Invasion of endothelial cells by *Neisseria meningitidis* requires cortactin recruitment by a phosphoinositide-3-kinase/Rac1 signalling pathway triggered by the lipo-oligosaccharide

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Summary

Type-IV-pilus-mediated adhesion of *Neisseria meningitidis* (also known as meningococcus) to human endothelial cells induces the formation of membrane protrusions leading to bacterial uptake. We have previously shown that these protrusions result from a Rho- and Cdc42-dependent cortical actin polymerization, and from the activation of the ErbB2 tyrosine-kinase receptor and the Src kinase, leading to tyrosine phosphorylation of cortactin. We report here that *N. meningitidis* mutants expressing a deglycosylated lipo-oligosaccharide are poorly invasive. These mutants show structurally altered actin polymerization. Moreover, although they efficiently recruit and activate ErbB2 and Src, these mutants are defective in the recruitment and phosphorylation of cortactin. We demonstrate that phosphorylated cortactin controls the cortical actin polymerization, which leads to membrane protrusion formation. In addition, we show that cortactin recruitment is dependent on the activation of a phosphoinositide-3-kinase/Rac1-GTPase signalling pathway, which is required for actin polymerization and internalization of *N. meningitidis*, and is not activated by the mutant strains. Altogether, these results define a new role for the lipo-oligosaccharide in triggering a phosphoinositide-3-kinase/Rac1 signalling required to elicit an efficient uptake of *N. meningitidis* in non-phagocytic cells.

Key words: Cortactin, Rac1, Phosphoinositide-3-kinase, *N. meningitidis*, Lipo-oligosaccharide, ErbB2

Introduction

Cortactin has recently emerged as a potentially crucial regulator of actin assembly (Olazabal and Machesky, 2001; Weed and Parsons, 2001). This multidomain actin-binding protein can bind and activate the actin-related protein 2/3 (Arp2/3) complex that is a key component in the formation of branched networks of actin filaments at the cell cortex (Uruno et al., 2001; Weaver et al., 2001; Weaver et al., 2002; Weaver et al., 2003). Through this interaction, cortactin is involved in the formation of actin-rich structures such as lamellipodia or membrane ruffles, and it therefore plays a major role in cell motility. In most cell types, cortactin localizes to the perinuclear region and translocates to the cell cortex in response to many stimuli, including growth factors or engagement of adhesion molecules (Weed and Parsons, 2001). The activation of the Rac-1 GTPase seems required for cortactin translocation to the cortical actin network and its subsequent tyrosine phosphorylation in response to growth-factor stimulation (Head et al., 2003; Weed et al., 1998). Although cortactin was first discovered as a prominent substrate for the Src protein tyrosine kinase, the effect of tyrosine phosphorylation on its activity is still unclear. Removal of the C-terminal domain, including the Src phosphorylation site, does not affect the ability of cortactin to activate the Arp2/3 complex (Uruno et al., 2001), whereas expression of a cortactin mutated in all three Src tyrosine phosphorylation sites decreases the rate of endothelial-cell migration in culture (Huang et al., 1998) and the metastatic potential of breast-cancer cells (Li et al., 2001) through an unknown mechanism.

We have recently shown that *Neisseria meningitidis*, an extracellular human-specific pathogen responsible for septicaemia and meningitis (also referred to as meningococcus), promotes the recruitment and tyrosine phosphorylation of cortactin at its site of entry, suggesting that cortactin might play a role in the invasion process of these bacteria (Hoffmann et al., 2001). Invasion of *N. meningitidis* through host barriers involves a complex series of events that require the dynamic rearrangements of the actin cytoskeleton to allow the formation of membrane protrusions, membrane fusion and vesicular formation (Nassif et al., 2002). Virulent encapsulated bacteria first adhere to epithelial or endothelial target cells through their type-IV pili, which are long filamentous protein structures, and proliferate locally to form

a colony at their site of attachment on the cell surface. When adhering to the apical membrane of epithelial cells, *N. meningitidis* induces the local elongation of microvilli towards the bacteria, leading to their engulfment and internalization (Merz et al., 1996; Pujol et al., 1997). Adhesion of *N. meningitidis* to endothelial cells promotes the local formation of membrane protrusions reminiscent of epithelial microvilli structures that surround bacteria and provoke their internalization within intracellular vacuoles (Eugene et al., 2002). This process was observed both in vitro and ex vivo, suggesting that it is essential for the crossing of human endothelium via a transcytosis pathway (Nassif et al., 2002).

The formation of membrane protrusions by encapsulated *N. meningitidis* stems from the organization of specific molecular complexes, referred to as cortical plaques, underneath bacterial colonies. Cortical plaques result from the recruitment of the molecular linkers ezrin and moesin, which cluster several membrane-integral proteins such as CD44 or ICAM-1, and from the localized polymerization of cortical actin (Eugene et al., 2002; Merz and So, 1997; Merz et al., 1999). Both RhoA and Cdc42 GTPases are crucial for the actin-cytoskeleton rearrangements induced by *N. meningitidis* in endothelial cells (Eugene et al., 2002). Moreover, we have shown that the pilus-mediated adhesion of encapsulated *N. meningitidis* onto human endothelial cells induces the clustering and tyrosine phosphorylation of the host-cell tyrosine-kinase receptor ErbB2 (Hoffmann et al., 2001). In turn, ErbB2 activates the Src protein tyrosine kinase and hence leads to tyrosine phosphorylation of cortactin. Although this signalling pathway appears to support bacterial entry into endothelial cells (Hoffmann et al., 2001), the mechanism involved in cortactin recruitment and the role of its tyrosine phosphorylation are still elusive.

The lipo-oligosaccharide (LOS) is one of the major surface molecules of neisseriae. It consists of two oligosaccharide chains attached to lipid A by the sequential action of glycosyl transferases and can be further modified by the addition of terminal sialic-acid moiety (Fig. 1). Meningococcal LOS is a well-known inflammatory mediator, owing to engagement of the lipid-A core with the macrophage CD14/Toll-like-receptor 4 (TLR4)/MD-2 receptor complex, leading to the production of proinflammatory cytokines and chemokines (Smirnova et al., 2003; Zughaier et al., 2004). Moreover, functional consequences of LOS sialylation on protection of *N. meningitidis* from the phagocytic activity of neutrophils, macrophages or dendritic cells are well documented (Estabrook et al., 1998; Moran et al., 1996; Unkmeir et al., 2002). Although live bacteria avoid phagocytosis, a role has been recently suggested for LOS production in the internalization of killed bacteria by specialized phagocytic cells. Indeed, interaction of sialylated LOS with sialic-acid-binding immunoglobulin-like lectins was shown to increase the uptake by macrophages of heat-killed bacteria (Jones et al., 2003), whereas the formation of complexes between LOS and the lipopolysaccharide-binding protein (LBP) seems to be required for rapid internalization by

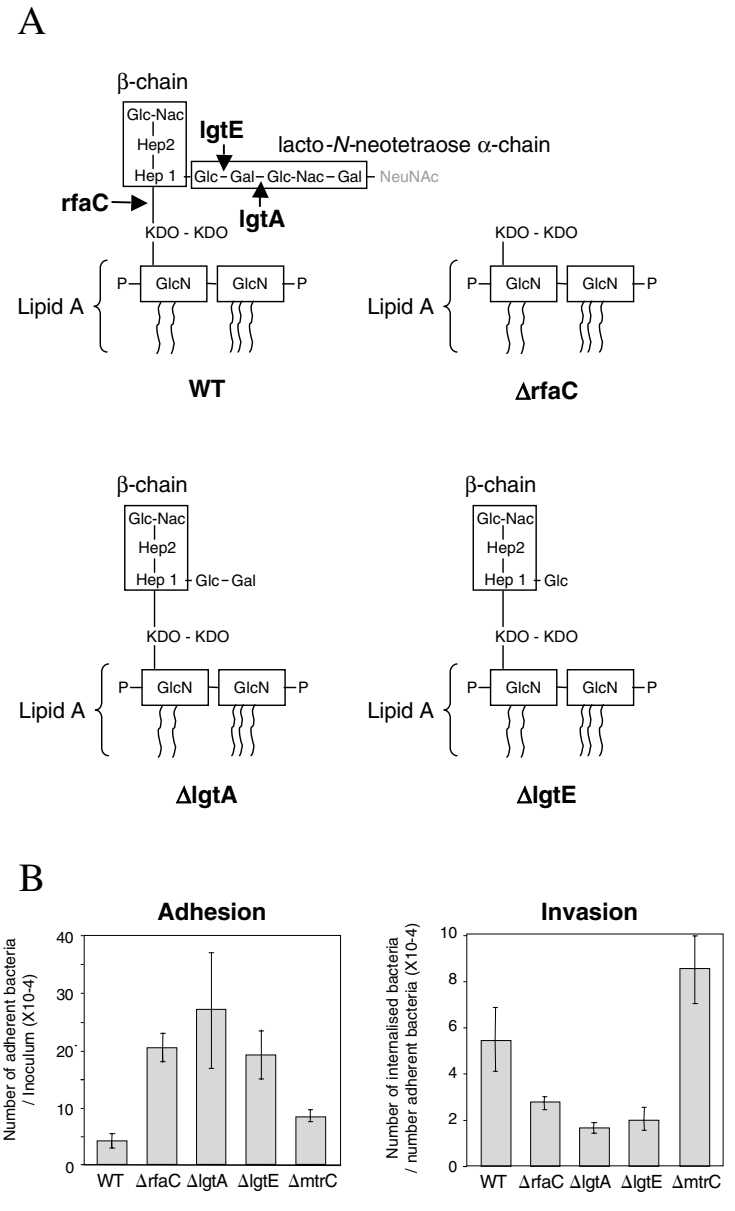

Fig. 1. LOS is required for the efficient internalization of *N. meningitidis* into human endothelial cells. (A) LOS structures of the wild-type, Δ*rfaC*, Δ*lgtA* and Δ*lgtE* mutant strains. The inner core structure of the LOS molecule of *N. meningitidis* consists of two molecules of 3-deoxy-D-manno-2-octulosonic acid (Kdo) attached to a lipid A. The oligosaccharide core consists of two heptose residues attached to the Kdo by the heptosyltransferase I RfaC and a lacto-*N*-neotetraose α-chain added by glycosyl transferases such as LgtA or LgtE. LOS can be further modified by the addition of terminal sialic-acid (NeuNAc) moieties. By inactivating *rfaC*, the Δ*rfaC* mutant strains produced contain only the lipid A moiety and the two Kdos of the LOS structure. Δ*lgtA* and Δ*lgtE* mutants express a truncated oligosaccharide structure. (B) HBMECs were infected with the wild-type (WT) or the isogenic *rfaC* (Δ*rfaC*), *lgtA* (Δ*lgtA*), *lgtE* (Δ*lgtE*) or *mtrC* (Δ*mtrC*) defective mutants of the 2C43 strain of *N. meningitidis*. After 30 minutes of contact, the number of adherent bacteria in relation to the number of bacteria contained in the inocula was determined (left). After 3 hours, the number of internalized bacteria in relation to the number of adherent bacteria was determined (right). Average values (± s.e.m.) from one representative experiment out of four independent experiments performed in triplicate are presented.

dendritic cells and their subsequent production of cytokines (Uronen-Hansson et al., 2004). However, the involvement of LOS in the internalization process of live *N. meningitidis* in non-phagocytic cells and the associated signalling events have not yet been explored.

In this study, we therefore investigated the contribution of LOS to the invasion of human endothelial cells by *N. meningitidis*. We provide evidence that LOS integrity is required to promote a phosphoinisitide-3-kinase (PI3K)/Rac1 co-stimulatory signal leading to the recruitment and tyrosine phosphorylation of cortactin at the bacterial adhesion site. Moreover, we show that phosphorylated cortactin plays a major role in the formation of actin structures allowing efficient bacterial invasion.

Materials and Methods

Antibodies and reagents

Antibodies against cortactin (p80/85) and p60$^{c\text{-}Src}$ were purchased from Upstate Biotechnology. Anti-phosphotyrosine antibody (4G10) was from Meditech. Polyclonal antibody directed against ErbB2 was purchased from Santa Cruz Biotechnology. Polyclonal antibody directed against ezrin was provided by P. Mangeat (CNRS UMR 5539, Montpellier, France). Rhodamine-phalloidin and the PI3K inhibitor wortmannin were from Sigma.

Bacterial strains

2C43 (formerly clone 12) is a pilus-bearing encapsulated Opa$^-$ variant of the serogroup-C meningococcal strain 8013 (Nassif et al., 1993; Pujol et al., 1999). ROU (group W135, ET37), is a pilus-bearing encapsulated isolate obtained from the cerebrospinal fluid of a 2-month-old human infant (Pron et al., 1997). Mutant strains used, kindly provided by V. Pelicic (Inserm U570, Paris, France), have been described previously (Geoffroy et al., 2003; Stojiljkovic et al., 1997). Bacterial strains were grown as described previously (Hoffmann et al., 2001).

Cell culture

Human bone-marrow endothelial cells (HBMECs) (Schweitzer et al., 1997) were kindly provided by B. B. Weksler (Weill Medical College, Cornell University, NY, USA) and were cultured in DMEM Glutamax (Life technologies) supplemented with 10% heat-inactivated foetal bovine serum, 7.5 μg ml^{-1} endothelial-cell growth supplement (Sigma), 7 IU heparin, 10 mM Hepes (pH 7.4). Before experiments, *N. meningitidis* was grown overnight on GCB solid medium [4% GC medium base (Difco, MD, USA), 1% agar, 0.4% glucose, 0.2 mg ml^{-1} thiamine, 0.0005% $Fe(NO_3)_3.9H_2O$, 0.01% L-glutamine] at 37°C under 5% CO_2. Several colonies were then selected and grown in DMEM Glutamax supplemented with 0.1% bovine serum albumin (BSA) for 2 hours and were finally diluted to approximately 10^7 wild-type bacteria per ml inoculum or 10^6 mutant bacteria per ml inoculum to obtain similar adhesion events. No differences were observed between the growth rates of the wild-type and mutant bacteria in suspension or at the endothelial-cell surface during the course of infection.

Infection and quantification of *N. meningitidis* adhesion to or entry into endothelial cells

Infections were performed as follows. Confluent HBMEC monolayers were starved for 18 hours before infection in DMEM Glutamax supplemented with 0.1% BSA (starvation medium) to lower the basal level of tyrosine phosphorylation. HBMECs were then overlaid for 30 minutes with bacterial inoculum in starvation medium. Cells were then washed three times with starvation medium to remove nonadherent bacteria and infection was allowed to proceed for various periods of time. When indicated, cells were pretreated for 2 hours with 100 ng ml^{-1} wortmannin before infection. Cells were then infected as above and the infection was allowed to proceed for 3 hours in the presence of the inhibitor. Wortmaninn treatment did not affect bacterial growth at the cell surface. For adhesion assays, cells grown to confluence in six-well plates were put in contact with bacterial inoculum in starvation medium for 30 minutes. Cell layers were extensively washed, collected by scraping into GCB liquid medium, serially diluted and spread onto GCB plates. Bacteria were grown overnight and colony-forming units were counted. Serial dilutions of bacterial inoculum were also spread onto GCB plates and the number of adherent bacteria in relation to the number of bacteria present in the inoculum was determined. To determine the number of internalized bacteria, cells were infected as above and the infection was allowed to proceed for 3 hours in the presence of wortmannin when indicated. Cells were then extensively washed, incubated with 150 μg ml^{-1} gentamicin at 37°C for 1 hour, washed extensively before scraping and plating onto GCB plates. The number of internalized bacteria in relation to the number of adherent bacteria was determined. Each condition was performed in triplicate and each experiment was carried out four times independently.

Immunoprecipitations and immunoblotting or kinase assays

Cells were washed once with cold PBS and lysed in a Nonidet P40-based buffer (1% Nonidet P40, 50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM $NaVO_4$, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin). The insoluble fraction was removed by centrifugation and the cleared lysates were used for immunoprecipitation with specific antibodies. Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell). After blocking for 1 hour in PBS containing 1% BSA and 0.05% Tween 20, filters were probed overnight with specific antibodies. Proteins were visualized with peroxidase-coupled secondary antibody using the ECL system (Amersham). Src-autophosphorylation/kinase assays were performed as previously described (Hoffmann et al., 2001).

Cell transfections

Vectors encoding full-length cortactin coupled to green fluorescent protein (GFP) or cortactin that had been mutated in its three Src tyrosine-phosphorylation sites and coupled to GFP, were kindly provided by X. Zhan (Holland Laboratory, Rockville, MD, USA). Vectors encoding wild-type or a dominant-negative form of Rac1 coupled to GFP were provided by J. Delon (Institut Cochin, Paris, France), the vector encoding the PH domain of the serine/threonine kinase Akt coupled to GFP was provided G. Bismuth (Institut Cochin, Paris, France) and the vector encoding the GTPase-binding domain of PAK1 coupled to GFP was provided by C. Gauthier-Rouvière (CRBM, Montpellier, France). HBMECs were transfected using the nucleofector system (Amaxa). Briefly, for optimal transfection of HBMECs, 10^6 cells were suspended in 100 μl solution V (provided by Amaxa) in the presence of 1 μg DNA and subjected to electroporation using program U15 of the nucleofector system. Cells were plated at the same density as cultures that had reached confluency on Permanox coverslips (Costar) at confluence in complete medium for 24 hours before infection and fixation for immunofluorescence analysis.

Confocal immunofluorescence microscopy and quantification of protein recruitment at bacterial entry site

HBMECs were grown on Permanox coverslips. After infection, cells

were fixed in 4% paraformaldehyde for 10 minutes, washed three times with PBS and permeabilized with 0.2% Triton X-100 in PBS for 10 minutes. Cells were incubated for 30 minutes with 3% BSA in PBS and then for 2 hours with the primary antibodies. After three washes with PBS, cells were incubated for 1 hour with CY2-, CY3- or CY5-conjugated anti-mouse or anti-rabbit IgG (Jackson Immunochemicals). Labelled preparations were mounted in Mowiol and analysed with a confocal microscope (Biorad Laboratories). For quantification analysis, the frequency of recruitment of the different proteins at the entry site of wild-type, Δ*rfaC* or Δ*lgtA* bacterial strains was determined by counting 30-50 cells. Recruitment was scored when the accumulation of the proteins in a honeycomb lattice structure, defined as a cortical plaque, was clearly visible.

Results

LOS integrity is required for an efficient internalization of *N. meningitidis* into endothelial cells

To determine the importance of an intact LOS structure on the ability of *N. meningitidis* to invade human endothelial cells, we have generated isogenic strains of the 2C43 and ROU strains of *N. meningitidis* that express LOS molecules lacking the lacto-*N*-neotetraose structure (Fig. 1A). By inactivating the gene encoding the heptosyltransferase I *rfaC* (Δ*rfaC* mutant strains), mutants containing only the lipid-A moiety and the two Kdo (3-deoxy-D-manno-2-octulosonic acid) moieties of the LOS were produced. Additional mutants expressing LOS molecules with a truncated oligosaccharide core were generated in the same way by inactivating the genes encoding the glycosyl transferase *lgtA* and *lgtE* (Δ*lgtA* and Δ*lgtE* mutants). Moreover, a mutant deleted in a gene unrelated to LOS biogenesis, encoding a membrane lipoprotein (Δ*mtrC* mutant) was used as control. In agreement with previous observations that LOS of neisseriae can produce an antiadhesive effect owing to the negatively charged carbohydrate sialic acid (Merz and So, 2000), we observed here that LOS truncation resulted in increased adherence of bacterial mutants to endothelial cells (Fig. 1B). In order to compare the number of internalized wild-type or mutant bacteria in relation to the number of adhering bacteria, our experiments were therefore performed with adjusted inocula to obtain similar adhesion events. We observed that the invasive abilities of the 2C43 Δ*rfaC*, Δ*lgtA* and Δ*lgtE* mutant strains were reduced by 50-60% compared with the wild-type strain, whereas deletion of *mtrC* did not reduce invasion (Fig. 1B). Similarly, the invasive ability of the ROU Δ*rfaC* strain was reduced by 60% in comparison with the ROU wild-type strain (not shown), strongly suggesting that the LOS efficiently increases the capacity of *N. meningitidis* to invade human endothelial cells.

LOS-dependent signalling controls cortical actin polymerization at the *N. meningitidis* entry site

N. meningitidis internalization into human endothelial cells depends on the dynamic assembly of actin filaments for the formation of membrane projections towards bacteria and their

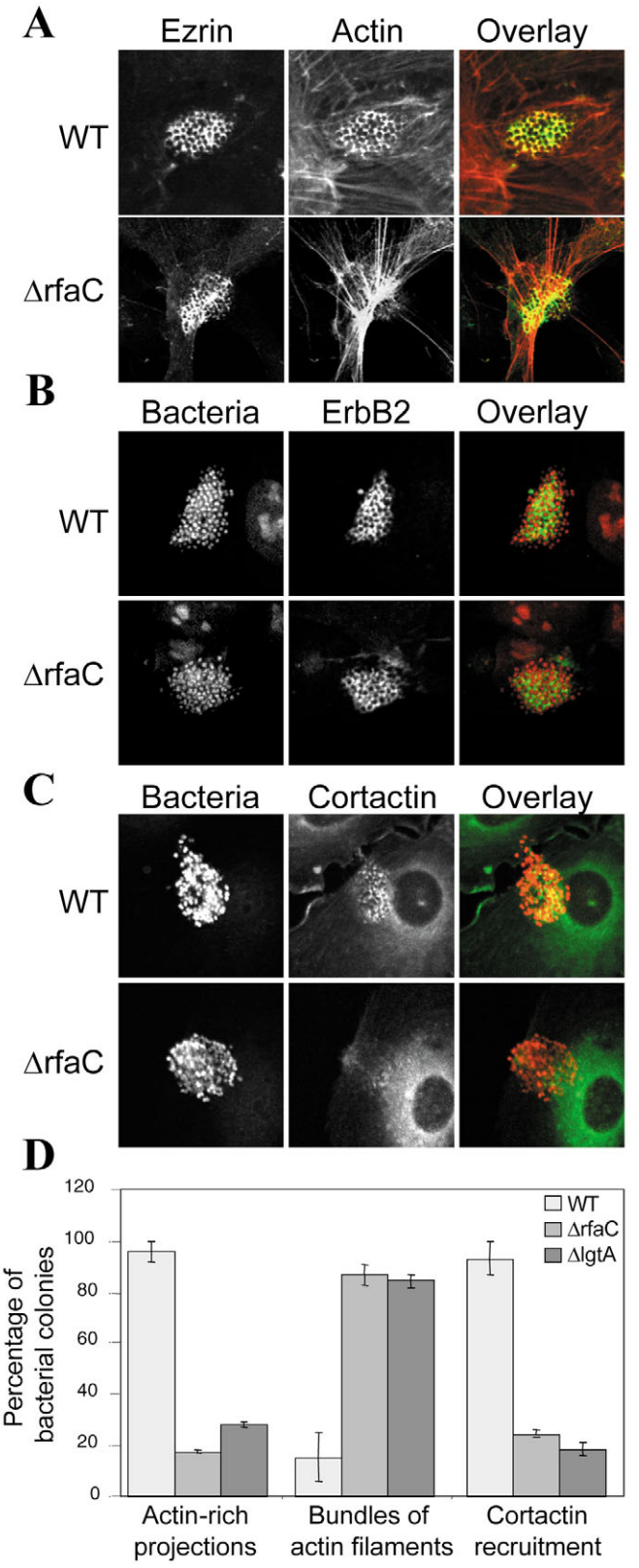

Fig. 2. LOS is required for the formation of actin-rich cell projections promoting bacterial uptake and for cortactin recruitment. HBMECs infected for 3 hours with the 2C43 wild-type strain of *N. meningitidis* (WT) or the isogenic *rfaC*-defective mutant strains (Δ*rfaC*) were double stained for actin (red) and ezrin (green) (A), for ErbB2 (green) and bacteria (red) (B) or for cortactin (green) and bacteria (red) (C), and were analysed by confocal microscopy. Merged images of the same fields are presented on the right-hand panels (overlay). (D) The frequency of formation of actin-rich cell projections or of bundle of actin filaments and of cortactin recruitment at the entry site of WT, Δ*rfaC* or Δ*lgtA* bacterial strains was determined by counting 50 cells. Average values (± s.e.m.) are presented from four independent experiments.

subsequent engulfment into intracellular vacuoles (Eugene et al., 2002). We therefore examined the reorganization of the actin cytoskeleton induced in cells infected by the Δ*rfaC* mutant strains. Ezrin, a molecular link between integral membrane proteins and actin cytoskeleton, which was previously shown to be recruited at the site of *N. meningitidis* adhesion, was normally recruited by Δ*rfaC* mutant strains (Fig. 2A), as well as the ezrin-binding transmembrane proteins CD44 and ICAM-1 (not shown). By contrast, cortical actin polymerization characteristic of the formation of cellular projections was much less frequently observed in cells infected by the Δ*rfaC* and Δ*lgtA* mutant strains than in cells infected by wild-type strains (Fig. 2D). Instead, a local formation of thick bundles of actin filaments was observed (Fig. 2A). These results indicate that a LOS-dependent signalling is essential for the development of the actin-rich cell projections that promote bacterial uptake.

LOS-dependent signalling is required for the recruitment and phosphorylation of cortactin at the *N. meningitidis* entry site

We next examined the potential role of the LOS-induced signalling in the activation of the ErbB2-Src-cortactin pathway, which is required for an efficient bacterial entry into endothelial cells (Hoffmann et al., 2001). Interestingly, we observed that, although Δ*rfaC* and Δ*lgtA* mutants induced ErbB2 recruitment as efficiently as the wild-type strains (Fig. 2B), they induced cortactin recruitment poorly (Fig. 2C,D). We therefore analysed whether the mutant strains were defective in activating the ErbB2-Src-cortactin pathway (Fig. 3A). As previously shown, the infection of human endothelial cells by *N. meningitidis* wild-type strains induced the tyrosine phosphorylation of ErbB2 and the downstream activation of the tyrosine kinase Src. Infection of endothelial cells by the Δ*rfaC* mutant strains induced ErbB2 tyrosine phosphorylation and Src activity to a similar extent to the wild-type strains, but only weakly induced the tyrosine phosphorylation of cortactin, which was reduced by 60-70% (Fig. 3C). Cortactin phosphorylation was decreased to a similar extent in cells infected with Δ*lgtA* or Δ*lgtE* mutant strains, while being slightly increased by the Δ*mtrC* mutant compared with the 2C43 wild-type strain (Fig. 3B,C). These observations indicate that LOS is required to induce both recruitment and tyrosine phosphorylation of cortactin in cell projections surrounding the bacteria.

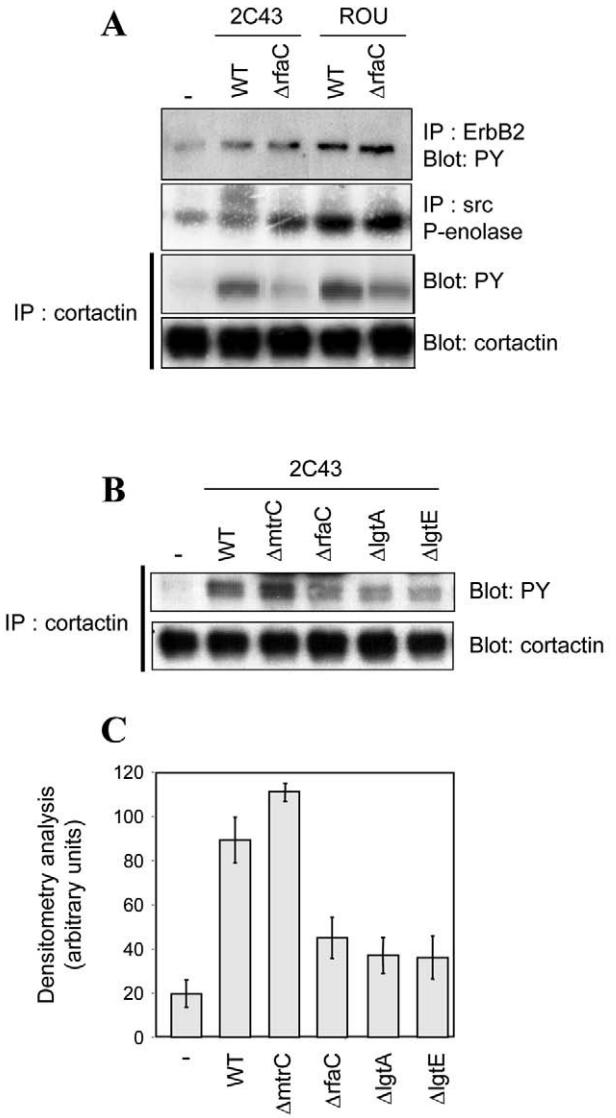

Fig. 3. LOS is required for cortactin tyrosine phosphorylation but not for ErbB2 or Src kinase activation. HBMECs (starved for 24 hours) were either not infected (–) or infected for 3 hours with the wild-type (WT) or isogenic *rfaC* (Δ*rfaC*), *lgtA* (Δ*lgtA*), *lgtE* (Δ*lgtE*) or *mtrC* (Δ*mtrC*) defective mutants of the 2C43 or ROU strains of *N. meningitidis*, as indicated. Inocula were adjusted to obtain similar adhesion events between wild-type and mutant bacteria. After lysis, ErbB2 receptor was immunoprecipitated and immunoblotted with an anti-phosphotyrosine antibody (PY) (A, top), Src was immunoprecipitated and subjected to an in-vitro kinase assay using acid-denatured enolase as a substrate (A, middle), or cortactin was immunoprecipitated and immunoblotted with an anti-phosphotyrosine antibody and the blot was reprobed with an anti-cortactin antibody to confirm that similar protein levels were immunoprecipitated (A, bottom, B). (C) Quantification by densitometry analysis (using NIH Image software) of cortactin phosphorylation induced by the wild-type and mutants of the 2C43 strain. Average values (± s.e.m.) are presented from four independent experiments.

Tyrosine phosphorylation of cortactin is required for the formation of the actin-rich cell projections that promote bacterial uptake

Cortactin is known to regulate the formation of dynamic actin networks, and so we investigated whether the induction of aberrant cortical actin polymerization by the bacterial mutant strains might be directly related to their deficiency in recruiting and phosphorylating cortactin. Actin polymerization induced by the 2C43 wild-type strain was analysed in endothelial cells expressing a GFP-tagged cortactin mutant deficient in all three tyrosine-phosphorylation sites (GFP-cortactin$_{Y/F}$) (Huang et al., 1998; Li et al., 2001), a GFP-tagged wild-type cortactin (GFP-cortactin) or GFP alone as control (Fig. 4). Infection of endothelial cells expressing GFP-cortactin led to the expected recruitment of both GFP-cortactin and ezrin, and to cortical actin polymerization associated with the formation of membrane protrusions. In cells expressing GFP-cortactin$_{Y/F}$,

infection induced its recruitment, in agreement with our observation that cortactin phosphorylation was not required for its translocation to sites of cortical actin polymerization (Hoffmann et al., 2001). Moreover, whereas ezrin recruitment was not affected in these cells compared with control cells, the formation of thick bundles of actin filaments, reminiscent of those induced by the Δ*rfaC* and Δ*lgtA* mutant strains, was observed at the site of bacterial adhesion. This effect was exacerbated when GFP-cortactin$_{Y/F}$ was expressed at a higher level, demonstrating that mutations preventing cortactin phosphorylation affect *N.-meningitidis*-induced actin-rich cell projections. In this condition, GFP-cortactin$_{Y/F}$ was not recruited to bacterial entry sites, in line with the capacity of cortactin to bind to cortical actin but not to actin bundles. As a control, no recruitment of GFP alone was observed in infected endothelial cells, excluding the possibility that GFP was nonspecifically sequestered in the plasma membrane folds induced by *N. meningitidis* wild-type strains. Moreover, GFP expression did not affect ezrin recruitment or cortical actin reorganization induced by *N. meningitidis.*

LOS-dependent activation of a PI3K/Rac1-GTPase signalling pathway

Cortactin translocation to the cortical actin network in response to growth-factor stimulation is dependent on the activation of the small GTPase Rac1 (Head et al., 2003; Weed et al., 1998). We therefore assessed whether Rac1 GTPase was recruited during *N. meningitidis* interaction with endothelial cells. Cells expressing a GFP-tagged wild-type form of Rac1 (GFP-Rac1) were infected by the wild-type and mutant strains of *N. meningitidis* and its recruitment at bacterial entry sites was analysed (Fig. 5A). Interestingly, infection by wild-type strains induced a massive recruitment of GFP-Rac1, whereas GFP-Rac1 recruitment by Δ*rfaC* (Fig. 5A) or Δ*lgtA* mutant strains (not shown) was barely detected, suggesting that only the wild-

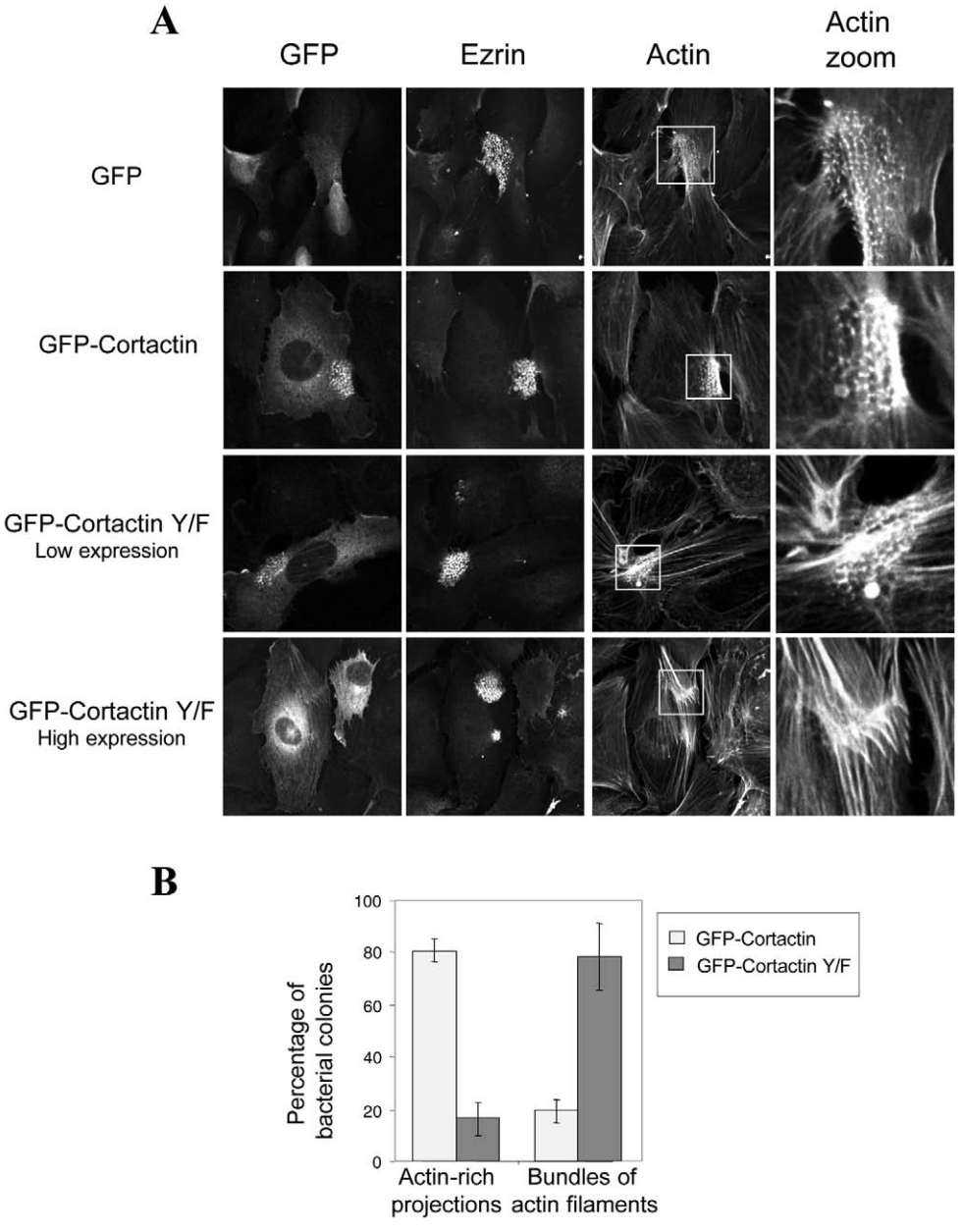

Fig. 4. Cortactin tyrosine phosphorylation induced by *N. meningitidis* is required for the formation of actin-rich cell projections promoting bacterial uptake. HBMECs transiently transfected with GFP alone, wild-type GFP-cortactin or a GFP-tagged cortactin mutant deficient in tyrosine phosphorylation (GFP-cortactin$_{Y/F}$), as indicated, were infected for 3 hours with the 2C43 wild-type strain of *N. meningitidis*. (A) Cells were then double stained for actin and ezrin, and analysed by confocal microscopy. (right) Higher magnification of the inset in the middle panels. (B) The frequency of formation of either actin-rich cell projections or bundles of actin filaments at bacterial entry sites in cells expressing high levels of both GFP-cortactin and GFP-cortactin$_{Y/F}$ was determined by counting 40 cells. Average values (± s.e.m.) are presented from four independent experiments.

type strains were able to recruit Rac1. Pull-down assays were performed to assess Rac1 activation directly, but the high level of Rac1 basal activity in uninfected cells prevented any clear evidence of Rac1 stimulation in infected cells. We therefore analysed and quantified the redistribution of a GFP-tagged form of the GTPase-binding domain of PAK1 (GFP-PAK1-PBD), a well-established downstream effector of both the Rac1 and the Cdc42 GTPases, which was shown to reflect the localization of activated Rac1 but not Cdc42 at the plasma membrane (Srinivasan et al., 2003). As shown in Fig. 5B,D, infection by wild-type strains induced a massive recruitment of GFP-PAK1-PBD at bacterial entry sites; by contrast, only a limited recruitment was observed in cells infected by either the Δ*rfaC* or Δ*lgtA* mutants, thus confirming a default in the activation of Rac1 by the bacterial mutant strains.

A key component in the activation of Rac1 by a wide range of membrane receptors is the PI3K that catalyses the production of plasma-membrane-associated phosphatidylinositol-(3,4,5)-trisphosphate [PtdIns(1,4,5)P_3] (Hall, 1998). In order to visualize the subcellular production of PtdIns(1,4,5)P_3, we therefore analysed the redistribution of a specific fluorescent probe containing the PtdIns(1,4,5)P_3-binding PH domain of Akt (GFP-Akt-PH), a well established PI3K effector (Gray et al., 1999). We observed that infection by the 2C43 wild-type strain induced the recruitment of GFP-Akt-PH (Fig. 5C,D), demonstrating the local activation of PI3K at bacterial entry sites. As expected, this recruitment was prevented in the presence of wortmannin, a selective inhibitor of PI3K (Fig. 5D). Moreover, wortmannin treatment prevented both GFP-Rac1 (not shown) and GFP-PAK1-PBD (Fig. 5D) recruitments induced by the wild-type strains, indicating that PI3K functions upstream of the activation of Rac1 GTPase. Interestingly, the recruitment of GFP-Akt-PH was substantially reduced in cells infected by the Δ*rfaC* or Δ*lgtA* mutant strain, indicating a default in the activation of PI3K by these bacterial mutants (Fig. 5C,D). Altogether, these results provide a strong evidence for the LOS-dependent activation by *N. meningitidis* of a PI3K/Rac1-GTPase signalling pathway.

LOS-dependent PI3K/Rac1 signalling pathway is required for cortactin recruitment and bacterial internalization

We therefore asked whether the PI3K/Rac1 signalling pathway was involved in cortactin translocation upon *N. meningitidis* infection. For this purpose, cells were transfected with a GFP-tagged dominant-negative form of Rac1 (GFP-Rac1-DN) and infected by the 2C43 wild-type strain. We observed that infection induced a massive recruitment of GFP-Rac1-DN, accompanied by a drastic inhibition of cortactin recruitment and a robust actin reorganization into bundles of actin filaments, similar to the actin polymerization structures induced by the Δ*rfaC* and Δ*lgtA* mutant strains (Fig. 6A, top; Fig. 6B, grey bars). As a control, in the adjacent untransfected cells (Fig. 6A, bottom; Fig. 6B, white bars), cortactin

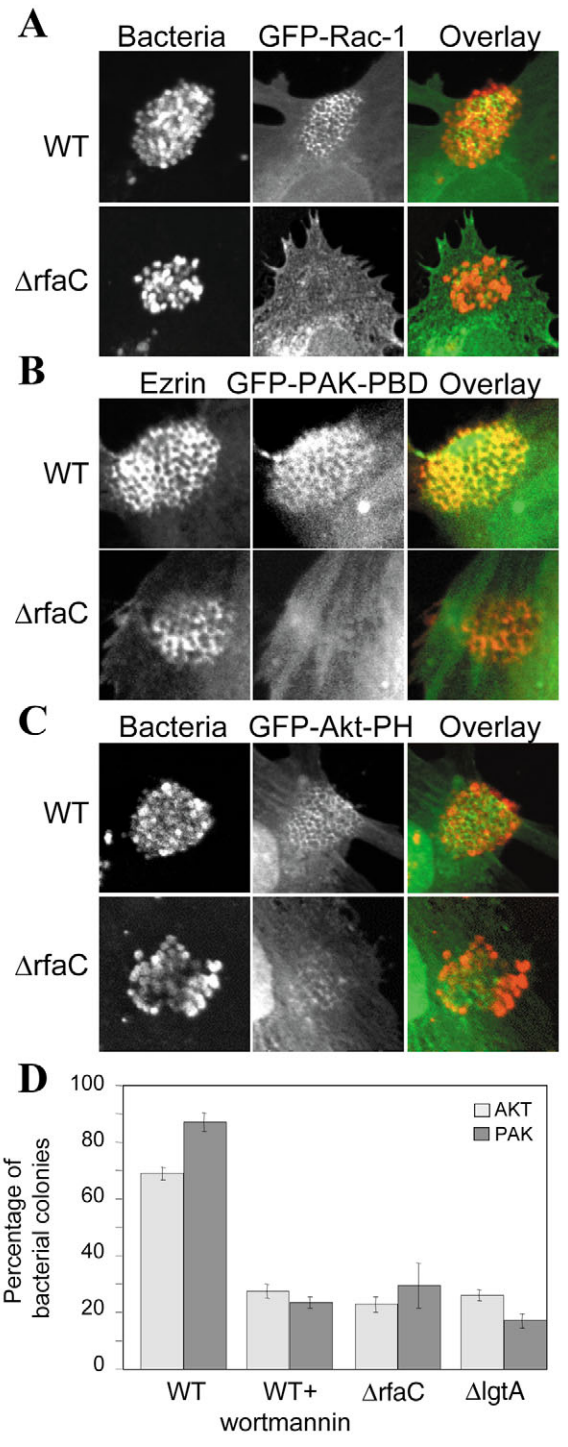

Fig. 5. LOS induces the activation of Rac1 GTPase and PI3K. HBMECs, transiently transfected with wild-type Rac1 coupled to GFP (GFP-Rac1), with the GTPase-binding domain of PAK1 coupled to GFP (GFP-PAK1-PBD) or with the PH domain of Akt coupled to GFP (GFP-Akt-PH) were infected for 3 hours with either the 2C43 wild type (WT) or the isogenic *rfaC* (Δ*rfaC*) or *lgtA* (Δ*lgtA*) defective mutants of *N. meningitidis*. When indicated (WT + wortmannin), HBMECs were pretreated for 2 hours with 100 ng ml^{-1} wortmannin and then infected with the 2C43 wild-type strain of *N. meningitidis* in the presence of the inhibitor. (A-C) Cells were then stained for bacteria or ezrin (red) and recruitment of the different GFP-tagged proteins at bacterial entry sites was analysed by confocal microscopy. (right) Merged images (overlay) of the same fields. (D) The frequency of recruitment of the different GFP-tagged proteins at the entry site of WT, Δ*rfaC* or Δ*lgtA* bacterial strains was determined by counting 50 cells. Average values (± s.e.m.) are presented from three independent experiments.

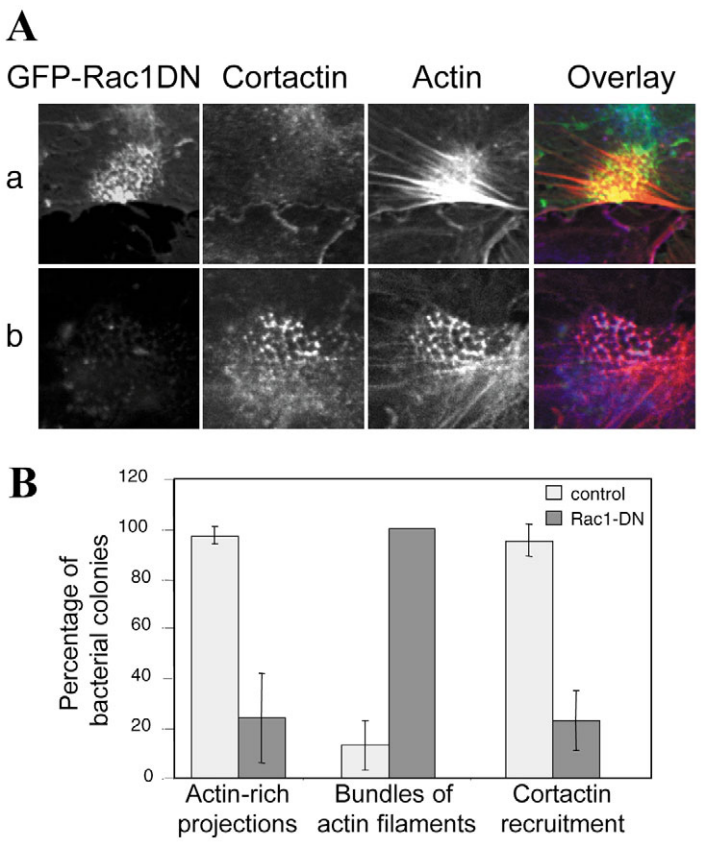

Fig. 6. The activation of Rac1 GTPase is required for cortactin recruitment and the formation of actin-rich cell projections promoting bacterial uptake. (A) The upper row (a) shows a cell expressing the GFP-Rac1-DN and the lower row (b) shows a nontransfected control cell. Both were infected with the bacterial wild-type strain. HBMECs transiently transfected with the dominant-negative form of Rac1 coupled to GFP (GFP-Rac1DN) were infected for 3 hours with the 2C43 wild-type strain of *N. meningitidis*. Cells were then double stained for actin (red) and cortactin (blue), and were analysed by confocal microscopy. (right) Merged images (overlay) of the same fields. (B) The frequency of formation of either actin-rich cell projections or bundle of actin filaments at bacterial entry sites in both control and Rac1-DN-expressing cells was determined by counting 40 cells. Average values (± s.e.m.) are presented from three independent experiments.

recruitment induced by the bacterial wild-type strains was not affected and normal cortical actin polymerization associated with the formation of cell projections was still observed. Moreover, inhibition of PI3K activation by wortmannin was also accompanied by an inhibition of the recruitment and tyrosine phosphorylation of cortactin (Fig. 7A,B), and was associated with an extensive actin reorganization into bundles of actin filaments, whereas ezrin recruitment was not affected (Fig. 7A). These results therefore establish the involvement of the LOS-dependent PI3K/Rac1 signalling pathway in cortactin recruitment and in the formation of actin structures and cell projections associated with bacterial invasion.

The potential role of the PI3K/Rac1 signalling pathway in bacterial internalization into endothelial cells was

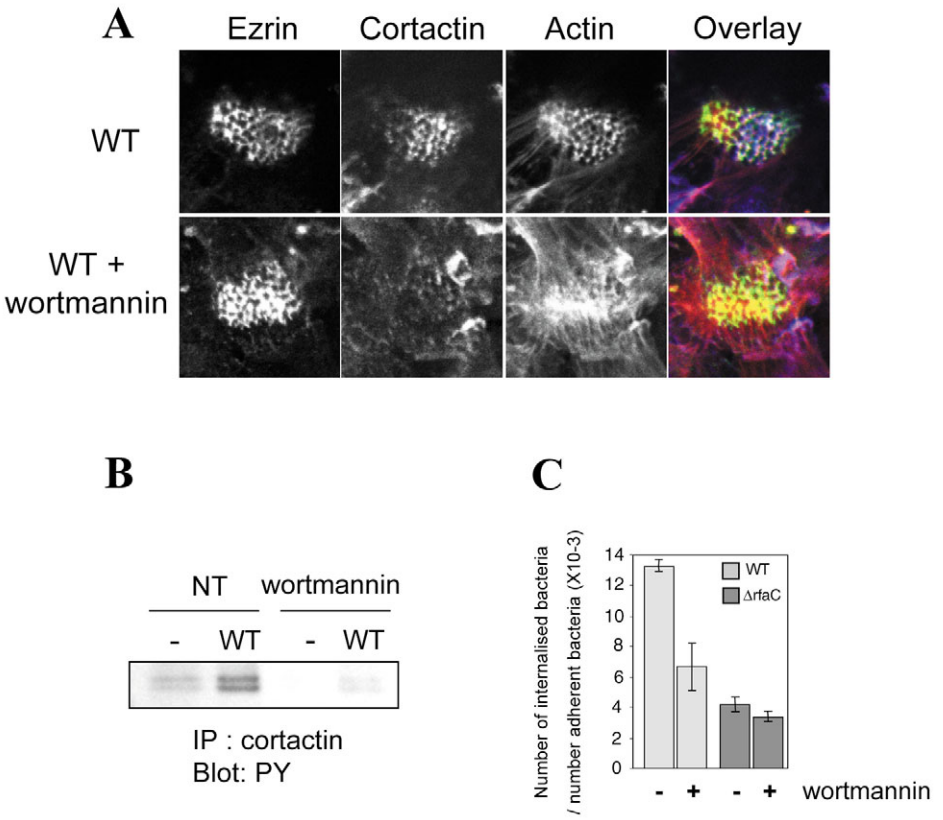

Fig. 7. Activation of PI3K is required for the recruitment and phosphorylation of cortactin and bacterial internalisation. (A,B) HBMECs were pretreated or not for 2 hours with 100 ng ml^{-1} wortmannin and then infected for 3 hours with the 2C43 wild-type strain of *N. meningitidis*. (A) Cells were triple stained for ezrin (green), cortactin (blue) and actin (red), and were analysed by confocal microscopy. (right) Merged images (overlay) of the same fields. (B) Cortactin was immunoprecipitated and immunoblotted with an anti-phosphotyrosine antibody (PY). (C) HBMECs were either left untreated (–) or pretreated for 2 hours with 100 ng ml^{-1} wortmannin (+) before infection with the 2C43 wild-type strain of *N. meningitidis* (WT) or with the isogenic *rfaC*-defective mutant strain (Δ*rfaC*) in the presence or absence of the inhibitor. After 3 hours, the number of internalized bacteria in relation to the number of adherent bacteria was determined. Average values (± s.e.m.) are presented from one representative experiment out of four independent experiments performed in triplicate.

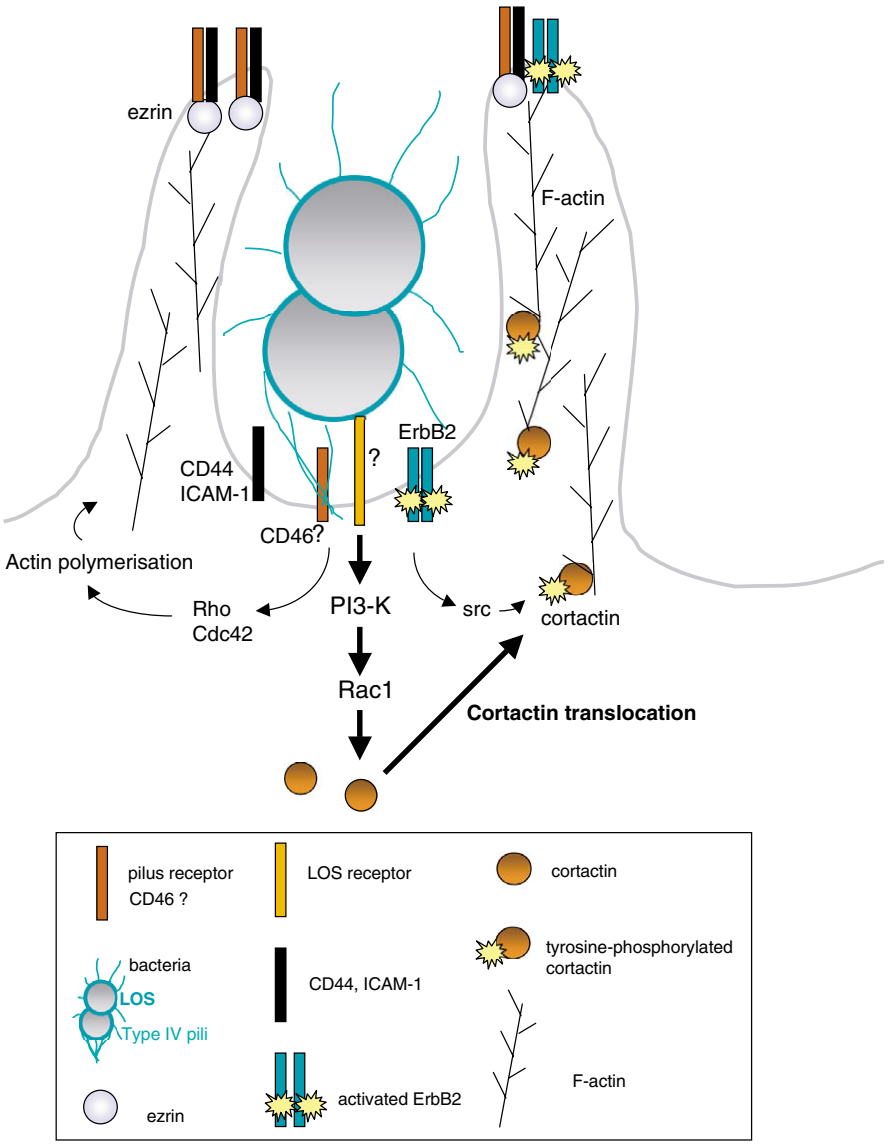

Fig. 8. Schematic representation of the signalling pathways activated by *N. meningitidis* and involved in bacterial entry into endothelial cells. Type-IV pili initiate the interaction of virulent, encapsulated *N. meningitidis* with human endothelial cells by interacting with a cellular receptor, possibly CD46 (Kallstrom et al., 1997). This pilus-dependent adhesion induces the recruitment of ezrin and the clustering of several transmembrane proteins: the ErbB2 tyrosine-kinase receptor and the ezrin-binding proteins CD44 and ICAM-1. The activation of both Rho and Cdc42 GTPases induces a local polymerization of cortical actin. ErbB2 clustering leads to the activation of Src tyrosine kinase. In parallel, LOS of *N. meningitidis*, by a mechanism which remains to be identified, provides a co-stimulatory signal leading to PI3K and Rac1 activation, and the subsequent translocation of cortactin to site of cortical actin rearrangements. When localized to the cell plasma membrane, cortactin is tyrosine phosphorylated by Src kinase and contributes to the formation of dynamic actin structures, leading to the formation of membrane projections that surround bacteria and provoke their internalization within endothelial intracellular vacuoles

therefore investigated. Cell treatment with wortmannin reduced the internalisation of the 2C43 wild-type strain by about 50%, to a level similar to that observed with Δ*rfaC* mutant strains (Fig. 7C), whereas it did not affect bacterial adhesion (not shown). Furthermore, in agreement with our conclusion that Δ*rfaC* mutant strains fail to activate the PI3K/Rac1 signalling pathway, wortmannin did not further reduce the internalization of these mutants. These experiments thus indicate that the LOS-dependent PI3K/Rac1 pathway is required for efficient internalization of *N. meningitidis* into endothelial cells.

Altogether, our results (summarized in Fig. 8) provide evidence that LOS plays a crucial role in meningococcal entry into endothelial cells by triggering a co-stimulatory signal that leads to PI3K and Rac1-GTPase activation. This signalling pathway is required for cortactin recruitment to the bacterial adhesion sites and its subsequent phosphorylation downstream of the ErbB2/Src pathway, contributing to the formation of actin structures and membrane protrusions associated with the internalization of *N. meningitidis*.

Discussion

This study provides evidence that LOS integrity is required for efficient internalization of *N. meningitidis* into human endothelial cells. LOS integrity is necessary for the activation of a PI3K/Rac1-GTPase signalling pathway allowing cortactin recruitment and tyrosine phosphorylation at bacterial adhesion sites. Moreover, the absence of tyrosine phosphorylation of cortactin was correlated with the formation of bundles of actin filaments and the lack of efficient bacterial internalization. We therefore conclude from our experiments that cortactin controls the formation of the actin-rich cell projections that promote bacterial uptake.

Cortactin has been shown in several situations to distribute to sites of dynamic actin assembly, including lamellipodia, podosomes, invadopodia and bacterial entry sites (Bowden et al., 1999; Weed and Parsons, 2001; Wu and Parsons, 1993). In particular, cortactin seems to be involved differently in cytoskeleton rearrangements occurring during the interaction of various bacterial pathogens with host cells. For example, interaction of enteropathogenic *Escherichia coli* with epithelial

cells results in cortactin recruitment to the bacterial adhesion site, where it seems to be required for F-actin accumulation in pedestal structures, without apparent changes in its tyrosine phosphorylation (Cantarelli et al., 2002). By contrast, invasion of epithelial cells by *Shigella flexneri* induces the tyrosine phosphorylation of cortactin by a Src-mediated signalling pathway (Dehio et al., 1995), whereas infection by *Helicobacter pylori* leads to cortactin dephosphorylation and actin rearrangement via Src inactivation (Selbach et al., 2003).

Because expression of cortactin mutant deficient in tyrosine phosphorylation drastically alters actin polymerization at *N. meningitidis* entry sites, we strongly suggest here that phosphorylated cortactin participate directly in the formation of the actin-rich cell projections that promote bacterial uptake. In vitro, cortactin can increase actin assembly by directly or indirectly stimulating the catalytic activity of the Arp2/3 complex (Uruno et al., 2001; Weaver et al., 2002) and by stabilizing actin filaments at a post-nucleation step (Weaver et al., 2001). However, it is not clear how tyrosine phosphorylation of cortactin modulates the architecture of the actin cytoskeleton, because it does not affect the ability of cortactin to bind F-actin and to activate the Arp2/3 complex. We observed here the formation of bundles of actin filaments in the absence of cortactin recruitment and/or phosphorylation at *N. meningitidis* entry sites, suggesting that phosphorylated cortactin might control the activity of an actin-bundling protein. These observations are consistent with previous investigations showing that dephosphorylation of cortactin appears to increase actin cross-linking in vitro (Huang et al., 1997). However the mechanism involved is still unclear and will be the subject of further studies.

The molecular mechanisms involved in cortactin translocation from cytosol to cortical actin network are also poorly described. We provide evidence here that cortactin recruitment at the site of formation of actin-rich cell protrusions promoted by *N. meningitidis* interaction with endothelial cells involves the activation of Rac1 GTPase. We further show that Rac1 is activated downstream of PI3K and production of PtdIns(1,4,5)P_3, a known essential factor for receptor-mediated activation of Rac1 in mammalian cells (Hall, 1998). We had previously shown that cortical actin polymerization induced by *N. meningitidis* was still observed in cells transfected with a dominant-negative form of Rac1, suggesting that Rac1 was unlikely to be involved in the formation of actin-rich cell protrusions (Eugene et al., 2002). We demonstrate here that, in the absence of Rac1 activation, *N. meningitidis* induces the formation of bundles of actin filaments that are clearly different from the actin-rich cell protrusions that promote bacterial uptake. Our results provide evidence that activation of the PI3K/Rac1 signalling pathway is required for cortactin recruitment and for *N. meningitidis* entry into endothelial cells. Activation of PI3K was previously reported in epithelial cells together with the Opa-dependent uptake of the related pathogen, non-piliated *Neisseria gonorrhoeae*, downstream of the interaction between the bacterial external membrane protein Opa and CEACAM cellular receptors (Booth et al., 2003). We conclude from these findings that piliated *N. meningitidis* and non-piliated strains of *N. gonorrhoeae* might similarly activate PI3K in host cells, albeit through different mechanisms. Interestingly, PI3K has also been implicated in the invasion of epithelial cells by several other bacterial pathogens, such as *Listeria monocytogenes* (Ireton et al., 1996), *H. pylori* (Kwok et al., 2002) and *Rickettsia conorii* (Martinez and Cossart, 2004). It is thus tempting to speculate, from the present study, that the PI3K/Rac1 signalling pathway is a general mechanism controlling cortactin translocation as well as cytoskeletal and membrane remodelling associated with cellular invasion by a range of bacterial pathogens.

Moreover, we noticed that a non-phosphorylatable cortactin mutant localized to the bacterial entry site, whereas inhibition of cortactin translocation by a PI3K inhibitor prevented its tyrosine phosphorylation; these findings indicate that cortactin translocation is required for its subsequent phosphorylation by Src. Interestingly, cortactin translocation in response to growth factor stimulation was also shown to require Rac1 activation and to be a prerequisite for its tyrosine phosphorylation (Head et al., 2003), suggesting that there are common mechanisms activated by both *N. meningitidis* and growth factors. By contrast, these mechanisms apparently differ from those involved during *Shigella* invasion, because it was recently shown that tyrosine phosphorylation of cortactin by Src precedes its relocalization to *Shigella* entry sites through interaction of phosphorylated cortactin with the SH2 domain of the adaptor protein Crk (Bougneres et al., 2004). Moreover, during *Shigella* invasion of epithelial cells, no actin foci developed in the absence of cortactin, and the expression of cortactin mutants deficient for binding to Arp2/3 drastically reduced actin polymerization, suggesting that cortactin is essential for the development of *Shigella*-induced actin-rich cell projections by directly participating in activation of the Arp2/3 complex (Bougneres et al., 2004). Although *Shigella* and *N. meningitidis* both exploit cortactin to modulate the architecture of the host actin cytoskeleton, they appear to use different strategies to recruit cortactin to their entry site, where it regulates actin polymerization and the formation of cellular protrusions by different mechanisms. These molecular differences might explain the diverse morphology of the cellular projections induced by these pathogens: *Shigella* promotes the formation of very large ruffles, whereas *N. meningitidis* triggers the formation of thin projections.

In the present study, we further demonstrate an essential role for LOS in the invasion of non-phagocytic cells by *N. meningitidis*, by showing that LOS is required for activation of the PI3K/Rac1 signalling pathway in endothelial cells, which leads to cortactin recruitment to bacterial adhesion sites. The addition of exogenous meningococcal LOS to endothelial cells infected by LOS mutant strains did not restore cortactin recruitment and phosphorylation (not shown). Similarly, addition of purified meningococcal LOS failed to restore the defective internalization of LOS-deficient bacteria into dendritic cells (Uronen-Hansson et al., 2004), suggesting the importance of the spatiotemporal engagement of membrane-bound LOS with bacterial and/or cellular partners for the association and internalization of *N. meningitidis* by both specialized and unspecialized phagocytic cells.

Although cortactin translocation is dependent on the activation of a PI3K/Rac1 signalling pathway promoted by the meningococcal LOS, the subsequent tyrosine phosphorylation of cortactin is dependent upon the ErbB2/Src kinase signalling pathway induced by the pilus-dependent adhesion of *N. meningitidis*. The formation of the actin-rich cell projections

promoting efficient internalization of *N. meningitidis* therefore results from two converging co-stimulatory signalling pathways, triggered by pili and LOS, most likely via interactions with distinct receptors on the surface of endothelial cells. Interestingly, it appears that, in line with our observations on *N. meningitidis*, the Opa-independent internalization process of piliated strains of the related pathogen *N. gonorrhoeae* into epithelial cells also involves both pili and LOS interactions with specific cell-surface receptors (Song et al., 2000). Although the signalling events have not been fully identified, the elongation of epithelial microvilli induced by *N. gonorrhoeae* required the interaction of the lacto-*N*-neotetraose moiety of LOS with the cell-surface asialoglycoprotein receptor (ASGP-R) (Harvey et al., 2001; Harvey et al., 2002). However, ASGP-R could not account for the effect of *N. meningitidis* LOS on human endothelial cells, because it is not expressed by endothelial cells (not shown). Therefore, *N. meningitidis* and *N. gonorrhoeae* appear to use distinct LOS-dependent molecular mechanisms for invasion. The meningococcal LOS can interact with several receptors, including the serum lipopolysaccharide-binding protein LBP and the CD14/TLR4 receptor complex on human macrophages and other host cells. However, whereas unglycosylated lipid A is sufficient for binding to and activation of the CD14/TLR4 pathway (Zughaier et al., 2004), our observations that the glycosylated moiety of LOS is required to facilitate *N. meningitidis* invasion suggest the involvement of another receptor, the identification of which will be the subject of a future study.

In conclusion, our results provide evidence for a requirement for LOS in the activation by *N. meningitidis* of a PI3K/Rac1 signal-transduction pathway in endothelial cells that regulates cortical actin rearrangements and bacterial uptake. Moreover, our results highlight the role of cortactin in this process, at the crossroad of the pilus- and LOS-dependent co-signalling pathways, underlining the complex sequence of signalling events induced by *N. meningitidis* to elicit its uptake in non-phagocytic cells.

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