Kinetics of activation of Cdc42 and Rac upon infection. HeLa cells in suspension were centrifuged for 20 seconds in DMEM alone (control lane) or with bacteria (other lanes), and incubated for the indicated times at 37°C. Cells were lysed and incubated with GST-CRIB. GST-CRIB-associated proteins were pulled-down using glutathione-Sepharose and analysed by western blotting using anti-Rac1 (A) and anti-Cdc42 (C) antibodies. Aliquots of total cell lysates were immunoblotted for total Rac1 (B) and total Cdc42 (D), showing that the total amount of each small GTPase was identical at all time points. Data are representative for four experiments.

Cdc42 and Rac act in concert to control actin polymerization

Knowing that Rac and Cdc42 are required for bacterial entry, we asked whether these proteins controlled actin polymerization. Cells transfected with dominant negative forms of Cdc42 or Rac where infected for 5 minutes as described earlier. Transfected cells were identified using anti-myc antibody followed with Cy^{TM}-5-labelled secondary antibody while the actin cytoskeleton was labelled using Alexa546 coupled phalloidin (Fig. 6). Following infection, several features were evident. First, actin patches, to which bacteria were associated, were observed in cells transfected

with dominant negative forms of Cdc42 or Rac, as in non-transfected cells, indicating that each of the small GTPases is not absolutely required for some actin polymerization. Second, both Cdc42N17 and RacN17 were recruited at the sites of actin polymerization, confirming the involvement of these small GTPases in the entry process. Third, in cells expressing RacN17 (and to a lesser extent Cdc42N17), the actin patches were larger than in control cells, indicating that these proteins are necessary for the harmonious reorganization of the actin cytoskeleton. When the same experiment was performed on cells overexpressing wild-type Rac the actin patches had a normal appearance, indicating that overexpression of the dominant negative form of the small GTPase was responsible for the aberrant actin organization (data not shown). Overexpressing wild-type Cdc42 induced extensive actin reorganization, precluding analysis during infection. Finally, actin patches and associated bacteria were still observed 15 minutes p.i. in cells overexpressing Cdc42N17 or RacN17. However, most patches had disappeared 45 minutes p.i., indicating that actin depolymerization was not blocked by overexpression of these mutants but occurred with kinetics similar to that in non-transfected cells (data not shown). Taken together, these experiments show that neither Cdc42 nor Rac is required to initiate actin polymerization, and that they are necessary for orchestrating the proper rearrangement of the cytoskeleton to allow for bacterial entry.

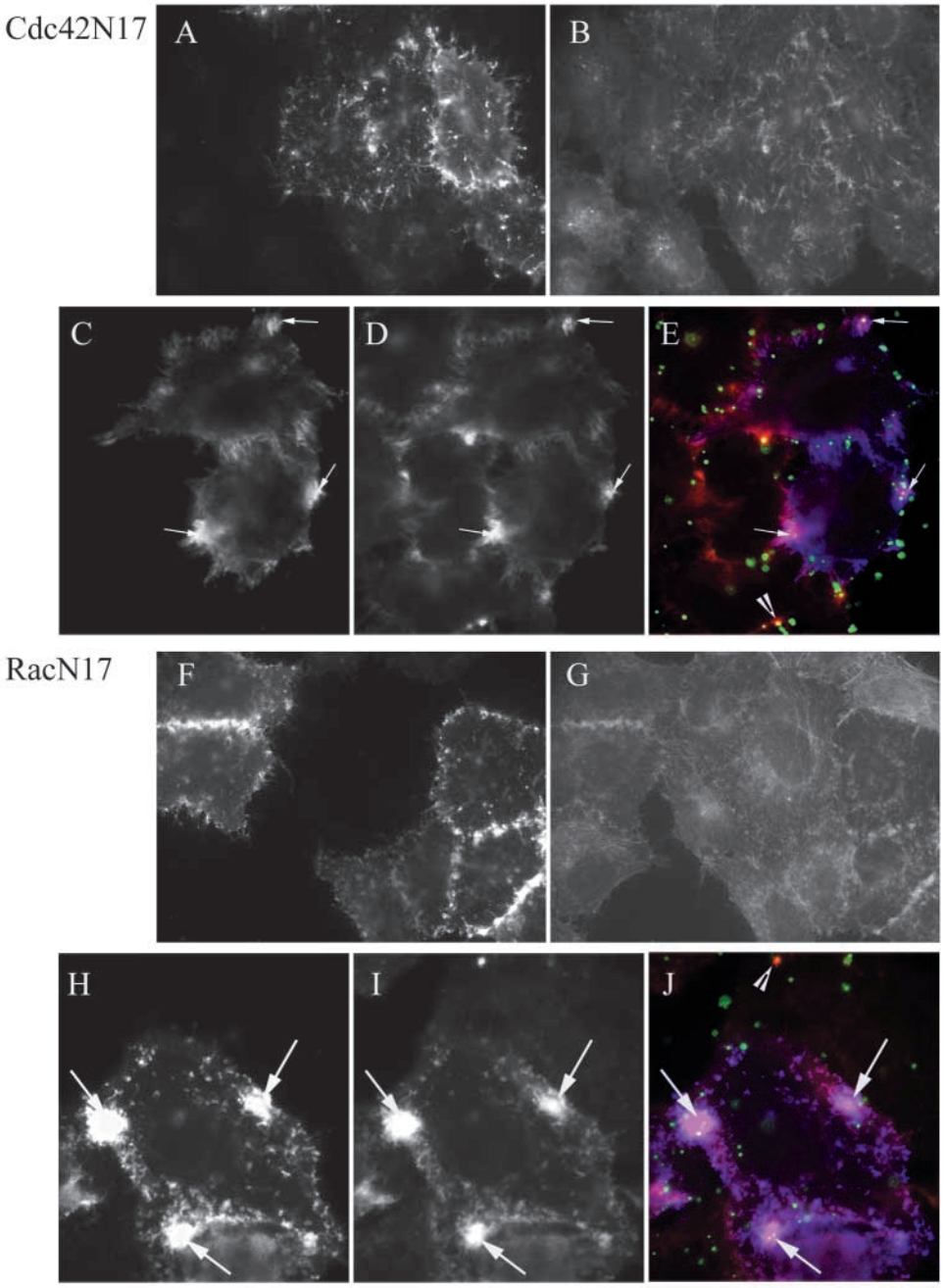

Fig. 6. Effect of the inhibition of Cdc42 and Rac on actin polymerization during bacterial entry. Cells, transfected for 18 hours with dominant negative Cdc42 (A-E) or Rac (F-J), were inoculated with culture medium alone (A,B,F,G) or with FITC-coupled bacteria (C,D,E,H,I,J). Five minutes p.i. the cells were fixed, and transfected cells were identified using anti-myc antibodies (A,C,F,H) while actin was observed using phalloidin (B,D,G,I). E and J show the superimposition of the 3 images collected from the infected samples with the transfected cells (blue), actin (red) and bacteria (green). Both Cdc42N17 and RacN17 were recruited to the sites of actin polymerization, with which bacteria were associated (arrows). Notice that in transfected cells actin patches were larger than in non-transfected cells (arrowheads).

C. caviae entry but not actin polymerization is blocked by PI 3-kinase inhibitors

Upstream as well as downstream effectors of Rho GTPases are the phosphatidylinositide (PI) 3-kinases, which catalyse D3-phosphorylation of phosphoinositides and are involved in several endocytic processes, including several steps of phagocytosis in macrophages (Simonsen et al., 2001). To test whether a PI 3-kinase activity was involved in *C. caviae* entry we determined the effect of two structurally unrelated PI 3-kinase inhibitors, wortmannin and LY294002, on this process. HeLa cells were pretreated for 30 minutes with 100 nM wortmannin or 50 μM LY294002 before infection. Four hours p.i. the cells were fixed and the number of internalized bacteria was evaluated by

differential staining of intracellular versus extracellular EBs as described in the Materials and Methods section. Wortmannin and LY294002 both reduced *C. caviae* invasiveness by 90% without affecting bacteria attachment, showing that a PI 3-kinase activity is required for the entry of the bacteria (Fig. 7A).

To investigate whether PI 3-kinase activity was required upstream of actin polymerization, we examined actin patches formed upon infection in cells treated or not with PI 3-kinase inhibitor (Fig. 7B). There was no obvious difference in the aspect of the actin patches between control cells and cells treated with LY294002. Altogether, these results indicate that PI 3-kinase signalling occurs downstream or independently of the activation of the small GTPases.

Discussion

Although chlamydial infections are mostly limited to mucosal surfaces and are quite specific in their host range, in culture they successfully invade a wide variety of cell types from many different species (Croy et al., 1975) and do so very efficiently (Byrne, 1978). These two properties suggest that EBs signal the host cell to initiate internalization, and that the cellular components and signalling pathways involved are ubiquitous. In this report we show that Cdc42 and Rac, two ubiquitous small GTPases of the Rho family, are activated upon infection with *C. caviae* and play a central role in its mechanism of entry. We also describe some of the early events that take place at the sites of bacterial invasion, which are shown schematically in Fig. 8.

To synchronize the entry step, we briefly centrifuged the bacteria on cells, before letting the internalization proceed for a short period of time at 37°C. In these conditions, mobilization of actin was easily observed in the form of small and dense actin patches surrounding invasive bacteria (Fig. 1). It is noteworthy that within the actin patches bacteria were not always accessible to antibodies, suggesting that actin polymerization was very dense around invasive bacteria. Actin polymerization, observed as early as 2 minutes p.i. (not shown) and abundant 5 minutes p.i., was transient, as the patches had disappeared 45 minutes after infection. This observation is very similar to the transient formation of microvilli observed by electron microscopy upon infection with *C. trachomatis* and *C. pneumoniae* (Carabeo et al., 2002; Coombes and Mahony, 2002). Therefore it is very unlikely that it is a consequence of the centrifugation step introduced in our protocol. As can be seen in Fig. 1, not all the bacteria that attach to HeLa cells upon centrifugation induce the formation of actin patches. Even with this protocol, entry remains somewhat asynchronous, and since we have observed that actin polymerization is transient, it is not possible to observe the induction of actin polymerization for all the invasive bacteria at a given time. Moreover, we do not know the proportion of bacteria that are infectious in the preparations used, and many of these 'inactive' bacteria may be non-infectious EBs.

Small GTPases of the Rho family, which are key regulators of actin polymerization, are obvious candidates to control infection-induced actin polymerization. This hypothesis was tested by measuring *C. caviae* entry in cells expressing dominant negative forms of Cdc42 or Rac (Fig. 4). Both mutants inhibited bacterial entry. Another method to inhibit Cdc42 and Rac activities consists of overexpressing protein domains that interact with the active GTP-bound forms of these proteins: GBD-WASP (which inhibits Cdc42 activity) and GBD-PAK (which preferentially inhibits Rac activity). Both proteins, when

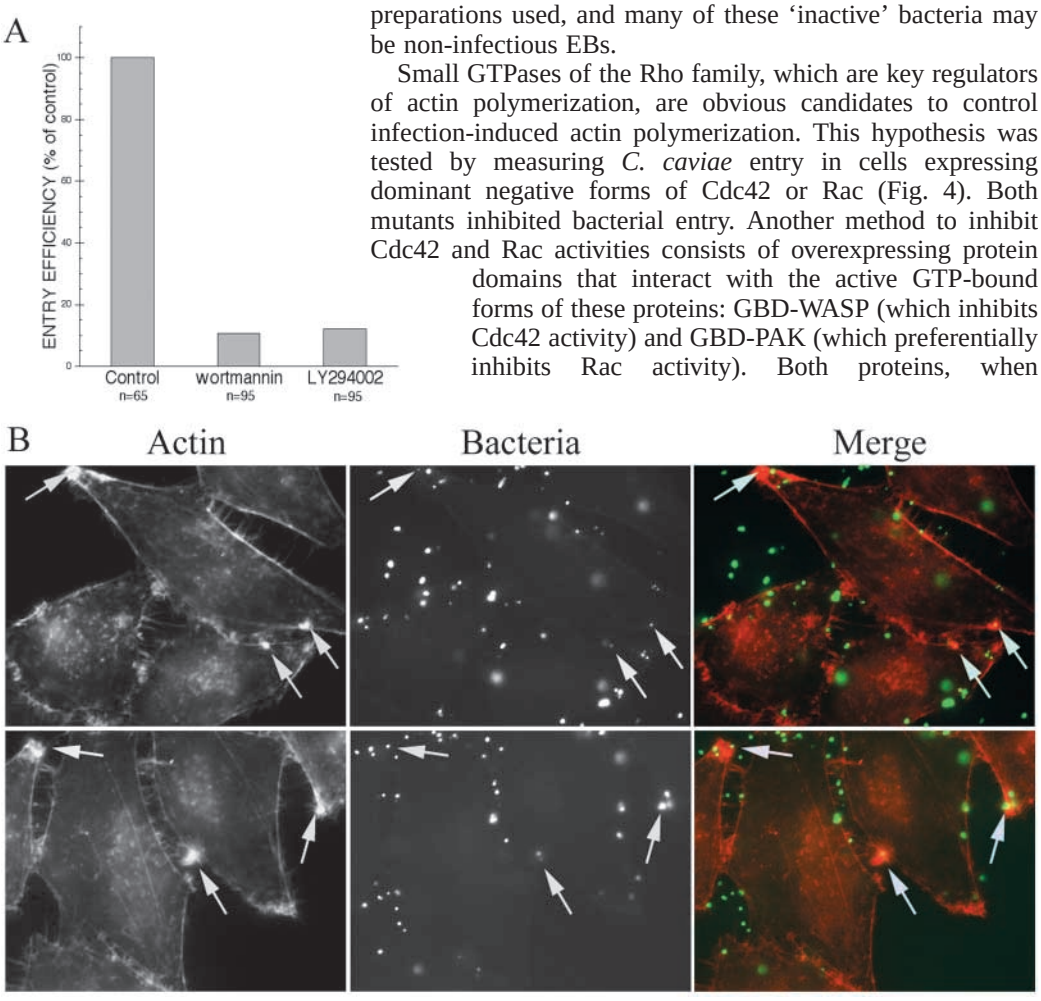

Fig. 7. Effect of PI 3-kinase inhibitors on bacterial entry and on actin polymerization. (A) Control HeLa cells or cells pretreated with 100 nM wortmannin or 50 μM LY294002 were infected with *C. caviae* GPIC. Four hours after infection the cells were fixed and extracellular and intracellular bacteria were labelled as described in the Materials and Methods section. The number of surface-associated and intracellular bacteria were counted in control and treated cells (*n*=number of cells) and the efficiency of entry (intracellular/total cell-associated) was calculated. Results are expressed as the efficiency of entry relative to that in control cells. This experiment is representative of two. (B) Control HeLa cells (top row) or cells pretreated for 30 minutes with 50 μM LY294002 (bottom row) were infected with FITC-coupled *C. caviae* GPIC, centrifuged and incubated for 5 minutes at 37°C before fixation and actin labelling using Alexa-546 phalloidin.

overexpressed, also inhibited *C. caviae* entry. The observation that none of these constructs resulted in more than 64% inhibition of entry may be due to the fact that the overexpression levels we obtained were not sufficient to fully inhibit the target proteins. In fact, the levels of inhibition we measured are similar to what was observed using these constructs to inhibit macrophage phagocytosis or other bacterial entry (Caron and Hall, 1998; Criss et al., 2001; Guzman-Verri et al., 2001; Martinez and Hultgren, 2002; Massol et al., 1998; Mounier et al., 1999).

Importantly, both Cdc42 and Rac were activated upon infection, as we showed using a fusion protein that specifically interacts with the activated form of these GTPases (Fig. 5). Maximal activation was observed 5 minutes p.i. and decreased thereafter until the basal level was reached, in about 45 minutes. This kinetics correlates perfectly with the kinetics of transient actin polymerization we observed by fluorescence microscopy.

Further evidence for the involvement of Cdc42 and Rac in *C. caviae* entry came from the observation that Cdc42N17 and RacN17 were recruited to the entry sites (Fig. 6). Recruitment of endogenous GTPases could not be observed, probably because there was a lot of background with the available antibodies (not shown). It may also be that the deficient forms of the GTPases accumulate at the entry sites, facilitating observation. In cells overexpressing dominant negative forms of Cdc42 or Rac, or both mutants simultaneously (data not shown), actin patches were still observed, indicating that none of these proteins is absolutely required to initiate some actin polymerization. However, the actin patches in transfected cells were larger than in non-transfected cells, especially in the case of RacN17 overexpression, suggesting that the small GTPases act in concert to regulate actin polymerization.

Two results indicate that Rho does not play a central role in *C. caviae* entry. First, expression of the dominant negative protein RhoN19 did nor affect *C. caviae* entry (Fig. 4), and actin patches observed at early times of infection in transfected cells had a normal aspect (data not shown). Second, treatment of the cells with EDIN, a protein with C3 transferase activity, which specifically inhibits Rho GTPase activity, had only a minor effect on bacterial entry (Fig. 4). In that respect, *C. caviae* entry mechanism resembles that of FcR-mediated phagocytosis in phagocytes, which relies mostly on Cdc42 and Rac activity (Caron and Hall, 1998; Underhill and Ozinsky, 2002).

Altogether our data show that upon bacteria contact with the host cell, Cdc42 and Rac get activated and orchestrate together the reorganization of the actin cytoskeleton to allow for bacteria entry. Thus *Chlamydiaceae* join the ever-growing list of micro-organisms which use the activity of small GTPases of the Rho family to invade eukaryotic cells (reviewed by Boquet and Lemichez, 2003). If invasive strategies developed by these micro-organisms all rely on small GTPase activities, they differ in the choice of timing, molecular partners and signalling cascades, leading to different morphological manifestations. Also, activation of the small GTPases can be achieved by different mechanisms, which are to date mostly unknown. For several Gram-negative bacteria, it has been shown that small GTPases were activated by the secretion of effector proteins from the bacteria into the host cell by a mechanism termed type III secretion. Effector proteins can be GTPase nucleotide exchange factors (GEFs), such as SopE from *Salmonella*,

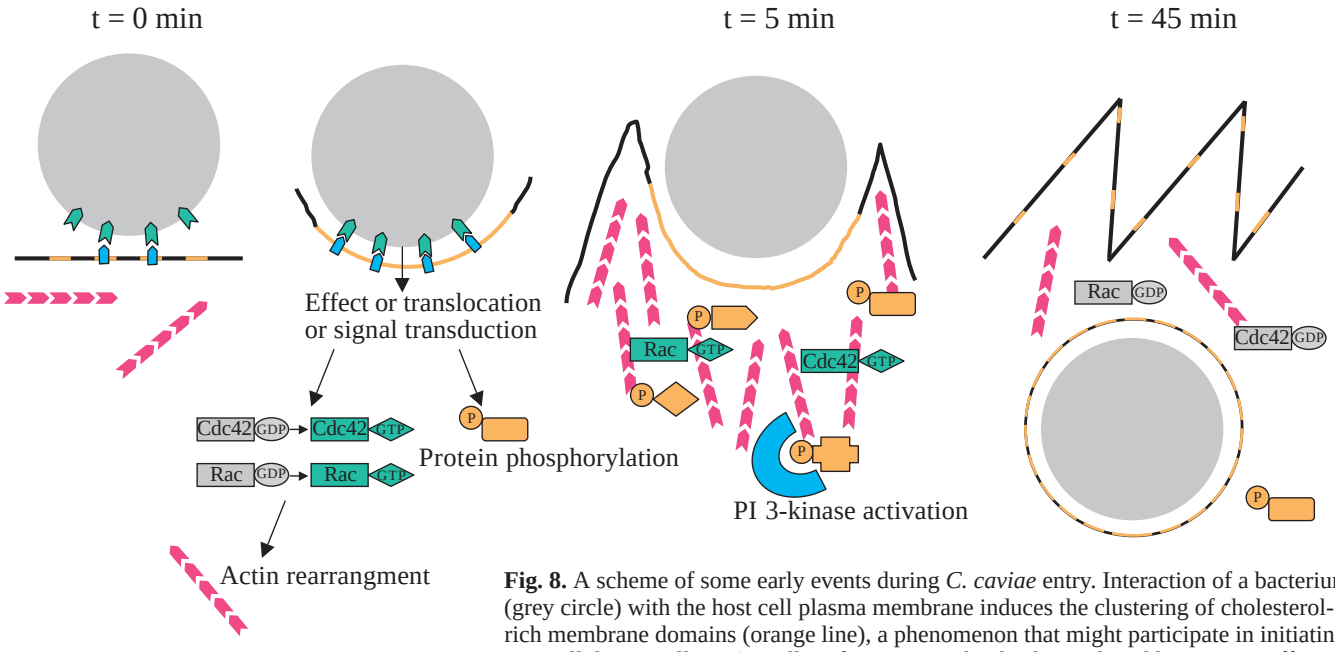

Fig. 8. A scheme of some early events during *C. caviae* entry. Interaction of a bacterium (grey circle) with the host cell plasma membrane induces the clustering of cholesterol-rich membrane domains (orange line), a phenomenon that might participate in initiating intracellular signalling. Signalling for entry might also be mediated by putative effector molecule(s) secreted by a type III mechanism. The small GTPases Cdc42 and Rac, which control proper actin polymerization, are rapidly activated. Abundant protein phosphorylation is detected and phosphorylated proteins (orange shapes) accumulate at the entry sites where they take part in the signalling cascade. In particular, several phosphorylated proteins can interact with the PI 3-kinase (blue), whose activity is required for *C. caviae* entry. Activation of Cdc42 and Rac is transient and is followed by the depolymerization of actin filaments, associated with a decrease in the accumulation of phosphorylated proteins around internalized bacteria. Although bacteria-associated actin patches have disappeared 45 minutes p.i., general remodelling of the cytoskeleton can be observed for longer times after infection.

which directly activates Rac and Cdc42 (Hardt et al., 1998), or can be proteins that indirectly promote small GTPase activation, such as IpaC from *Shigella* (Van Nhieu et al., 1999). Since *Chlamydiaceae* possess a type III apparatus, which is present on the EBs (Fields et al., 2003), proteins secreted via this mechanism may play a role in activating Cdc42 and Rac. Alternatively, attachment of bacteria to the host cell may induce intracellular signalling, which would eventually lead to the activation of the small GTPases. The association of the bacteria with DRMs and subsequent clustering of these domains observed here, which have been shown to be sites of signal transduction in several systems, may initiate the cascade that eventually leads to the entry of the pathogen into the cell.

Whereas the participation of GTPases of the Rho family is a common theme in pathogen entry, the involvement of PI 3-kinases is more restrictive, since PI 3-kinase inhibitors do not affect the entry of a number of pathogens (Mecsas et al., 1998). Even among *Chlamydiaceae*, there are different requirement for PI 3-kinase activity: PI 3-kinase inhibitors impaired *C. caviae* (Fig. 7A) and *C. pneumoniae* (Coombes and Mahony, 2002) entry, but not *C. trachomatis* LGVL2 entry (Carabeo et al., 2002). Which step in *C. caviae* entry is controlled by PI 3-kinase activity? In macrophages, PI 3-kinases have been shown to be involved in a number of steps. For example, with the development of specific probes which allow the visualization of the different products of PI 3-kinases, Vieira et al. were able to distinguish distinct roles of class I and class III PI 3-kinases in phagosome formation and maturation (Vieira et al., 2001). In another study, Cox et al. showed that in macrophages, PI 3-kinase activity was not required for FcγR-mediated actin assembly (Rac1-dependent pathway) but was required for pseudopod extension along ingested particles (Cox et al., 1999). They suggested that PI 3-kinase activity was necessary for coordinating membrane recruitment from intracellular pools and pseudopod extension. Similarly, our data show that PI 3-kinase activity may be involved downstream of the activation of the small GTPases, or in an independent signalling pathway. Indeed, PI 3-kinase inhibition did not prevent actin polymerization (Fig. 7B), or recruitment of Cdc42N17 or RacN17 to the entry sites (data not shown). Because of the small size of EBs, it is unlikely that membrane recruitment from intracellular pools is a limiting step as it is in the case of particle ingestion. However, there may be other steps controlled by PI 3-kinase activity, such as the closure of the vesicle containing the bacteria.

The ability to synchronize entry by centrifugation allowed us to observe very early events that had not been studied microscopically before. DRMs clustering was visible very early upon infection (5 minutes p.i., Fig. 2), at the sites of actin aggregation. Similar clustering of lipid raft markers, including GM1, has been observed in the process of entry of FimH-expressing (type 1 fimbriated) *E. coli* in bone marrow-derived mast cells, but the participation of the actin cytoskeleton was not addressed in the investigation (Shin et al., 2000). GM1 clustering during entry is a process too discrete to establish its precise timing. However, our observations suggest that GM1 clustering and actin polymerization occur in the same time frame, as we were not able to dissociate the two events at the shorter post-infection times examined (2 minutes p.i., data not shown). A close link between DRM clustering and actin polymerization has been established in lymphoid cells in which raft clustering by cross-linking of some of the raft components leads to local actin polymerization (Harder and Simons, 1999). Moreover, it was shown that tyrosine phosphorylated proteins accumulated in raft patches, and that the accumulation of actin may be dependent on phosphotyrosine accumulation. We also observed that tyrosine-phosphorylated proteins accumulated 5 minutes p.i. where actin patches formed (Fig. 3), suggesting that invasive bacteria are able to initiate local phosphorylation events. This accumulation of phosphorylated proteins was transient and was no longer observed 45 minutes p.i. (data not shown). In cells transfected with dominant negative forms of Cdc42 or Rac, which inhibit entry to a large extent, tyrosine phosphorylation upon infection seemed unaffected, suggesting that initiation of phosphorylation precedes entry (data not shown).

In this paper we report some of the early events that take place upon infection by *C. caviae* (Fig. 8). This study raises important questions concerning the nature of the host receptor and its participation in signal transduction, the involvement of bacteria-secreted effectors, the links between the different partners we analysed, and the other signalling molecules involved. We know that some of the signalling pathways diverted by pathogens to promote their entry are shared by several micro-organisms, while others are unique. We anticipate that among the *Chlamydiaceae*, which have evolved to adapt to different niches, further studies will reveal some diversity in the strategies developed to ensure efficient entry in the host cell.

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