Early signaling events involved in the entry of *Rickettsia conorii* into mammalian cells

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Accepted 24 June 2004
Journal of Cell Science 117, 5097-5106 Published by The Company of Biologists 2004
doi:10.1242/jcs.01382

Summary

Rickettsia conorii*, the causative agent of Mediterranean spotted fever, is able to attach to and invade a variety of cell types both in vitro and in vivo. Although previous studies show that entry of *R. conorii* into non-phagocytic cells relies on actin polymerization, little else is known about the molecular details governing *Rickettsia*-host cell interactions and actin rearrangements. We determined that *R. conorii* recruits the Arp2/3 complex to the site of entry foci and that expression of an Arp 2/3 binding derivative of the WASP-family member, Scar, inhibited bacterial entry into Vero cells, establishing that Arp2/3 is an active component of this process. Using transient transfection with plasmids expressing dominant negative versions of small GTPases, we showed that Cdc42, but not Rac1 is involved in *R. conorii* invasion into Vero cells. Using pharmacological approaches, we show that this invasion is dependent on phosphoinositide (PI) 3-kinase and on protein tyrosine kinase (PTK) activities, in particular Src-family kinases. C-Src and its downstream target, p80/85 cortactin, colocalize at entry sites early in the infection process. *R. conorii* internalization correlated with the tyrosine phosphorylation of several other host proteins, including focal adhesion kinase (FAK), within minutes of *R. conorii* infection. Our results reveal that *R. conorii* entry into non-phagocytic cells is dependent on the Arp2/3 complex and that the interplay of pathways involving Cdc42, PI 3-kinase, c-Src, cortactin and tyrosine-phosphorylated proteins regulates Arp2/3 activation leading to the localized actin rearrangements observed during bacterial entry. This is the first report that documents the mechanism of entry of a rickettsial species into mammalian cells.

Key words: *Rickettsia conorii*, Invasion, Arp 2/3, Cdc42, Tyrosine phosphorylation, Actin

Introduction

Rickettsiae are obligate intracellular gram-negative pathogens that are transmitted to humans via arthropod vectors (Hackstadt, 1996). Based upon the antigenicity of their lipopolysaccharide (LPS) and the differences in the diseases that they cause, members are divided into two groups, the spotted fever group (SFG) and the typhus group (TG) (Vishwanath, 1991). Both groups have been classified by the National Institute of Allergy and Infectious Diseases (NIAID) as 'select agents' for bioterrorism (http://www2.niaid.nih.gov/biodefense/bandc_priority.htm). *Rickettsia prowazekii*, the causative agent of epidemic typhus, is transmitted via the human body louse whereas *Rickettsia conorii*, the causative agent of Mediterranean spotted fever, is transmitted primarily via tick bite inoculation of bacteria into the skin of the human host. The genomes of both *R. conorii* (Ogata et al., 2001) and *R. prowazekii* (Andersson et al., 1998) have been sequenced; however, as there are no genetic tools currently available, the utilization of sequence data is severely limited.

Subsequent proliferation of SFG rickettsiae at the site of inoculation, typically in endothelial cells, results in the characteristic dermal and epidermal necrosis known as 'eschar' or 'tache noire' (Walker et al., 1988). Injury to the vascular endothelium leads to an increase in vascular permeability and leakage of fluid into the interstitial space, resulting in the characteristic dermal rash (Hand et al., 1970; Walker et al., 1988). Bacteria can then spread via lymphatic vessels to the lymph nodes and via the bloodstream to various other tissues including the lungs, spleen, liver, kidneys and heart (Walker and Gear, 1985). Although advances in modern medicine have reduced the rate of fatality in infected patients, there is still an estimated 4-5% of infected patients, especially children, who die of Mediterranean spotted fever (Yagupsky and Wolach, 1993).

Adherence to the target endothelial cells is a critical step in the establishment of a successful infection. SFG rickettsiae putative adhesins, rOmpA (Li and Walker, 1998) and rOmpB (Uchiyama, 2003) have been described; however, currently there are no genetic tools available for rickettsial species to determine whether or not these proteins are necessary and sufficient in mediating internalization. Subsequent to adherence, rickettsiae, like some other pathogenic bacteria, enter into non-phagocytic host cells and then quickly lyse the phagocytic vacuole (Hackstadt, 1996). Within the cytoplasm, rickettsiae begin to divide and in some cases are able to polymerize host actin filaments to propel themselves intra- and intercellularly (Gouin et al., 2004; Gouin et al., 1999; Heinzen et al., 1993; Teysseire et al., 1992).

Interactions of TG and SFG rickettsiae with various cultured cells show that internalization is associated with a phospholipase A_2 activity and host actin polymerization (Silverman et al., 1992; Walker et al., 2001; Walker, 1984).

However, little more is known about the interactions between SFG rickettsiae and cultured cells, in particular the mechanism(s) by which *R. conorii* invades non-phagocytic cells. An initial investigation of proteins that could control actin dynamics during *R. conorii* invasion revealed that the Arp2/3 complex is recruited to the entry site. We then utilized various approaches to disrupt signaling pathways that have been previously demonstrated to activate the Arp2/3 complex directly or indirectly. We found that *R. conorii* uses pathways involving Cdc42, PI 3-kinase, c-Src and other PTK activities to enter non-phagocytic cells and that signals from these pathways may be coordinated to ultimately activate the Arp2/3 complex.

Materials and Methods

Cell lines and bacterial strains

The African green monkey kidney epithelial cell line, Vero (ATCC CRL 1587), was cultured in DMEM (Gibco-BRL) supplemented with 10% fetal calf serum (Valbiotech) at 37°C, 10% CO_2 and cells were used between passages 8-18. *Rickettsia conorii* was grown in Vero cells with DMEM supplemented with 10% fetal calf serum at 32°C, 5% CO_2 and purified and stored at –80°C as previously described (Gouin et al., 1999).

Plasmids, antibodies and other reagents

The cDNAs encoding dominant negative Rac1 (N17Rac1-GFP) and Cdc42 (N17Cdc42-GFP) in pEGFP were kind gifts from Dr Philippe Chavrier (Institut Curie, Paris, France). The cDNAs encoding c-Myc epitope-tagged full length Scar (Scar FL) and the Scar WA domain in pRK5 were kindly provided by Dr Laura Machesky and have been described (Machesky et al., 1999).

Rabbit polyclonal antisera to human p85α and monoclonal antibodies against phosphotyrosine (clone 4G10), c-Src (clone GD11) and cortactin (clone 4F11) were purchased from Upstate Biotechnology (UBI). Monoclonal antisera against actin (clone AC-40), polyclonal horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG antisera used for immunoblotting were obtained from Sigma. For immunofluorescence studies, Alexa488-phalloidin, Alexa488-conjugated goat anti-rabbit IgG and goat anti-mouse IgG, Cy3-conjugated goat anti-rabbit IgG, Cy5-conjugated goat anti-rabbit IgG and goat anti-mouse IgG and Alexa546-conjugated goat anti-mouse IgG antisera were purchased from Molecular Probes. Monoclonal antibody against the c-Myc epitope tag (clone 9E10) and Cdc42 (clone B-8) and rabbit polyclonal antibody against c-Src (SRC 2) and FAK (A-17) were obtained from Santa Cruz Biotechnology. Rabbit polyclonal anti-*R. conorii* antisera (R47) (Gouin et al., 1999) and mouse polyclonal anti-*R. conorii* serum (S1) have been described (Gouin et al., 2004). Rabbit polyclonal Arp3 antisera has also been described (David et al., 1998).

Protein phosphatase 1 (PP1) was obtained from Calbiochem and genistein, cytochalasin D and wortmannin were obtained from Sigma. Complete protease inhibitor cocktail was purchased from Boehringer Mannheim.

Bacterial internalization assay

Vero cells were seeded onto sterile glass coverslips in 24-well plates (Costar) 24 hours prior to use (approximately 1.2×10^5 cells/well). On the day of the experiment, cells were washed three times with DMEM and serum starved in DMEM for 4 hours at 37°C, 5% CO_2. 30 minutes prior to infection, cells were incubated in DMEM containing PP1, genistein, wortmannin or cytochalasin D at the indicated concentrations. As a control, Vero cells were incubated with DMEM containing 0.5% DMSO. Vero cells were infected with *R. conorii* at a multiplicity of infection (MOI) of 10-15, centrifuged for 5 minutes at 200 ***g*** at room temperature and then shifted to 37°C, 5%CO_2 for 30 minutes to induce bacterial internalization. Cells were washed, fixed for 20 minutes in 3.5% paraformaldehyde at room temperature and then processed for immunofluorescence.

For immunofluorescence staining of extracellular bacteria, infected Vero cells were incubated with rabbit anti-*R. conorii* antisera (R47, 1:500 in 1× PBS, 2% BSA) for 1 hour at room temperature and then incubated with goat anti-rabbit IgG-Cy3 (1:500 in 1× PBS, 2% BSA) for 45 minutes at room temperature. In order to stain total bacteria, cells were permeabilized for 5 minutes in 0.1% Triton X-100 in 1× PBS and re-incubated with the R47 antisera and Alexa488 anti-rabbit IgG (1:500) for 45 minutes at room temperature as described above. Cells were rinsed in PBS and then glass coverslips were mounted in Mowiol mounting medium. Preparations were viewed on a Zeiss Axiovert 135 fluorescence microscope and images were captured and processed using the Metamorph software package (Universal Imaging). Invasion indices are presented as intracellular bacteria versus total bacteria per cell. Approximately 150 infected cells were counted for each experiment and performed at least in duplicate. Pre-incubation with pharmacological inhibitors had no effect on bacterial adherence or on cellular viability. The data presented are representative of at least three different experiments.

Examination of the cell cytoskeleton and colocalization of host proteins

Vero cells were seeded onto sterile 12 mm glass coverslips and then infected with *R. conorii* as described above. After 15 minutes, cells were washed with ice-cold PBS, fixed in 3.5% PFA, 1× PBS and extracellular bacteria were stained with rabbit anti-*R. conorii* (R47) and anti-rabbit IgG-Cy3 conjugated antibodies as described above. Actin filaments were visualized with Alexa488-conjugated phalloidin (1:500 in 1× PBS, 2% BSA). To visualize *R. conorii* and the distribution of Cdc42, c-Src and cortactin in infected Vero cells (15 minutes post-infection), cells were incubated with R47 antisera (1:500) and Alexa488-conjugated anti-rabbit IgG. To visualize *R. conorii* and the distribution of phosphotyrosine proteins, extracellular bacteria were stained as described except that the secondary antiserum used was Cy3-conjugated anti-rabbit IgG. Cells were permeabilized with 0.1% Triton X-100 in PBS and then incubated with the indicated antiserum (4 μg/ml Cdc42; 5 μg/ml c-Src; 5 μg/ml 4G10 or 10 μg/ml cortactin) and Cy3-conjugated anti-mouse IgG (1:500) or Alexa488-conjugated anti-mouse IgG.

To visualize endogenous Arp3, *R. conorii*-infected Vero cells were incubated with mouse anti-*R. conorii* polyclonal antiserum (1:100) and anti-mouse IgG Alexa488-conjugated antibodies (1:500). Cells were then permeabilized with 0.1% Triton X-100 in 1× PBS and total bacteria were labeled with mouse anti-*R. conorii* (S1) and anti-mouse IgG-Cy5 conjugated antibodies. Arp3 staining was performed using rabbit polyclonal Arp3 antibodies (1:500) and anti-rabbit IgG Alexa546-labelled antisera. After immune staining, cells were mounted in Mowiol mounting media and images were captured as described above.

Transient cell transfection

Vero cells were seeded (approximately 1.2×10^5 cells/well) onto sterile glass coverslips in 24-well plates (Costar) 24 hours prior to transfection. On the day of the experiment, cells were washed twice with PBS and then transfected with 1 μg of the indicated DNA per well using the Lipofectamine 2000 reagent according to manufacturer's instructions. 24 hours post transfection, cells were washed with DMEM and infected with *R. conorii* (MOI ~20), centrifuged for 5 minutes at room temperature at 200 ***g*** and then incubated at 37°C and 10% CO_2 for 30 minutes. Infected cells were washed with ice-cold PBS, fixed with 3.5% PFA in PBS for 5 minutes

at room temperature and then processed for immunofluorescence. Briefly, extracellular and total bacteria were stained with rabbit anti-*R. conorii* antisera (R47) as described above except that after cell permeabilization, total bacteria were detected with anti-rabbit IgG Cy5-conjugated secondary antisera. Transfected cells were identified with anti-cMyc epitope antisera (1:500) and Alexa546 anti-mouse IgG (1:500) or through the expression of the GFP fusion protein. For each experiment, at least 50 transfected cells associated with bacteria were counted in duplicate. Data are presented as internal bacteria versus total bacteria per transfected cell and are representative of at least two independent experiments.

Immunoprecipitation and western immunoblotting

Vero cells were seeded onto six-well plates (Costar) in DMEM with 10% FBS overnight at 37°C and 5% CO_2 (approximately 3.0×10^5 cells/well). On the day of the experiment, cells were washed twice with DMEM and serum starved for 4 hours at 37°C and 5% CO_2. Cells were either left uninfected or infected with *R. conorii* (MOI 50), centrifuged at 200 ***g*** for 5 minutes at room temperature and quickly shifted to 37°C, 5% CO_2 for the indicated time. After each time point, cells were washed three times with ice-cold PBS and then lysed in 500 μl 1% NP-40 lysis buffer (1% NP-40, 20 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 20 mM NaF, 3 mM Na_3VO_4, 1× Complete Protease Inhibitor cocktail). Lysates were centrifuged at 15,000 ***g*** for 15 minutes (4°C) to pellet insoluble matter. Samples were adjusted for equal protein content and immunoprecipitated with anti-phosphotyrosine antisera (2 μg 4G10/sample) or anti-FAK antisera (2 μg/sample) overnight at 4°C. Immune complexes were captured with 30 μl 50% protein A-Sepharose slurry for 1 hour at 4°C, washed three times with 1% NP-40 lysis buffer and then boiled in SDS sample buffer. Immune complexes were resolved by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose. For western immunoblotting, membranes were incubated for 1 hour at room temperature in 1× TBST with 3% BSA containing anti-phosphotyrosine (1 μg/ml), anti-p85α (1 μg/ml) antibodies or anti-c-Src antibody (1 μg/ml, SRC 2) as indicated and then incubated in anti-rabbit IgG-HRP (Sigma, 1:5000) or anti-mouse IgG-HRP (Sigma, 1:5000) where appropriate. In some experiments, equal amounts of pre-immune precipitated lysates were separated on SDS-PAGE and immunoblotted with the indicated antisera as described above to serve as a protein loading control.

Proteins were visualized with Super Signal WestPico enhanced chemiluminescence system (Pierce) and exposure to film. In some cases, films were scanned on a SNAPSCAN 1236 scanner (AGFA) and subsequent densitometric analysis of protein bands was performed using the ImageQuant software package (Molecular Dynamics). Bands were normalized against the amount of protein present in pre-immune precipitated lysates as described above. In some experiments, blots were stripped with Restore stripping solution (Pierce) and reblotted with the indicated antisera to demonstrate equal protein loading in each lane. Blots shown are representative of at least three different experiments.

Results

Internalization of *R. conorii* into Vero cells is dependent on actin polymerization.

Electron micrographs of the *R. conorii* invasion process demonstrated that bacterial entry requires host cytoskeletal alterations at the site of infection (Gouin et al., 1999) probably involving localized actin rearrangements. The effect of bacterial adherence on the host cell cytoskeletal network was visualized using Vero cells that were infected with *R. conorii* for 15 minutes. As shown in Fig. 1A, F-actin colocalized with

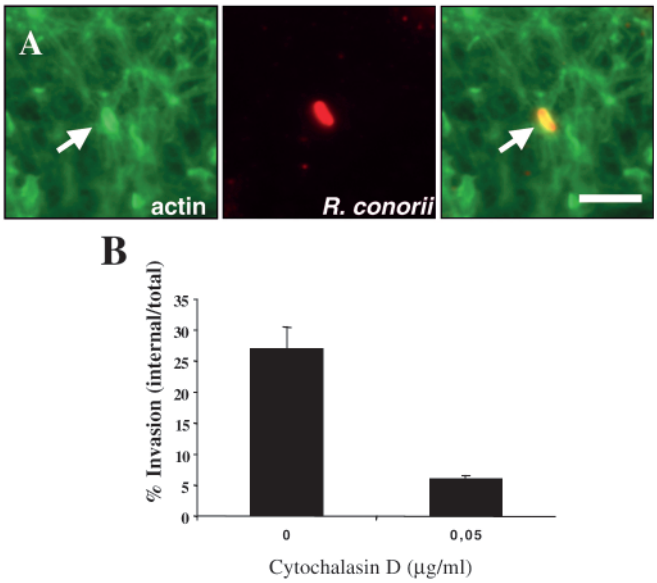

Fig. 1. Invasion of *Rickettsia conorii* is dependent on host actin polymerization. (A) In Vero cells infected with *R. conorii*; F-actin (green, left panel) colocalizes with invading bacteria (red) within 15 minutes of infection, as shown in the merged image (yellow, right panel). (B) Preincubation of Vero cells with cytochalasin D diminished the ability of *R. conorii* to invade. Bar, 2 μm.

adherent bacteria suggesting that this recruitment of actin was involved in uptake (arrows). To investigate the role of the actin polymerization in the entry process, we used a specific pharmacological inhibitor of actin polymerization (cytochalasin D) in conjunction with a fluorescence-based internalization assay (see Materials and Methods). Inhibition of actin polymerization blocked *R. conorii* invasion (Fig. 1B), but had no effect on bacterial adherence (data not shown), confirming the important role of actin rearrangements in the invasion process.

Involvement of the Arp2/3 complex in *R. conorii* invasion.

To identify proteins that modulate the actin cytoskeleton and that are potentially involved in *R. conorii* invasion, we first focused on the Arp2/3 complex. The Arp2/3 complex has a direct role in initiating actin nucleation and the branching of actin filaments (Bear et al., 2001; Bear et al., 2002). Initially, to investigate the role of the Arp2/3 complex in *R. conorii* entry, Vero cells were infected with *R. conorii* for 15 minutes and then processed for immunofluorescence using a polyclonal anti-Arp3 antisera (David et al., 1998). As shown in Fig. 2A, Arp3 strongly colocalizes with bacteria at the cell surface (arrow), but not with bacteria that are already internalized (arrowhead), suggesting that the Arp2/3 complex could play a role in *R. conorii* entry.

The Wiskott-Aldrich syndrome protein (WASp) family member Scar1, directly binds to and activates the actin nucleating activity of the Arp2/3 complex and affects Arp2/3-dependent actin reorganization (Machesky et al., 1999). Therefore, if Arp2/3 is required for *R. conorii* invasion, overexpression of Scar1 and an Arp2/3 binding derivative (Scar

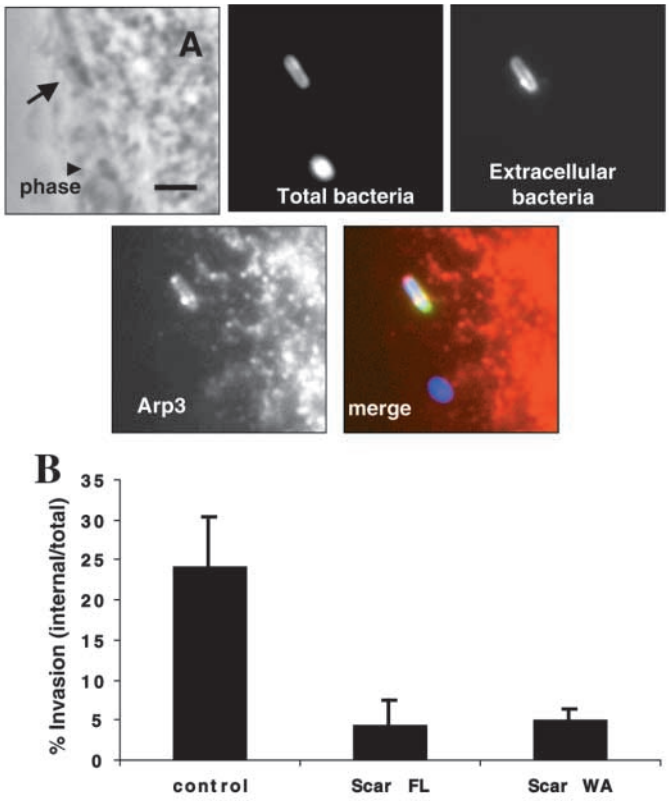

Fig. 2. The Arp2/3 complex is required for efficient bacterial internalization. (A) Extracellular *Rickettsia conorii* (arrow), but not intracellular bacteria (arrowhead) strongly colocalizes with Arp3, suggesting that Arp2/3 recruitment plays an essential role in the uptake process in Vero cells. (B) Transfection of the WASp-related protein, Scar (Scar FL), and the Arp2/3 binding domain (Scar WA) inhibits the ability of *R. conorii* to enter Vero cells, but has no effect on bacterial adherence (data not shown). Bar, 2 μm.

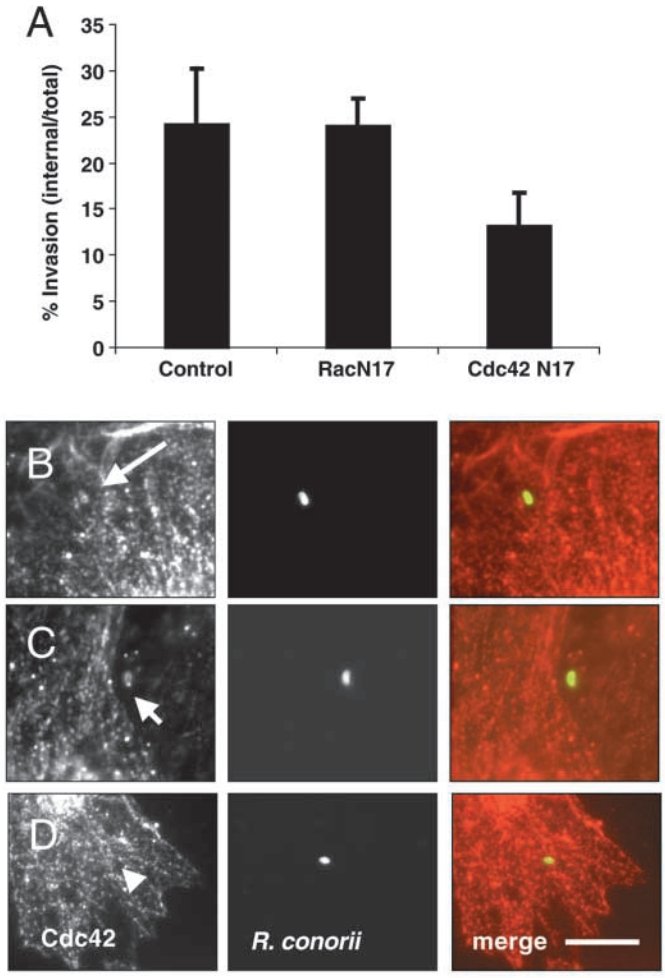

Fig. 3. Cdc42 but not Rac1 governs actin rearrangements associated with *Rickettsia conorii* invasion. (A) Expression of dominant negative N17Cdc42, but not N17Rac1 in Vero cells inhibited *R. conorii*-mediated invasion when compared to non-transfected (Control) cells as assessed by a fluorescence-based invasion assay (see Materials and Methods). Expression of either construct had no effect on bacterial adherence (data not shown). (B,C) Extracellular *R. conorii* were found to colocalize with endogenous Cdc42 (arrows) in non-transfected cells within 15 minutes of bacterial infection. (D) Colocalization of Cdc42 with bacteria is not caused by antibody crossreactivity with bacteria (arrowhead). Bar, 4 μm.

WA) could titrate endogenous Arp2/3 complexes and inhibit uptake into Vero cells. As shown in Fig. 2B, expression of full-length Scar1 (Scar FL) and the Scar1 WA domain (Scar WA) inhibited *R. conorii* entry into Vero cells. These results suggest that the recruitment and activation of the Arp2/3 complex at the site of bacterial entry may be a crucial, early event leading to the localized actin polymerization necessary for bacterial invasion.

Involvement of the small GTPase Cdc42, but not Rac1 in *R. conorii* entry

Small GTP-binding proteins, namely Rho family members Cdc42 and Rac and other members of the Ras superfamily of GTPases, act as guanine nucleotide exchange switches to regulate diverse cellular processes within host cells (Olson et al., 1995; Ridley and Hall, 1992; Ridley et al., 1992) including the reorganization of the actin cytoskeleton in migrating cells (Hall, 1998) and during the phagocytosis of pathogens (Alrutz et al., 2001; Galan and Zhou, 2000; Martinez and Hultgren, 2002; Tran Van Nhieu et al., 1999). It has also been demonstrated that Cdc42 and Rac utilize proteins of the WASp family as downstream effector proteins to activate Arp2/3 during cortical actin polymerization (Higgs and Pollard, 2001), suggesting that activation of Cdc42 and Rac could indirectly lead to Arp2/3-mediated cytoskeletal changes. We utilized transfection of dominant negative versions of Cdc42 and Rac1 (N17Cdc42 and N17Rac1, respectively) coupled with a fluorescence-based internalization assay to assess the effects of these proteins on bacterial entry. As shown in Fig. 3A, expression of N17Cdc42-GFP, but not N17Rac1-GFP, into Vero cells resulted in an inhibition of *R. conorii* entry (~45% inhibition compared to untransfected control). To investigate the putative role of Cdc42 in the entry process, non-transfected Vero cells were infected with *R. conorii* for 15 minutes and then processed for immunofluorescence microscopy using a monoclonal anti-Cdc42 antibody. *R. conorii* colocalized with

endogenous Cdc42 at bacterial entry sites (Fig. 3B,C, arrows). This recruitment appeared to be specific to invading bacteria as not all cell-associated *R. conorii* colocalized with endogenous Cdc42 (arrowhead in Fig. 3D). Taken together, these results suggest that activation of Cdc42 contributes to the localized actin polymerization that occurs at bacterial entry foci and may contribute to the activation of Arp2/3 during *R. conorii* invasion.

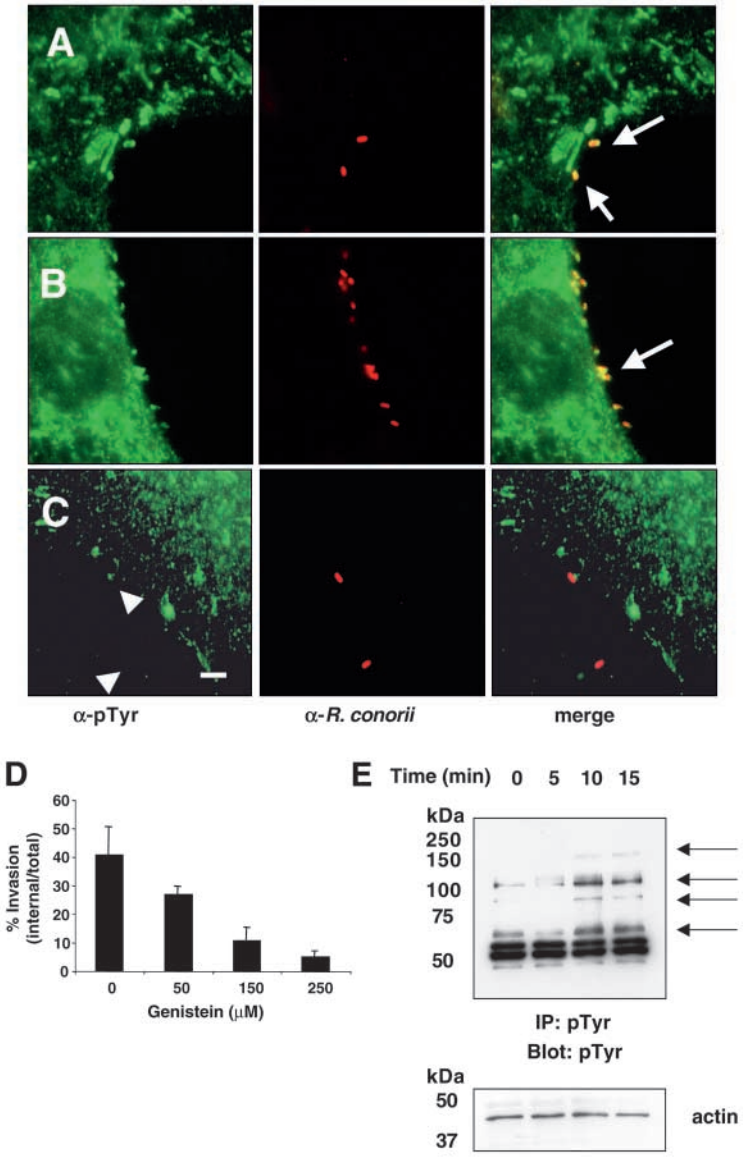

Fig. 4. Involvement of host protein tyrosine phosphorylation in the internalization of *Rickettsia conorii* into Vero cells.
(A,B) Immunofluorescence microscopy of infected cells demonstrates that extracellular *R. conorii* recruits tyrosine phosphoproteins (pTyr) to sites of adherence within 15 minutes of infection (arrows point to bacteria that have unambiguously recruited tyrosine phosphoproteins). (C) Specificity of pTyr protein colocalization with adherent *R. conorii* is not caused by antibody crossreactivity to bacterial antigens (arrowheads). (D) Pre-incubation of Vero cells with a PTK inhibitor, genistein, blocks *R. conorii* entry in a concentration-dependent fashion, highlighting a role for PTKs in bacterial entry. (E) Immunoprecipitation and western immunoblot analysis using anti-phosphotyrosine antisera (pTyr) revealed that *R. conorii* infection of Vero cells induces the phosphorylation of host proteins (arrows) 10 minutes after infection, with strong phosphorylation of a p125/130 protein. Immunoblot analysis of pre-immune precipitated cellular lysates with anti-actin antisera serves as a protein loading control. Bar, 3 μm.

Evidence for the involvement of protein tyrosine kinase (PTK) and PI 3-kinase activities in *R. conorii* entry

Several studies of other pathogens have demonstrated that bacterial attachment can lead to the activation of host signal transduction cascades, predominantly through tyrosine phosphorylation of proteins, that contribute to the internalization of pathogens into host cells (Finlay and Cossart, 1997; Finlay and Falkow, 1997). Infection of Vero cells with *R. conorii* induced the recruitment of tyrosine phosphoproteins to some (Fig. 4A,B, arrows), but not all (arrowheads in Fig. 4C) sites of entry within 15 minutes of infection. The involvement of host protein tyrosine phosphorylation in the *R. conorii* invasion pathway was further investigated using genistein, a potent general tyrosine kinase inhibitor. As shown in Fig. 4D, pre-incubation of Vero cells with genistein inhibits *R. conorii* entry in a concentration-dependent fashion, but has no effect on bacterial adherence (data not shown) highlighting the importance of PTK activity in the entry process.

The internalization of *R. conorii* into Vero cells appears to be very rapid, within 5-15 minutes post infection (Gouin et al., 1999; Teysseire et al., 1995), therefore signaling events including tyrosine phosphorylation of host proteins should be detectable within this early time frame. Vero cells were infected and changes in the phosphorylation state of host proteins after different time points were monitored by immunoprecipitation and western immunoblotting with anti-phosphotyrosine antisera. As shown in Fig. 4E, *R. conorii* induced the tyrosine phosphorylation of several host proteins (arrows), including a p125/130 protein with a peak of phosphorylation after 10 minutes of infection.

Tyrosine phosphorylation has previously been shown to be involved in PI 3-kinase activation, following the binding of the p85 subunit to proteins containing phosphotyrosine (Carpenter et al., 1993; Carpenter and Cantley, 1996). Class I PI 3-kinases are heterodimeric proteins, consisting of a non-catalytic p85 subunit and a catalytic p110 subunit, which catalyze the generation of 3-phosphoinositides PtdIns(3)*P*, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (also called PIP3) (Leevers et al., 1999). Lipid products such as PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are important secondary messengers within the host cell and can directly or indirectly modulate the actin cytoskeletal dynamics (Dekker and Segal, 2000; Hartwig et al., 1995; Vanhaesebroeck and Waterfield, 1999). Several gram-negative and gram-positive invasive bacteria, such as uropathogenic *E. coli* (UPEC) and *Listeria monocytogenes*, have evolved mechanisms with which to exploit the activity of PI 3-kinase to induce changes in the cellular cytoskeleton that in turn, facilitates the internalization of the

pathogen into the host cell (Ireton et al., 1996; Martinez et al., 2000). Thus, the role of PI 3-kinase in the *R. conorii* invasion pathway was investigated using wortmannin, a fungal toxin that specifically inhibits PI 3-kinase activity. Treatment of Vero cells with wortmannin reduced invasion in a concentration-dependent manner when compared to non-treated controls (Fig. 5A), but had no effect on bacterial adherence (data not shown). The mechanism of PI 3-kinase activation involved in *R. conorii* entry was addressed by immunoprecipitation of tyrosine phosphoproteins and western immunoblotting with p85α antisera. As shown in Fig. 5B, infection of mammalian cells with *R. conorii* resulted in a fivefold increase in the amount of p85α that co-immunoprecipitates with tyrosine phosphoproteins after bacterial infection for 10 minutes. Taken together, these results suggest that the activation of PI 3-kinase may be an early, key signal involved in cellular entry.

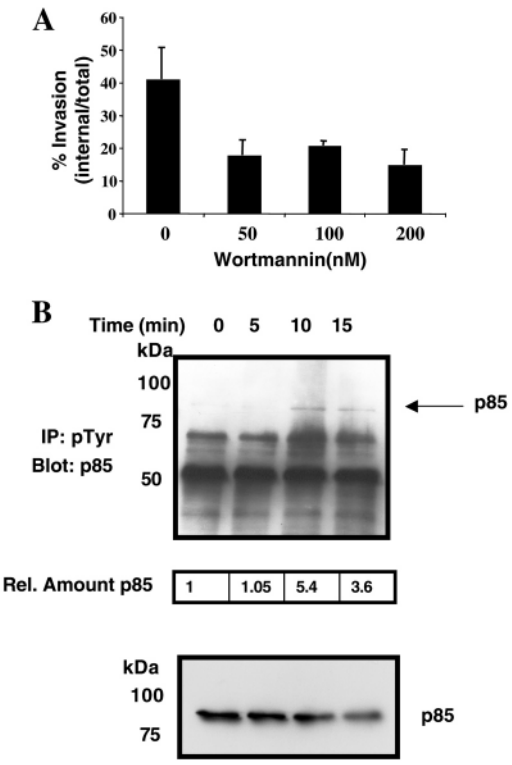

Fig. 5. PI 3-kinase activity is involved in *Rickettsia conorii* uptake. (A) A specific inhibitor of PI 3-kinase activity (wortmannin) blocks *R. conorii* invasion, with ~64% inhibition at 200 nM compared to levels in control cells. (B) *R. conorii* infection of Vero cells leads to co-immunoprecipitation of p85α with tyrosine phosphoproteins within minutes of infection (arrow), suggesting that PI 3-kinase activation is an important event in the entry process. Densitometric analysis of scanned blots indicates the relative amount of p85α that co-immunoprecipitates with pTyr proteins during *R. conorii* infection. Immunoblot analysis of cellular lysates with anti-p85 antibodies.

Focal adhesion kinase (FAK) is tyrosine phosphorylated in response to bacterial entry

As demonstrated above, *R. conorii* invasion of Vero cells correlates with a robust phosphorylation of a 125/130 kDa protein among other host proteins. Interestingly, pre-incubation of cells with chemical inhibitors of invasion, namely genistein, wortmannin and cytochalasin D, diminished the induced tyrosine phosphorylation of this p125/130 protein albeit at different levels (Fig. 6A). The 125 kDa protein, focal adhesion kinase (FAK), has been demonstrated to have a key role in remodeling the actin cytoskeleton during cellular migration (Richardson and Parsons, 1996) and is implicated in the entry of invasive pathogens into non-phagocytic cells (Martinez et al., 2000; Persson et al., 1997). Infection of Vero cells with *R. conorii* induced the tyrosine phosphorylation of FAK (Fig. 6B). Taken together, these results suggest that tyrosine phosphorylation of host proteins, including FAK, and recruitment of phosphotyrosine-containing proteins to bacterial entry foci may be key signaling events in the entry process.

c-Src and cortactin may play key roles during the *R. conorii* entry process

We next focused our efforts on c-src, a non-receptor PTK of approximately 60 kDa, which along with FAK promotes the tyrosine phosphorylation of several focal adhesion associated proteins including pp80/85 cortactin (Wu and Parsons, 1993) and has been shown to be involved in regulating cytoskeletal changes during the uptake of various invasive pathogens including *Shigella flexneri* (Dumenil et al., 2000), *Staphylococcus aureus* (Agerer et al., 2003) and *Chlamydia trachomatis* (Fawaz et al., 1997). As shown in Fig. 7A, *R. conorii* entry is associated with an accumulation of c-Src at some (arrows), but not all (arrowheads) points of bacterial entry. In addition, pharmacological inhibition of Src family tyrosine kinases by PP1 drastically reduced the ability of *R. conorii* to enter Vero cells in a concentration-dependent fashion (Fig. 7B), but did not affect either bacterial adherence or cellular viability (data not shown). Multiple mechanisms have been shown to activate pp60 c-src, including the interaction of c-src with phosphotyrosine containing proteins (Thomas et al., 1998). Infection of Vero cells with *R. conorii* results in a transient increase in the amount of pp60 c-src that co-immunoprecipitates with proteins containing phosphotyrosine, suggesting that association with tyrosine-phosphorylated proteins could be a mechanism for c-src activation during bacterial entry (Fig. 7C). Interestingly, *R. conorii* infection was further correlated with the colocalization of the c-src substrate, cortactin, at bacterial entry foci, further highlighting the importance of src-family PTK activity during the bacterial invasion process (Fig. 7D,E).

Discussion

Although invasion into host cells is critical for subsequent bacterial growth and establishment of a successful infection within the target host, the signal transduction events leading to *R. conorii* uptake have not yet been addressed. This is the first report that describes the host cell signaling cascades involved in the entry of a rickettsial species. We have shown that, as for other well-characterized invasive pathogens, *R. conorii* entry is dependent on a dynamic host cell actin cytoskeleton. Furthermore, our results demonstrate that *R. conorii* exploits the complex interplay between host signaling molecules

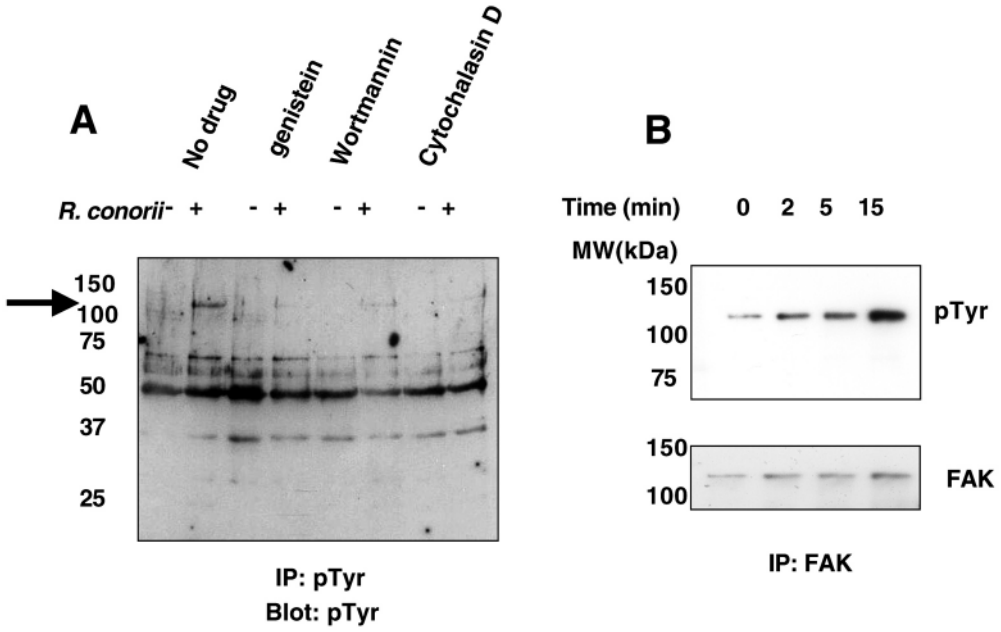

Fig. 6. *Rickettsia conorii* infection of Vero cells induces the tyrosine phosphorylation of pp125FAK. (A) Pre-incubation of Vero cells with the indicated inhibitors of bacterial invasion, blocks the induced tyrosine phosphorylation of at least one host protein (p125/130) after 5 minutes of bacterial infection (arrow). (B) Immunoprecipitation of pp125FAK and immunoblot analysis with anti-phosphotyrosine (pTyr) demonstrates an induced phosphorylation of FAK during the infection process, suggesting that this protein may play a key role in the entry process.

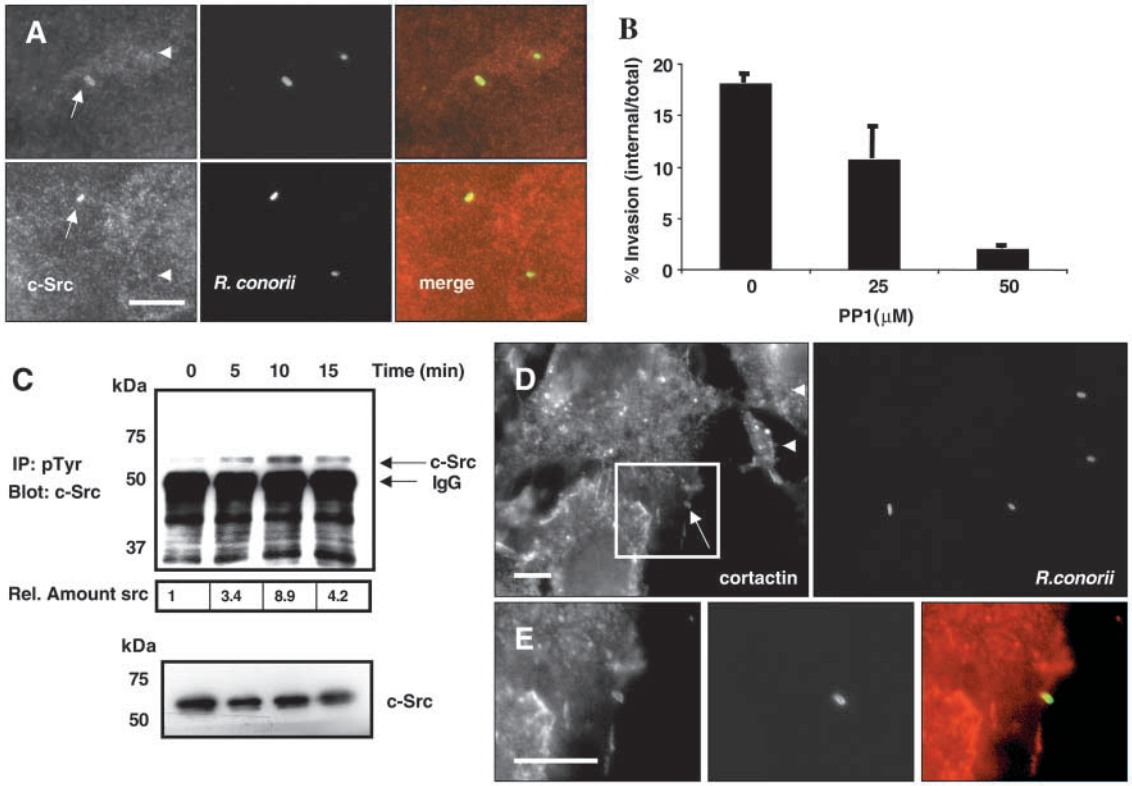

Fig. 7. Involvement of c-src and downstream effector, cortactin, in *Rickettsia conorii* invasion of mammalian cells. (A) Immunofluorescence microscopy reveals that *R. conorii* colocalizes with endogenous c-src within 15 minutes of infection (arrow). Colocalization is not caused by antibody crossreactivity as not all adherent bacteria recruit c-src (arrowheads). (B) Pharmacological inhibition of Src-family PTKs with protein phosphatase 1 (PP1), diminishes bacterial entry but has no effect on bacterial adherence or cellular viability (data not shown). (C) *R. conorii* infection induces the co-immunoprecipitation of c-Src with phosphotyrosine proteins, suggesting that this may be a mechanism for activating c-src during the invasion process. Densitometric analysis of scanned immunoblots indicates the relative amount of c-src that co-immunoprecipitates with pTyr proteins in response to bacterial infection. Immunoblot analysis of pre-immune precipitated cellular lysates serves as a loading control. (D) The c-src kinase substrate, cortactin, colocalizes with some (arrows), but not all (arrowheads) extracellular *R. conorii* within 15 minutes of infection, suggesting that specific cortactin recruitment plays a role in the uptake process. (E) The boxed area in (D) has been enlarged to show detail. Bars in (A,E), 6 μm; (D), 3 μm.

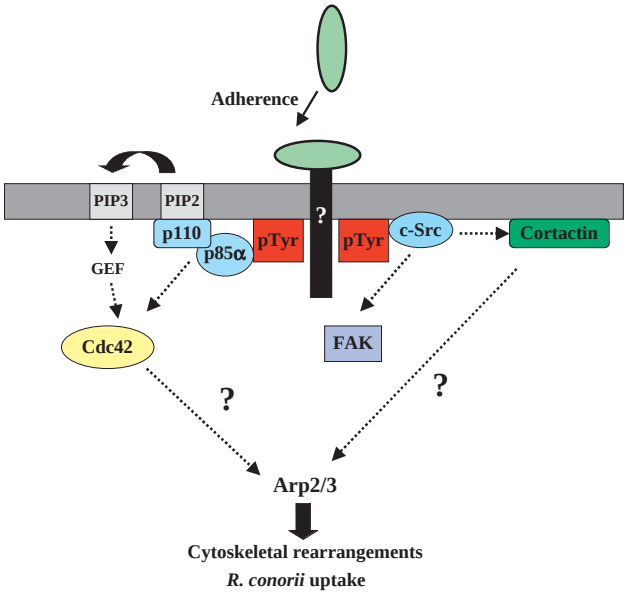

Fig. 8. Model of putative interactions involved in the uptake of *Rickettsia conorii* in non-phagocytic mammalian cells. Various signaling pathways can activate the Arp2/3 complex independently or in synergy. Internalization of *R. conorii* most likely involves the interaction of *R. conorii* surface protein(s) with an unidentified host cell receptor(s) (black rectangle with question mark), which serves to activate multiple pathways involving Cdc42, PI 3-kinase, Src, FAK and cortactin, ultimately leading to Arp2/3-dependent actin cytoskeletal changes that drive bacterial entry (see Discussion). Putative associations of proteins during bacterial entry are indicated by question marks and dotted arrows.

including Cdc42, PI 3-kinase, Src-family and other PTK activities to activate the Arp 2/3 complex to direct the localized changes in the actin cytoskeleton necessary for bacterial entry. How the different signals are integrated during the *R. conorii* invasion process to coordinate actin cytoskeletal changes remains to be elucidated.

The Arp2/3 complex has been previously shown to mediate signals generated through the Cdc42/N-WASp pathway (Rohatgi et al., 1999) as well as the Rac/IRSp53/Scar1/WAVE pathway (Machesky et al., 1999) to induce changes in the cortical actin cytoskeleton. Because Rac does not appear to be involved in the entry process and attempts to colocalize Scar1/WAVE to *R. conorii* entry sites have scored negative (Martinez and Cossart, unpublished observations), utilization of downstream Rac1 effectors such as Scar1/WAVE seems unlikely. We thus favor a working model (Fig. 8) in which active GTP-bound Cdc42 may directly bind to N-WASp allowing the acidic C-terminal (A) domain of N-WASp to recognize the Arp2/3 complex (Miki et al., 1998; Rohatgi et al., 2000; Rohatgi et al., 1999). However, whether or not N-WASp is involved in *R. conorii* entry is unclear as we have not been able to detect N-WASp at *R. conorii* entry foci. Interestingly, *Yersinia pseudotuberculosis* has been shown to utilize a Rac1-dependent, Cdc42-independent entry pathway into non-phagocytic cells, but the role of N-WASp has not been definitely established (Alrutz et al., 2001; McGee et al., 2001). The role of Cdc42 during the entry process also remains to be elucidated.

Although inhibition of Arp2/3 by overexpression of Scar1 constructs (ScarFL and ScarWA) strongly diminished *R. conorii* entry into mammalian cells, transfection of dominant negative Cdc42 (Cdc42N17) has a modest effect (approximately 45% inhibition) on entry. These results suggested that although Cdc42 is important for entry, another pathway may be utilized with Cdc42 or independently during entry to activate Arp2/3 leading to the observed actin polymerization at entry sites. Previous work has shown that although N-WASp and cortactin can function cooperatively to induce Arp2/3-dependent actin polymerization, they are also independent activators of Arp2/3 (Weaver et al., 2002). Our results suggest that *R. conorii* may induce a pathway involving the association of c-src with tyrosine-phosphorylated proteins, such as FAK, in order to relocalize c-src to the periphery (Schaller et al., 1999) and to sites of bacterial entry. Activation of Src early in the infection process (within 10 minutes of infection) could then recruit and potentially phosphorylate cortactin, allowing cortactin to interact directly with and subsequently activate the Arp 2/3 complex, leading to de novo actin polymerization as previously shown (Weaver et al., 2002) (Fig. 8). Interestingly, it has been demonstrated that the internalization of invasive pathogens *Shigella flexneri* (Dehio et al., 1995) and *Chlamydia trachomatis* (Fawaz et al., 1997) also correlated with an increase in cortactin tyrosine phosphorylation. Whether or not *R. conorii* invasion involves Src-mediated tyrosine phosphorylation of cortactin requires further investigation. These results suggest that *R. conorii* utilizes several pathways either in concert or independently to activate the Arp2/3 complex leading to bacterial entry.

Bacterial pathogens have been shown to use at least two morphologically and mechanistically distinct mechanisms, termed ‘trigger’ and ‘zipper’, to enter non-phagocytic cells. In the case of the trigger mechanism, invasive bacteria such as *Shigella* and *Salmonella* species, use a specialized secretion machinery called a type III secretion system to inject bacterial effector proteins into the host cell cytoplasm. These effectors can go on to modulate the activity of small GTPases, Rac and Cdc42, leading to intense actin-rich membrane ruffles and bacterial uptake (Galan and Zhou, 2000; Yoshida et al., 2002). In contrast, in the case of the zipper mechanism, invasive bacteria such as *L. monocytogenes*, UPEC and *Y. pseudotuberculosis*, use a strategy whereby the host cell membrane wraps or zippers around adherent bacteria in response to signals generated by the sequential interactions of specific host receptors with bacterial adhesin molecules. Electron micrographs of entry (Gouin et al., 1999; Teysseire et al., 1995) along with the results shown here suggest that the entry of *R. conorii* morphologically and mechanistically resembles a zipper-like invasion strategy. However, unlike *L. monocytogenes* which utilizes internalin (InlA) and E-cadherin as well as internalin B (InlB) and the hepatocyte growth factor receptor (HGFR or c-Met) (reviewed by Cossart et al., 2003) and unlike *Y. pseudotuberculosis* which utilizes invasin and β1-integrins (reviewed by Isberg and Barnes, 2001) for entry, the bacterial adhesin(s) and host receptor(s) for *R. conorii* are not known. Identification of host receptor(s) and bacterial adhesin(s) mediating *R. conorii* invasion is now a priority and is currently being investigated. With the threat of the potential use of rickettsiae as agents for biological terrorism, understanding the complex interplay between the pathogen and

host cell receptor(s) is very important and may provide insight into the development of more efficacious therapies for the treatment and prevention of SFG rickettsial diseases.

We would like to thank Dr Hélène Bierne and Dr Stéphanie Seveau along with other members of the Cossart lab for helpful discussions and critical reading of this manuscript. We would also like to thank Dr Philippe Chavrier (Institut Curie, Paris, France) for the kind gift of GFP-N17Rac1 and GFP-N17Cdc42 expression constructs and Dr Laura Machesky for the kind gift of c-Myc tagged Scar1 (ScarFL, Scar WA) constructs. This work was supported by the Institut Pasteur and a grant from Délégation Générale pour l'Armement (DGA contract 01-34-068). This work was also supported in part by an EMBO Longterm Post-Doctoral Fellowship (ALTF-171-2001), a post-doctoral fellowship from the Institut Nationale de la Recherche Agronomique (INRA) and a post-doctoral fellowship from the Fondation pour la Recherche Medicale (FRM) to J. J. Martinez. P. Cossart is an international research scholar of the Howard Hughes Medical Institute.

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