

Type IV pili of pathogenic *Neisseriae* elicit cortical plaque formation in epithelial cells

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Summary

The pathogenic *Neisseriae* *Neisseria meningitidis* and *Neisseria gonorrhoeae*, initiate colonization by attaching to host cells using type IV pili. Subsequent adhesive interactions are mediated through the binding of other bacterial adhesins, in particular the Opa family of outer membrane proteins. Here, we have shown that pilus-mediated adhesion to host cells by either meningococci or gonococci triggers the rapid, localized formation of dramatic cortical plaques in host epithelial cells. Cortical plaques are enriched in both components of the cortical cytoskeleton and a subset of integral membrane proteins. These include: CD44v3, a heparan sulphate proteoglycan that may serve as an Opa receptor; EGFR, a receptor tyrosine kinase; CD44 and ICAM-1, adhesion molecules known to mediate inflammatory responses; f-actin; and ezrin, a component that tethers membrane components to the actin cytoskeleton. Genetic analyses reveal that cortical plaque formation is highly adhesin specific. Both *pilE* and *pilC* null mutants fail to induce cortical plaques, indicating that neisserial type IV pili are required for cortical plaque induction. Mutations in *pilT*, a gene required for pilus-mediated twitching motility, confer a partial defect in cortical plaque formation. In contrast to type IV pili, many other neisserial surface structures are not involved in cortical plaque induction, including Opa, Opc, glycolipid GgO₄-binding adhesins, polysialic acid capsule or a particular lipooligosaccharide variant. Furthermore, it is shown that type IV pili allow gonococci to overcome the inhibitory effect of heparin, a soluble receptor analogue, on gonococcal invasion of Chang and A431 epithelial cells. These and other observations strongly suggest that type IV pili play an active role in initiating neisserial

infection of the mucosal surface *in vivo*. The functions of type IV pili and other neisserial adhesins are discussed in the specific context of the mucosal microenvironment, and a multistep model for neisserial colonization of mucosal epithelia is proposed.

Introduction

The closely related Gram-negative bacteria *Neisseria gonorrhoeae* (gonococci, GC) and *Neisseria meningitidis* (meningococci, MC) are causative agents of gonorrhoea and meningitis (Meyer *et al.*, 1994; Nassif and So, 1995). Both species initiate infection by colonizing mucosal epithelial cells, a process that has been studied intensively using organ and cell culture systems. Several adhesins have been identified in GC and MC, and receptors have been identified for a subset of these. Type IV pili are fibrous structures that mediate the initial adhesion of GC and MC to epithelial cells and may be required for pathogenicity *in vivo* (Kellogg *et al.*, 1963; 1968; Swanson, 1973; Buchanan *et al.*, 1977; Nassif *et al.*, 1993; Seifert *et al.*, 1994; Cannon *et al.*, 1996). GC pili are implicated in twitching motility, which requires both pilus assembly and the *pilT* locus (Wolfgang *et al.*, 1998a,b). Neisserial pili exhibit high-frequency phase (on/off) and antigenic (primary structure) variation (Seifert, 1996). Both antigenic variation at *pilE* and phase variation at *pilC* can result in the assembly of pili with altered binding properties (Meyer *et al.*, 1994; Nassif and So, 1995; Seifert, 1996). Human CD46 (membrane cofactor protein, MCP) has been identified as a host receptor for neisserial type IV pili (Kallstrom *et al.*, 1997).

The Opa outer membrane proteins are encoded by a family of unlinked genes that are independently phase variable (Meyer *et al.*, 1994; Nassif and So, 1995; Dehio *et al.*, 1998a). This paper uses the unified Opa nomenclature suggested recently by Achtman *et al.* (Dehio *et al.*, 1998a; Malorny *et al.*, 1998). Particular Opa variants confer the ability to invade certain epithelial cell lines. One set of invasion-associated Opas, exemplified by Opa30 from GC strain MS11, contains putative surface-exposed loops rich in basic amino acids. These Opa variants bind to a variety of polyanionic molecules, including heparan sulphate proteoglycans on the host cell surface, and to vitronectin. Moreover, soluble heparin, heparan sulphate and DNA bind to these Opas and potently inhibit Opa-mediated adhesion and invasion by non-piliated GC (Swanson, 1992a,b;

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1994; Chen *et al.*, 1995; van Putten and Paul, 1995; Gomez-Duarte *et al.*, 1997; van Putten *et al.*, 1997; Dehio *et al.*, 1998b). Many other Opa variants expressed by either GC or MC have recently been shown to bind receptors in the CD66 family, which includes biliary glycoprotein, carcinoembryonic antigen and non-specific cross-reacting antigen (Chen and Gotschlich, 1996; Virji *et al.*, 1996a,b; Bos *et al.*, 1997; Chen *et al.*, 1997; Gray-Owen *et al.*, 1997a,b; Wang *et al.*, 1998). GC, MC or recombinant *Escherichia coli* expressing the appropriate Opa proteins can invade cells that express large amounts of CD66 (Dehio *et al.*, 1998a; Simon and Rest, 1992). There is also evidence for Opa binding to neisserial lipooligosaccharides (Blake *et al.*, 1995) and to serum components (Virji *et al.*, 1994; Duensing and van Putten, 1997; 1998; Gomez-Duarte *et al.*, 1997; Dehio *et al.*, 1998b). Opc, a protein weakly related to Opa, is present in some MC strains. A similar protein appears to be encoded in the genome of GC strain FA1090. Opc permits invasion of endothelial cells by non-encapsulated MC and can bind human vitronectin (Virji *et al.*, 1994). Other potential adhesins have been identified in GC and MC, including lipooligosaccharide (Porat *et al.*, 1995a,b) and multiple glycolipid-binding adhesins (Paruchuri *et al.*, 1990). These and other components await further characterization.

In several cases, bacteria appear to initiate adhesion cascades, ordered processes in which multiple adhesins sequentially engage different receptors on a single host cell (Isberg, 1991; Hoepelman and Tuomanen, 1992; Hultgren *et al.*, 1993). These bacteria may exploit not only host cell receptors, but also signalling pathways and dynamic functions normally used by host cells to establish adhesion to other cells and to substrates. For example, in both normal metazoan cell adhesion and microbe–host cell interactions, attachment is often accompanied by the formation in the host cell of structures containing cytoskeletal components, tyrosine-phosphorylated proteins and signalling molecules. In addition, subsets of membrane-associated proteins and glycolipids may become highly concentrated at these sites, processes known as clustering, capping or plaque formation (Singer, 1992; Yamada and Geiger, 1997; Adams and Nelson, 1998). Cortical rearrangements can be a prerequisite for subsequent events, such as the initiation of T-cell receptor signalling (Singer, 1992; Dustin, 1998; Monks *et al.*, 1998), the full activation of integrin-mediated signalling from focal adhesions (Yamada and Geiger, 1997) or *fimH*-mediated survival of *Escherichia coli* within macrophages (Baorto *et al.*, 1997).

GC and MC interactions with host cells may also follow a multistep cell adhesion pathway, in which initial attachment of the bacteria triggers host cell responses that facilitate subsequent adhesive interactions. Genetic studies indicate that GC and MC type IV pili inhibit bacterial invasion of some cell types but enhance the invasion of others

(Makino *et al.*, 1991; Virji *et al.*, 1995; Merz *et al.*, 1996; Pujol *et al.*, 1997). These results suggest that pili modulate the function of other neisserial adhesins in a manner that depends on the host cell.

Electron microscopy data are also consistent with a multistep adhesion process. GC or MC initially attach via pili and form small aggregates or microcolonies. At this stage, host microvilli often appear to be elongated and can be seen contacting the bacterial cell envelope. At later times, the bacteria and host cell surfaces become tightly apposed, a stage referred to as 'close association'. At this stage, the host and bacterial membranes are only a few nanometres apart, the host cell cortex immediately beneath the bacteria is amorphous and electron dense, microvilli have largely disappeared from the host cell and the bacteria adhere as individual organisms rather than as microcolonies (Ward and Watt, 1972; McGee *et al.*, 1983; Shaw and Falkow, 1988; Stephens and Farley, 1991; Pujol *et al.*, 1997).

More recent studies using molecular markers and fluorescence microscopy are consistent with the electron microscopy data. At early stages, the bacteria attach as microcolonies, accompanied by microvillus elongation and rearrangements of cortical actin filaments (Grassme *et al.*, 1996; Merz and So, 1997; Pujol *et al.*, 1997; Giardina *et al.*, 1998), as well as the accumulation of tyrosine-phosphorylated proteins at sites of bacterial attachment (Merz and So, 1997). Accumulations of actin and phosphotyrosine occur with piliated (P^+) Opa⁻ GC or MC or with non-piliated (P^-) Opa30⁺ GC, and are thus potentiated by *Neisseriae* that adhere via at least two distinct adhesin–receptor combinations (Merz and So, 1997). Furthermore, recent reports indicate that GC adhering via different adhesins trigger the activation of different signal transduction systems in host cells (Grassme *et al.*, 1997; Hauck *et al.*, 1998; Kallstrom *et al.*, 1998). At later times after infection, bacteria adhere as individual organisms that lack immunodetectable pili (Pujol *et al.*, 1997), and microvilli disappear from the host cell surface (Merz and So, 1997).

In this report, we present two sets of observations that support the hypothesis that neisserial type IV pili are the initiators of a multistep adhesion cascade. First, we demonstrate that the attachment of P^+ GC or MC to epithelial cells triggers rapid rearrangements within the host cell cortex. These rearrangements result in the formation of plaques comprising both components of the cortical cytoskeleton and a specific subset of transmembrane glycoproteins, including receptors known to be involved in cell adhesion, signal transduction and the inflammatory response. Cortical plaque formation is shown to be adhesin specific. Plaque formation occurs only in the presence of type IV pili and is partially dependent on the *pilT* locus, suggesting a role for pilus-mediated twitching motility. Plaque formation does not require several other neisserial surface structures, including Opa. Second, invasion assays indicate that P^+

GC are at least 100-fold more resistant than P⁻ GC to the effects of heparin, a potent inhibitor of Opa-mediated invasion of A431 and Chang epithelial cells. Together, these results demonstrate that neisserial adhesion via type IV pili triggers specific rearrangements at the host cell surface that may have modulatory effects on subsequent *Neisseria*-host interactions.

Results

Piliated GC cause major rearrangements in the epithelial plasma membrane

In previous work, the attachment of GC and MC to cultured epithelial cells was shown to trigger the assembly of f-actin and phosphotyrosine-rich structures at the sites of bacterial attachment (Merz and So, 1997). These results raised the question of whether rearrangements elicited by GC and MC are confined to the region beneath the host cell plasma membrane, or whether changes also occur within the plasma membrane itself. To address this question, the subcellular localization of several transmembrane glycoproteins was examined after GC and MC infection. A431, Chang or HEC-1-B human epithelial cells were grown on coverslips, infected with P⁺ GC or MC, fixed, and processed for indirect immunofluorescence microscopy, as described in *Experimental procedures*.

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase. EGFR associates with the cortical cytoskeleton, is present at the medium-exposed surface of A431 cells grown on plastic or glass and is a major phosphoprotein of A431 cells (Landreth *et al.*, 1985; den Hartigh *et al.*, 1992; van Bergen en Henegouwen *et al.*, 1992). Indirect immunofluorescent staining of uninfected A431, HEC-1-B and Chang epithelial cells demonstrated EGFR on microvilli, at the leading edge of cell protrusions and at lateral cell-cell junctions, as reported previously (Lichtner and Schirrmacher, 1990; van Bergen en Henegouwen *et al.*, 1992). The amount of EGFR observed at lateral cell-cell junctions correlated with the degree of culture confluence, but EGFR was observed on the medium-exposed surfaces of all three cell lines even in confluent cells. When epithelial cells were infected with MC8013.6 (P⁺ Opa⁻ *opc*) or GC

MS11A (P⁺ Opa⁻), dramatic clusters of EGFR were found associated with microcolonies of bacteria (Fig. 1A). Some EGFR clusters were visible within 1 h after infection and, by 4 h after infection, >90% of adherent microcolonies were associated with EGFR accumulations. The *Neisseria*-associated EGFR clusters were very similar in appearance to 'caps' formed in antibody-mediated cross-linking experiments (Khrebtukova *et al.*, 1991; Singer, 1992). Identical results were obtained using either monoclonal or polyclonal antibodies against EGFR, and using A431, Chang or HEC-1-B cells (see *Experimental procedures*). These results are consistent with our previous work demonstrating that phosphotyrosine-containing proteins are recruited to the sites of GC and MC attachment (Merz and So, 1997).

CD44 comprises a broadly expressed family of variant transmembrane proteins in mammalian cells that are synthesized from differentially spliced mRNA transcripts from a single genetic locus (Sherman *et al.*, 1994). CD44 variants are extensively and differentially glycosylated and have functions in cell-cell and cell-matrix adhesion, in the presentation of growth factors and chemokines and in the induction of inflammation (Sherman *et al.*, 1994). The localization of the entire population of CD44 molecules was examined using two different monoclonal antibodies that recognize epitopes common to all CD44 variants (panCD44). In uninfected cells, panCD44 had a subcellular distribution similar to that of EGFR, with the highest concentrations at lateral cell-cell junctions and at cell protrusions, but with reactivity visible over the entire surface of most cells. Immunofluorescent staining of infected cells revealed dramatic concentrations of panCD44 associated with adherent microcolonies of GC MS11A or MC8013.6 (Figs 1B and 2A). panCD44 accumulations were observed by 1 h after infection and, by 4 h after infection, >90% of adherent microcolonies were associated with CD44 accumulations. Identical results were obtained with A431, Chang or HEC-1-B cells (see *Experimental procedures*).

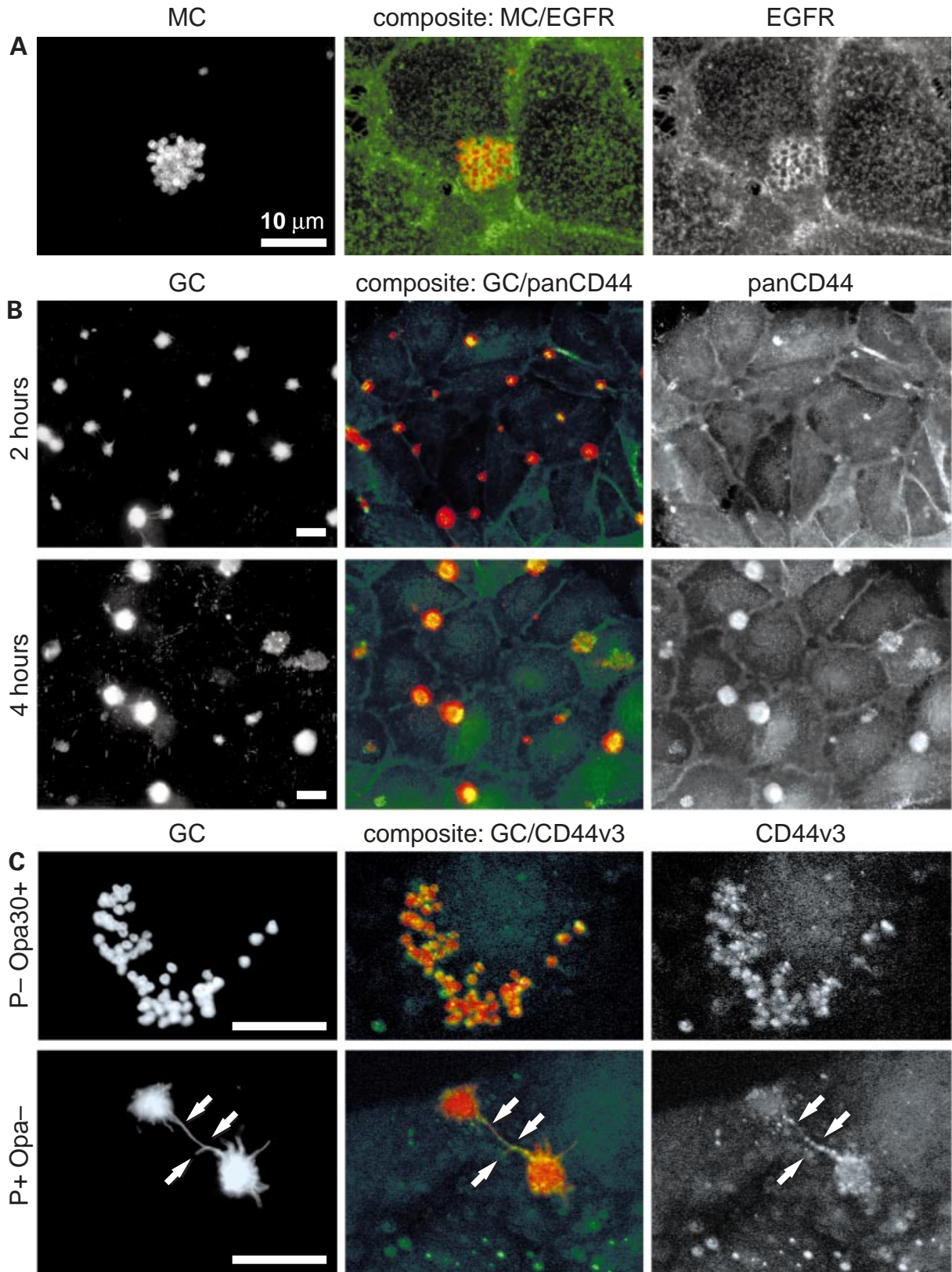
In both human keratinocytes and the A431 cervical carcinoma cells used in the present experiments, a subset of CD44 molecules contains the variant 3 (v3) exon. In these variants, the v3 domain of the mature protein is decorated with both heparan and chondroitin sulphate (Milstone *et*

Fig. 1. Pathogenic *Neisseriae* trigger rearrangement of EGFR and CD44 variants.

A. Distribution of EGFR. HEC-1-B cells were infected for 4 h with MC 8013.6 (P⁺ Opa⁻ *opc*), fixed and processed for double immunofluorescence using anti-MC antiserum and mAb EGF(R)-(528). Optical sections were acquired by confocal microscopy and assembled into extended focus projections as described in *Experimental procedures*.

B. Wide-field views of distribution of panCD44 in A431 cells infected with GC MS11A (P⁺ Opa⁻) for 2 h (top row) or 4 h (bottom row). panCD44 was detected using mAb H4C4. Note that almost all microcolonies of GC are associated with panCD44 accumulations. These images were acquired by conventional wide-field immunofluorescence microscopy.

C. Distribution of CD44v3 in A431 cells infected with GC MS11 AM13.1 (P⁻ Opa³⁰⁺; top row) or GC MS11A (P⁺ Opa⁻; bottom row). CD44v3 was detected using mAb 3C5. Note localization of CD44v3 with linear structures presumed to be type IV pili (arrows) and with microcolonies of ≈10 GC cells. Individual bacterial cells are not rendered distinctly in this micrograph because the photomultiplier gain was increased to render pili clearly. These images are single optical sections obtained by confocal microscopy.



al., 1994; Bennett *et al.*, 1995; Jackson *et al.*, 1995). Multiple heparan sulphate proteoglycans have been shown to serve as receptors for a subset of Opa proteins, including MS11 Opa30, and to be recruited to the sites of GC attachment when Opa30 is produced (Chen *et al.*, 1995; van Putten and Paul, 1995; van Putten *et al.*, 1997). To determine whether CD44v3 is recruited by GC in A431 cells, indirect immunofluorescence was performed using a specific monoclonal antibody directed against the v3 domain. The results (Fig. 1C) show that CD44v3 variants form clusters beneath adherent P⁻ Opa30⁺ GC MS11 AM13.1, as would be predicted for a known heparan sulphate proteoglycan. However, clusters of CD44v3 were also observed beneath P⁺ Opa⁻ GC MS11A. CD44v3 clusters were observed beneath both microcolonies and linear structures that stain with anti-GC antiserum. These linear fluorescent structures are very likely to be type IV pili, because such structures are only rarely observed with *pilC* null mutants and are never observed with *pilE* null mutants. *pilC* and *pilE* null mutants have few or no pili when examined by transmission electron microscopy (Meyer *et al.*, 1994; Nassif and So, 1995). The above results suggest that cortical rearrangements triggered by piliated GC result in the recruitment of at least one host molecule that subsequently interacts with a GC outer membrane adhesin.

ICAM-1, an adhesion molecule induced by inflammatory stimuli, is upregulated in cultured epithelial cells infected with *Salmonella typhimurium* (Huang *et al.*, 1996). In cells infected for 4 h with GC (P⁺ Opa⁻), ICAM-1 staining was visible in bright, punctate clusters beneath adherent GC microcolonies (Fig. 2B). The staining pattern of the ICAM-1 clusters was similar to that observed with panCD44 and EGFR; however, the overall distribution of ICAM-1 appeared to be somewhat more punctate. Similar results were obtained using both A431 (not shown) and Chang cells (Fig. 2B).

Transferrin receptor (TfR) is the transmembrane glycoprotein receptor for the serum protein transferrin. TfR cycles rapidly and continuously between the plasma membrane and early endosomes. In marked contrast to CD44, EGFR and ICAM-1, little or no co-localization of TfR was observed with adherent GC (Fig. 3). Less than 20% of

adherent P⁺ Opa⁻ GC MS11A was associated with accumulations of TfR. Similar results were obtained with MC and when cells were observed by conventional wide-field or confocal fluorescence microscopy. The absence of TfR clusters at the bacterial attachment sites indicates that the GC- and MC-induced plaques comprise only a specific subset of the proteins in the epithelial membrane.

Together, these experiments demonstrate that binding to host epithelial cells by P⁺ Opa⁻ GC MS11A triggers the recruitment of specific proteins within the plasma membrane. GC-induced clusters of transmembrane panCD44 and EGFR were observed when infected cells were not treated with detergent or were fixed using a different protocol, indicating that the observed structures are not artifacts of the fixation or detergent treatments used (see *Experimental procedures*). Furthermore, experiments performed with multiple cell lines yielded similar results, suggesting that the clustering of transmembrane proteins may be a general consequence of the attachment of piliated GC and MC. We refer to these clusters of proteins as cortical plaques because of their resemblance to cortical plaques that form during other eukaryotic cell adhesion events.

Neisseria-triggered membrane protein clustering correlates with cytoskeletal rearrangements and is not prevented by cytochalasin D

P⁺ Opa⁻ GC and MC trigger actin accumulation at sites of bacterial attachment (Merz and So, 1997), and EGFR, CD44 and ICAM-1 have all been identified as proteins that interact with the cortical cytoskeleton. In contrast, TfR, which does not associate with adherent GC or MC, is not thought to associate with the cytoskeleton. Double-label immunofluorescence microscopy was therefore used to compare the distribution of f-actin with that of panCD44, ICAM-1 and EGFR in infected epithelial cells. At 3 h after infection, dramatic clusters of panCD44, EGFR and ICAM-1 were associated with adherent microcolonies of P⁺ Opa⁻ GC MS11A (Fig. 2A–C). In each case, cortical f-actin accumulations were found to coincide with the membrane protein-enriched plaques at sites at which GC had attached (Fig. 2A and C; data not shown).

Fig. 2. Co-localization of *Neisseria*-triggered transmembrane and cytoskeletal protein clusters.

A. Co-localization of panCD44 and f-actin clusters in A431 cells infected with GC MS11A (P⁺ Opa⁻) for 3 h. panCD44 was detected using mAb H4C4, and f-actin was detected using BODIPY 581/591-phalloidin.

B. Co-localization of ICAM-1 and ezrin in Chang epithelial cells. ICAM-1 was detected with mAb P2A4, and ezrin was detected using antiserum B22. Images are extended focus projections of sets of optical sections acquired by confocal microscopy. Arrows show the locations of GC microcolonies, as determined by phase-contrast microscopy.

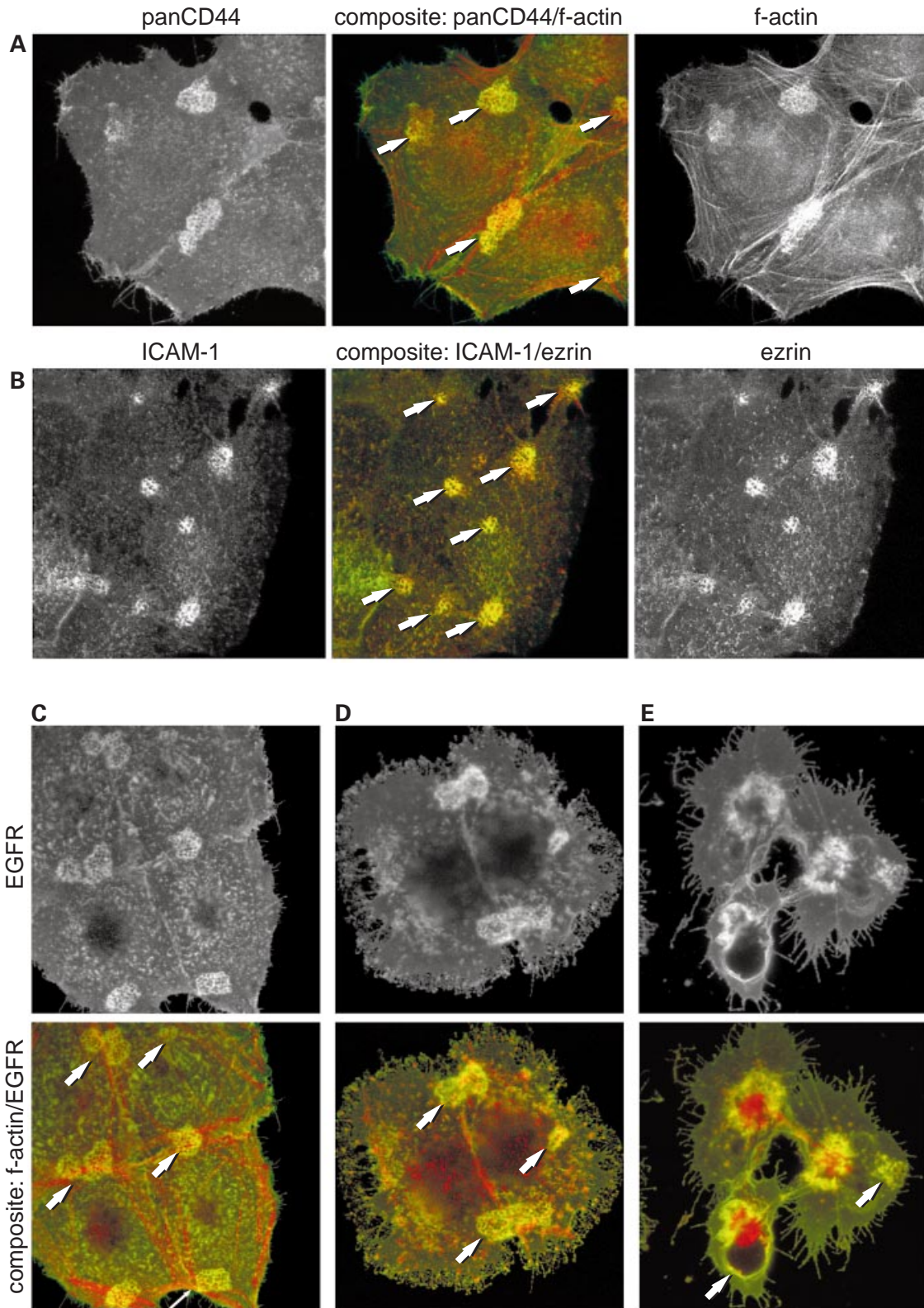
C–E. Effects of CCD (cytochalasin D; 5 µg ml⁻¹ final concentration) on GC MS11A-induced EGFR clusters in A431 cells.

C. Control infection (3 h) in the presence of DMSO vehicle.

D. CCD present during the final 20 min of a 3 h infection.

E. CCD added 20 min before a 3 h infection.

Images are extended focus projections of sets of optical sections acquired by confocal microscopy. Arrows indicate the positions of GC microcolonies, as determined by phase-contrast microscopy.



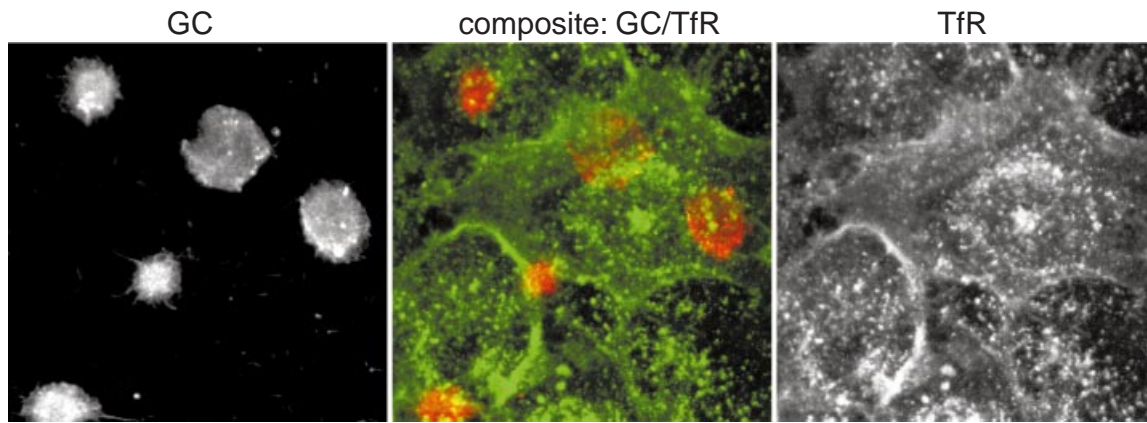


Fig. 3. Distribution of TfR in A431 cells infected with GC MS11A ($P^+ Opa^-$) for 4 h. Confocal optical sections were acquired at $1 \mu\text{m}$ intervals and assembled into extended focus projections as described in *Experimental procedures*. Identical results were obtained by conventional wide-field fluorescence microscopy.

The distribution of the cortical cytoskeleton component ezrin was also examined. Ezrin is expressed in epithelial cells and is concentrated at the cell cortex in microvilli and in other cell protrusions. It is thought to serve as a physical link between the f-actin cytoskeleton and many transmembrane and membrane-associated proteins, including panCD44 and ICAM-1, and binds to several signalling molecules (Bretscher *et al.*, 1997). When infected cells were examined using indirect immunofluorescence, dramatic foci of ezrin staining were observed beneath adherent microcolonies of P^+ GC (Fig. 2B) or MC (not shown). In double-label immunofluorescence experiments, ezrin colocalized closely with ICAM-1 (Fig. 3B), panCD44 and EGFR (not shown) at the sites of bacterial attachment. Thus, at least two components of the cortical cytoskeleton, f-actin and ezrin, are concentrated in cortical clusters associated with adherent P^+ Opa^- GC, together with a subset of transmembrane glycoproteins.

EGFR has been shown to associate with the cortical cytoskeleton, possibly through direct binding to f-actin (Landreth *et al.*, 1985; den Hartigh *et al.*, 1992; van Bergen en Henegouwen *et al.*, 1992). These data suggested that disruption of the actin cytoskeleton might alter the morphology of GC-triggered cortical plaques (Fig. 2C–E). We first tested whether preformed clusters of EGFR were sensitive to the f-actin disrupting agent cytochalasin D (CCD). Treatment with $5 \mu\text{g ml}^{-1}$ CCD during the last 20 min of a 3 h GC MS11A ($P^+ Opa^-$) infection resulted in the disassembly of all f-actin stress fibres (Fig. 2D; Merz and So, 1997). In addition, the distribution of EGFR was altered. In the control cells, the distribution of EGFR was somewhat punctate as a result of the presence of microvilli (Fig. 2C) but, after 20 min of CCD treatment, the distribution of EGFR was much more homogeneous over the cell surface (Fig. 2D). However, although CCD treatment produced changes in

the subcellular localization of both f-actin and EGFR, neither the preformed EGFR clusters nor the f-actin accumulations beneath adherent GC were disrupted by this treatment. Thus, CCD treatment did not result in the disassembly of preformed GC-triggered f-actin or EGFR clusters.

Experiments were also performed to determine whether CCD could inhibit the formation of new EGFR clusters by beginning CCD treatment 20 min before a 3 h infection (Fig. 2E). The results indicated that EGFR still accumulates beneath adherent GC, even though the actin cytoskeleton is massively perturbed by CCD treatment (Fig. 2E; Merz and So, 1997). We conclude from these results that either GC-triggered cortical rearrangements of EGFR are f-actin independent, or that the rearrangements involve a CCD-insensitive pool of f-actin (Morris and Tannenbaum, 1980). This contrasts with GC internalization by host cells, a process strongly inhibited by the same concentration of CCD (Shaw and Falkow, 1988).

Neisserial components involved in cortical plaque formation

To define the bacterial requirements for cortical plaque formation, a panel of GC and MC strains was evaluated for the ability to elicit cortical plaques (Table 1). To simplify scoring of the samples, EGFR and panCD44 were chosen as diagnostic markers for plaque formation. These molecules are abundant at the A431 cell surface and form especially bright, visible clusters under adherent GC MS11A or MC 8013.3. Cells were infected for 3–4 h because at these times nearly all adherent microcolonies of GC MS11A or MC8013.6 were associated with concentrations of panCD44 and EGFR (see Figs 1B and 4A for examples). The criteria used to score cortical plaque formation are described in *Experimental procedures*.

Table 1. Ability of *Neisseria* strains to trigger epithelial membrane protein clustering in A431 cells.

Strain	Genotype and/or phenotype	Clustering panCD44	EGFR	Strain source or reference
<i>N. gonorrhoeae</i>				
15063G	P ⁺ Opa ⁺	+	+	Waldbeser <i>et al.</i> (1994)
FA1090	P ⁺ Opa ⁻	+	+	
MS11A	P ⁺ Opa ⁻	+	+	Segal <i>et al.</i> (1986)
MS11A (+ chloramphenicol)	P ⁺ Opa ⁻ (non-adherent)	-	-	
MS11A (heat-killed)	P ⁺ Opa ⁻ (non-adherent)	-	-	
MS11A (+ 50 µg ml ⁻¹ heparin)	P ⁺ Opa ⁻	+	+	
MS11 24-1	P ⁺ Opa ⁻ GgO ₄ -binding ⁻	+	+	Paruchuri <i>et al.</i> (1990)
MS11-306	P ⁻ (Δ <i>pilE1</i> Δ <i>pilE2</i>) Opa ⁻ (low adherence, MOI 200)	-	-	Merz <i>et al.</i> (1996)
MS11 AM1	P ⁺ (Δ <i>pilC1::Erm</i>) Opa ⁻	+	+	Merz and So (1997)
MS11 AM12	P ⁺ (Δ <i>pilC2::Cat</i>) Opa ⁻	+	+	Merz and So (1997)
MS11 AM13	P ⁻ (<i>PilC</i> ⁻) Opa ⁻ (low adherence)	-	-	Merz and So (1997)
MS11 AM13.1	P ⁻ (<i>PilC</i> ⁻) Opa30 ⁺	-	-	Merz and So (1997)
MS11 AM13.1R	P ⁻ (<i>PilC</i> ⁻) Opa ⁻ (low adherence)	-	-	Merz and So (1997)
N400	MS11 VD300 (<i>recA6</i>) P ⁺ Opa ⁻	+	+	Wolfgang <i>et al.</i> (1998a)
GT103	N400 P ⁺ (<i>pilT::mTnErm</i>) Opa ⁻	(+)	(+)	Wolfgang <i>et al.</i> (1998a)
GT102	N400 P ⁺ (<i>pilT</i> Δ <i>QSL 318-320</i>) Opa ⁻	(+)	(+)	Wolfgang <i>et al.</i> (1998a)
<i>N. meningitidis</i>				
8013.6	P ⁺ Opa ⁻ <i>opc</i> (serogroup C capsule ⁺)	+	+	Nassif <i>et al.</i> (1993)
8013 <i>pilE::Km</i>	P ⁻ Opa ⁻ (non-adherent)	-	-	Nassif <i>et al.</i> (1993)
8013 <i>pilC1::Erm</i>	P ⁺ Opa ⁻ (low adherence, MOI 200)	+	+	Nassif <i>et al.</i> (1994)
8013 <i>pilC2::Km</i>	P ⁺ Opa ⁻	+	+	Nassif <i>et al.</i> (1994)

panCD44 and EGFR clustering was scored as '+' if at least 70% of adherent microcolonies were associated with clusters, and as '-' if less than 30% of adherent microcolonies were associated with clusters. The GC *pilT* mutants are scored as '(+)' because the panCD44 and EGFR clusters associated with these strains appeared to be less intense than with the parent strain (see Fig. 4). Samples were fixed and stained at 3–4 h after infection, and a positive control (usually GC MS11A) was included in every experiment. All experiments were performed at least twice on different days.

P⁺ neisserial strains that do not produce detectable Opa or Opc proteins (GC MS11A and FA1090, and MC 8013.6) caused cortical plaque formation (Table 1). These results indicate that Opa and Opc are not necessary for cortical plaque formation.

The P⁺ GC strain MS11A24-1 carries defined mutations in at least two loci that encode glycolipid-binding adhesins and is defective in binding to GgO₄ (gangliotetraosyl ceramide) in an *in vitro* overlay assay (Paruchuri *et al.*, 1990). This strain induced cortical plaques similar to those elicited by its isogenic wild-type parent MS11A (Table 1). Therefore, the GgO₄-binding adhesins are not required for cortical plaque formation. Additional loci homologous to those mutated in MS11A24-1 have been detected in GC and MC by DNA hybridization and in DNA sequence database analyses (Paruchuri, 1990; unpublished results). At present, these other loci cannot be excluded as participants in the formation of cortical plaques.

P⁺ strains with different lipooligosaccharide structures (e.g. GC MS11A and FA1090) and both capsulated (MC 8013.6) and non-encapsulated *Neisseriae* (all GC strains used) all caused plaque formation (Table 1). These results suggest that particular carbohydrate structures present on the neisserial surface are neither required for, nor inhibitory of, cortical plaque formation.

Several GC and MC strains carrying mutations in loci important for type IV pilus assembly and function were

assayed. Null mutations in *pilE*, which encodes pilin, the major pilus subunit, result in a non-piliated (P⁻) phenotype. GC MS11-306 is P⁻ (Δ *pilE1* Δ *pilE2*) and Opa⁻ and is 20- to 50-fold reduced in adherence to epithelial cells relative to its P⁺ parent strain. At very high multiplicities of infection (MOI; 200), significant numbers of GC MS11-306 adhered to host cells, probably because of the presence of minor subpopulations of Opa⁺ phase variants in the inocula (Makino *et al.*, 1991). However, clusters of EGFR or panCD44 were not associated with the adherent GC under these conditions (Table 1). These results suggest that, even if GC adhere via non-pilus adhesins, *pilE* is required for cortical plaque formation.

Two *pilC* loci are present in GC and in MC. These loci are functionally redundant in pilus biosynthesis, because strains with defects in either *pilC* locus are P⁺, while double mutants have few or no pili. Because mutation of MC *pilC1* results in P⁺ organisms with reduced adherence, a high MOI of 200 was used in assays with the MC *pilC1* mutant. Under these conditions, all four P⁺ single mutants tested (GC *pilC1* and *pilC2*; MC *pilC1* and *pilC2*) triggered clustering of EGFR and panCD44 (Table 1). In contrast, the P⁻ strain GC MS11 AM13.1, with mutations in both *pilC* loci, did not cause panCD44 or EGFR clustering (Table 1). This strain produces Opa30, and its adherence efficiency is equivalent to its P⁺ Opa⁻ parent MS11A (data not shown). Although this strain did cause clustering of

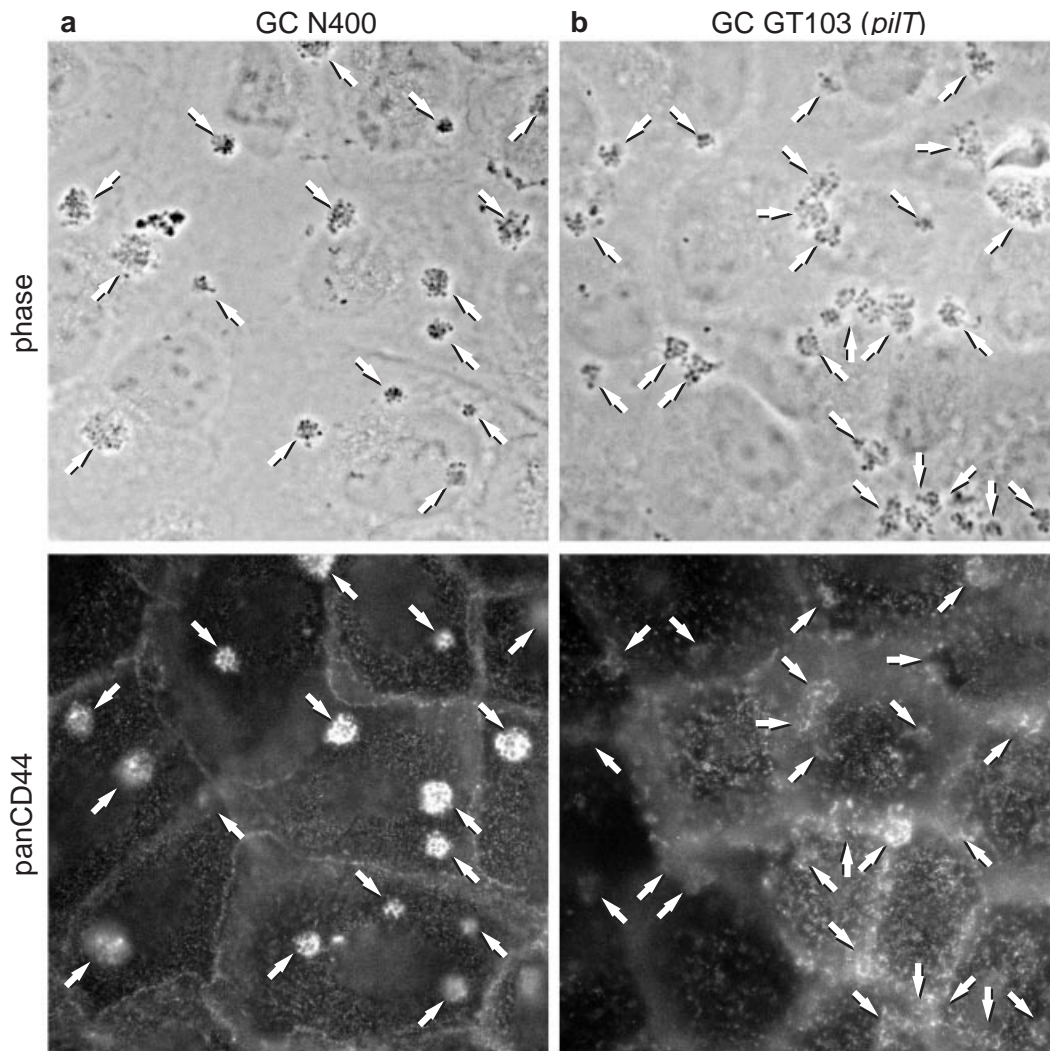


Fig. 4. GC *pilT* mutants exhibit a partial loss of function in the plaque formation assay. Wide-field views of A431 cells infected for 3 h with (A) GC N400 (*pilT*⁺ P⁺ Opa⁻) or (B) GC GT 103 (*pilT*::mTnErm P⁺ Opa⁻). Phase-contrast images are shown at the top, and the same fields of cells stained for panCD44 are shown at the bottom. Arrows indicate the positions of GC microcolonies. Note that GC N400 and GC GT103 adhere in similar numbers. Identical results were obtained using GC GT 103 (*pilT*::mTnErm) or GC GT102 (*pilT*_{Δ*QSL* 318–320}), and similar results were obtained when cells were stained for EGFR, ICAM-1 or ezrin.

CD44v3, a minor CD44 population decorated with heparan sulphate (Fig. 1C), it did not cause clustering of EGFR or of the entire (pan)CD44 population (Table 1). This result demonstrates that a P⁻ strain with mutations in both *pilC* loci cannot trigger cortical plaque formation, even in the presence of Opa30, an outer membrane adhesin that facilitates efficient attachment via heparan sulphate proteoglycans.

Taken together, these results show that two different types of mutations that abolish or strongly diminish piliation (*pilE* null or *pilC* null) also eliminate the ability to trigger cortical plaques, suggesting strongly that pili are required for plaque formation. These results also indicate that the individual *pilC* loci of GC and MC are interchangeable in

the induction of plaque formation. Although the *pilC1* locus in MC is specifically required for efficient adhesion to epithelial cells, plaque formation was still triggered by the MC *pilC1* mutant, provided that it was present at a high enough MOI to compensate for its low adhesion efficiency. In addition, strains carrying different *pilE* alleles (MC 8013.6 and GC FA1090, MS11A and MS11N400) all triggered cortical plaque formation, indicating that plaque formation does not depend on the expression of a unique pilin antigenic variant.

The GC *pilT* locus is required for twitching motility but not for pilus assembly, autoaggregation or attachment to host cells (Wolfgang *et al.*, 1998a). Two different GC *pilT* mutants were assayed, one generated by a mTnEGNS

insertion and one with a non-polar in frame deletion that eliminates a short stretch of residues within the putative ATP-binding cassette of PilT. Both mutants caused clustering of EGFR and panCD44 (Table 1 and Fig. 4B), as well as of ezrin and ICAM-1 (not shown). However, the intensity of the clusters formed was strikingly reduced in comparison with the wild-type parent strain GC MS11 N400 (Fig. 4A). We were unable to quantify this reduction in staining intensity. Mutation of *pilT* therefore confers a partial defect in cortical plaque formation.

Taken together, our data strongly suggest that type IV pilus-mediated adherence is required for GC and MC to form cortical plaques and indicate that twitching motility (or a related function) strongly enhances the efficiency of plaque formation. Our results also show that Opa, Opc, glycolipid GgO₄-binding adhesin, specific lipooligosaccharide structures and capsule are not involved in the neisserial triggering of cortical plaque formation in A431 cells.

Invasion of Chang and A431 cells by piliated GC is not inhibited by heparin

The observation that P⁺ GC and MC cause relatively rapid rearrangements in the host cell cortex suggested that pili might influence the course of events later in colonization.

In particular, the observation that P⁺ GC recruited a heparan sulphate proteoglycan to the attachment site, even in the absence of Opa expression, suggested that pili might facilitate Opa–receptor interactions. To test this prediction, the well-characterized Chang cell model for GC invasion was used (see *Experimental procedures*).

Chang cells are invaded efficiently by non-piliated GC MS11 that express Opa30 (Makino *et al.*, 1991; Kupsch *et al.*, 1993). When Chang cells are infected with P⁻ Opa⁻ GC, spontaneously arising Opa30⁺ phase variants are highly enriched in the gentamicin-protected fraction (Makino *et al.*, 1991; Kupsch *et al.*, 1993). These and other experiments indicate that, even if the GC inoculum is predominantly (>95%) Opa⁻, invasion of Chang cells is mediated mainly through Opa30. Opa30⁺ GC bind to heparan sulphate proteoglycans present on the epithelial surface, and invasion by these organisms is potently inhibited by soluble polyanions such as heparin. To address whether similar mechanisms govern the interactions of P⁺ and P⁻ GC with Chang cells, isogenic P⁺ and P⁻ GC were subjected to gentamicin protection assays in the presence of increasing concentrations of heparin.

As expected, the results indicate that, with P⁻ Opa⁻ GC MS11-306, heparin inhibits entry into Chang cells by 50% at $\approx 1 \mu\text{g ml}^{-1}$ and by 90% at $\approx 10 \mu\text{g ml}^{-1}$ (Fig. 6A). In contrast, invasion by the isogenic piliated GC strain MS11A

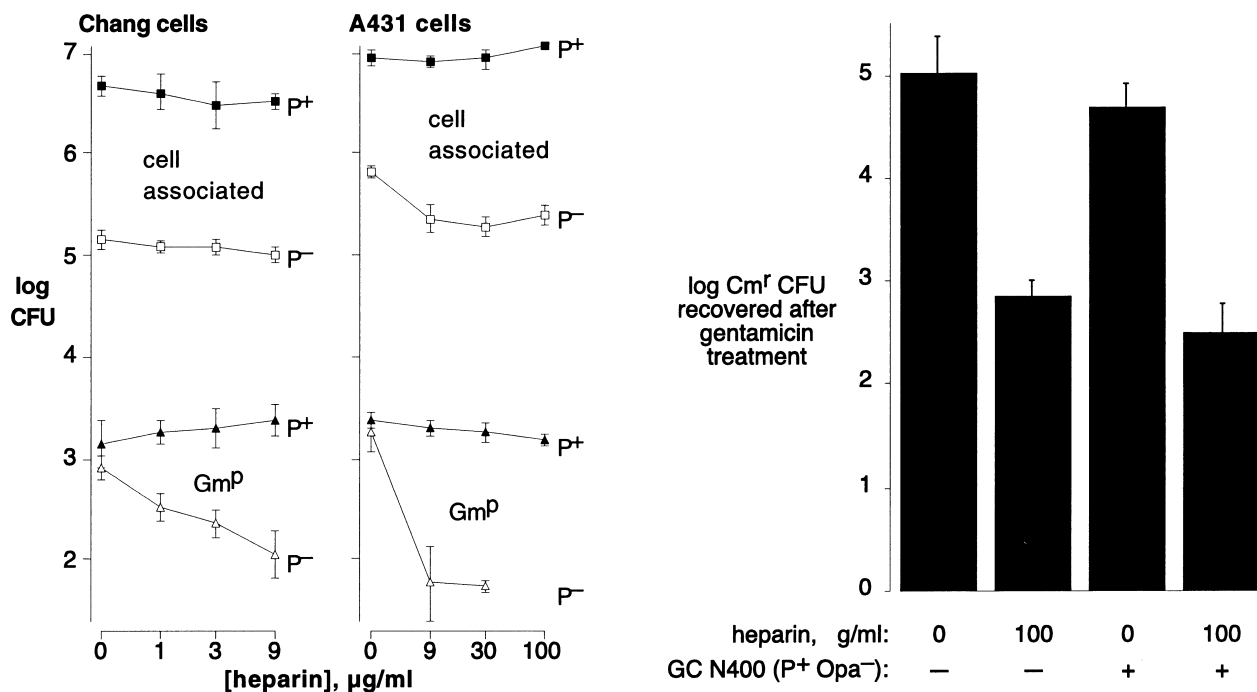


Fig. 5. Invasion of epithelial cells by P⁺ and P⁻ GC in the presence and absence of heparin. A. Adhesion (cell-associated) and invasion (Gm^P) of GCMS11A (P⁺) and GC MS11-306 (P⁻) in the presence of increasing concentrations of heparin. Infections were carried out for 8 h. Plots show mean \pm SD of triplicate determinations from a representative experiment. B. Invasion of Chang cells by MS11AM13.1 (P⁻ Opa⁺) in the presence or absence of heparin and in the presence or absence of P⁺ GC MS11 N400. Infection was carried out for 4 h. Bars indicate mean \pm SD of triplicate determinations from a representative experiment.

was undiminished when heparin was present at identical concentrations (Fig. 5A). Even at $100\ \mu\text{g ml}^{-1}$ heparin, invasion by the P^+ strain was reduced by less than 50%. Identical data were obtained using A431 cells (Fig. 5A). These experiments show that pili allow GC to overcome the inhibitory effects of heparin on GC invasion of Chang and A431 cells over a 100-fold range of heparin concentrations.

Pili could influence the invasion process in two ways. *In trans*, P^+ GC could help P^- GC to invade host cells in the presence of heparin. *In cis*, pili could confer this phenotype only upon the bacteria that produce them. To discriminate between these possibilities, P^- , Opa30⁺ GC MS11 AM13.1 were assayed for invasion of Chang cells in the presence or absence of $100\ \mu\text{g ml}^{-1}$ heparin and in the presence or absence of piliated MS11 N400 (Fig. 5B). MS11 AM13.1 carries a selectable marker, allowing the invasion of this strain to be measured in the presence of the P^+ strain N400. N400 was used because it carries the *recA6* allele, which renders it transformation defective and eliminates the possibility that marker transfer might cause the P^+ strain to be confused with the P^- strain. In both the presence and the absence of piliated organisms, heparin inhibited invasion by MS11 AM13.1 by ≈ 2.3 logs. Similar results were obtained using a predominantly Opa⁻ inoculum in place of MS11 AM13.1 (not shown). These results demonstrate that P^+ GC do not help P^- GC to invade Chang cells in either the presence or the absence of heparin.

Discussion

Neisserial type IV pili are complex organelles that mediate bacterial adhesion, autoaggregation, twitching motility and DNA transformation (Meyer *et al.*, 1994; Nassif and So, 1995; Seifert, 1996). Recent experiments also suggest a role for neisserial pili in the invasion and traversal of epithelial or endothelial monolayers, which may be distinct from their role in adhesion *per se* (Virji *et al.*, 1995; Merz *et al.*, 1996; Pujol *et al.*, 1997). The studies reported here reveal two new phenotypes conferred by neisserial type IV pili. First, the adhesion of P^+ GC and MC results in the formation of clusters, or plaques, of proteins within and immediately subjacent to the epithelial cell surface. These cortical plaques contain known signal transducers as well as molecules involved in cell adhesion and in the induction of inflammation. Cortical plaque formation depends on the presence of type IV pili, but not on Opa or Opc expression. Second, neisserial type IV pili neutralize the inhibitory effect of heparin on GC invasion of two epithelial cell lines, suggesting that pilus-mediated host cell responses may promote or stabilize interactions between other neisserial adhesins and their host receptors.

Cortical actin rearrangements occur in host cells after

neisserial attachment via either type IV pili or Opa (Grassme *et al.*, 1996; Merz and So, 1997; Pujol *et al.*, 1997; Giardina *et al.*, 1998). In addition, phosphotyrosine-containing proteins aggregate beneath GC and MC after attachment mediated by either type IV pili or GC Opa30 (Merz and So, 1997). In the present report, these observations are extended to show that the cortical rearrangements triggered by P^+ GC and MC occur within as well as beneath the plasma membrane, and that at least some of these rearrangements occur only in the presence of type IV pili.

Experiments using genetically defined bacterial mutants establish a strong correlation between pilus-mediated neisserial adhesion and large-scale cortical rearrangements (e.g. of EGFR and panCD44; Table 1). In contrast to the apparent requirement for type IV pili, many well-studied neisserial surface structures do not influence cortical plaque formation (Table 1). These results are consistent with the interpretation that pili are required for cortical plaque formation. However, we have been unable to induce plaque formation using pili or purified pilus preparations, either in solution or coated onto latex beads (data not shown). Therefore, we cannot formally exclude the possibility that plaque formation involves some uncharacterized function that is coupled to type IV pilus biogenesis. Consistent with this interpretation, P^+ GC strains carrying either of two defined *pilT* mutations were partially defective in cortical plaque formation (Table 1 and Fig. 4A). Although this result rules out an absolute requirement for twitching motility in cortical plaque formation, it indicates that a PilT-dependent function strongly increases the efficiency of plaque formation.

The mechanism by which PilT acts is not known, but circumstantial evidence implies that twitching motility in *Pseudomonas* and GC occurs through PilT-dependant pilus retraction (Bradley, 1974; Wolfgang *et al.*, 1998b). Interestingly, biophysical experiments have demonstrated directly that mechanical tension increases the efficiency of adhesion plaque formation in mammalian cells (Wang *et al.*, 1993). It is tempting to speculate that the observed PilT-dependent enhancement of cortical plaque formation occurs by analogous mechanisms and is a consequence of mechanical tension generated by PilT-dependent pilus retraction. A different possibility is that neisserial type IV pili participate in the export of secreted effectors, as suggested recently for the type IV pili of *Pseudomonas aeruginosa* (Lu *et al.*, 1997).

Neisseria-induced cortical rearrangements may modify interactions among host cells. CD44 and ICAM-1 are involved in the migration and activation of immune cells, and CD44 and EGFR are involved in cellular responses to growth factors. Ezrin, another component of pilus-induced cortical plaques, binds to both CD44 and ICAM-1 and can also bind to and modulate the function of additional

signalling molecules (Bretscher *et al.*, 1997). By modifying the adhesive properties of epithelial surfaces, the signalling properties of epithelial cells, or both, the cortical plaques triggered by piliated *Neisseriae* may directly influence the course of the mucosal inflammatory response. Recent data are consistent with this interpretation. Experimental infection of male volunteers with a large dose (10^6 cfu) of a non-reverting P^- GC mutant resulted in colonization of the male urethra, but failed to elicit the typical inflammatory response normally associated with infection at this site (Cannon *et al.*, 1996). Interestingly, enteropathogenic *Escherichia coli* carrying a mutation in *bfpF*, a structural and functional homologue of *pilT*, could colonize human volunteers but was 200-fold attenuated in virulence (Bieber *et al.*, 1998).

The observation that GC and MC attachment triggers adhesin-specific clustering of various host cell membrane proteins and cytoskeletal components is reminiscent of other eukaryotic cell adhesion processes, in which the ligation and/or cross-linking of different plasma membrane proteins results in the assembly of specific cortical structures (Singer, 1992; Wang *et al.*, 1993; Yamada and Geiger, 1997; Monks *et al.*, 1998). This similarity suggests that neisserial colonization of host cells may involve a similar type of multistep adhesion cascade, in which pilus-mediated attachment 'primes' the host cell surface to facilitate subsequent adhesin-receptor interactions. An observation that apparently contradicts this hypothesis is that P^- Opa⁺ GC adhere efficiently to and invade certain epithelial cell lines. However, the *in vitro* assays generally used to measure adhesion and invasion do not mimic certain aspects of attachment *in vivo*. Organisms colonizing mucosal epithelia *in vivo* are immersed in mucosal fluids. In this milieu, they are surrounded by high concentrations of nucleic acids, lipids and soluble glycoproteins, including both proteoglycans and CD66 family members (Moghissi, 1973; Krause, 1980; Asseo *et al.*, 1986; Neutra and Forstner, 1987; Fujii *et al.*, 1988; Nanbu *et al.*, 1988; Briese *et al.*, 1989; Mack and Sherman, 1991; Tabak, 1995; Widdicombe, 1995). In addition, the female reproductive tract is periodically washed with menstrual blood and tissue debris. Many components present in such mixtures bind to Opas and, in a few cases, these have been demonstrated to inhibit Opa-mediated adhesion or invasion (Swanson, 1992a,b; 1994; van Putten *et al.*, 1997).

Because numerous such components are present at high concentrations, they would be expected to compete with, and therefore inhibit, Opa-mediated binding to receptors on host cells (van Putten *et al.*, 1997). Indeed, a major function attributed to mucus is inhibition of microbial adhesion by soluble decoys that compete with or block adhesin-receptor interactions (Neutra and Forstner, 1987; Mack and Sherman, 1991; Tabak, 1995; Widdicombe, 1995). For example, low concentrations ($1-10 \mu\text{g ml}^{-1}$) of DNA or heparin substantially inhibit adhesion or invasion by P^- GC (Swanson, 1992b; Chen *et al.*, 1995; van Putten and Paul, 1995; van Putten *et al.*, 1997). At the far higher concentrations of competitors present in mucus, discrimination between *bona fide* host cell receptors and soluble decoys might pose an insurmountable obstacle to adhesion or invasion mediated solely by Opa or other outer membrane adhesins. Moreover, carbohydrate structures present on GC and MC surfaces *in vivo*, including capsule and sialylated lipooligosaccharide, are known to interfere with the function of outer membrane adhesins (Virji *et al.*, 1993; 1995; van Putten *et al.*, 1995).

We propose that pilus-mediated adhesion and the subsequent Opa-independent cell surface rearrangements documented here allow GC or MC to establish secondary adhesin-receptor interactions in the 'difficult' conditions that predominate *in vivo*. A simplified model of such a cascade is depicted in Fig. 6. In the presence of a single outer membrane adhesin such as Opa30, adhesion and invasion in tissue culture medium are efficient processes (Fig. 6A). But when decoys are present, they bind to the adhesin, blocking interaction with receptors on the cell surface (Fig. 6B). Adhesion via a primary adhesin such as pili partially overcomes this blockade by bringing the bacterium close to the host cell surface and raising the local concentration of receptors for the outer membrane adhesin (Fig. 6C). Moreover, if pilus-mediated attachment initiates a signal that triggers clustering of host receptors for the outer membrane adhesin, the local receptor concentration is increased further (Fig. 6D). In combination, pilus-mediated bacterial proximity and receptor clustering could increase the effective receptor concentration by several orders of magnitude. This is expected to have at least two consequences.

First, increased local receptor concentration should allow the bacterium to discriminate more effectively

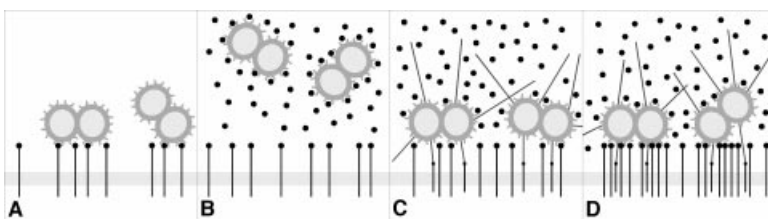


Fig. 6. Model for type IV pilus function in neisserial colonization of host cells. See text for discussion.

between host cell receptors and soluble decoys (Wickham *et al.*, 1995). Such an effect is difficult to measure empirically, but is consistent with our data showing that pilus expression allows GC to overcome the inhibitory effect of heparin on invasion of Chang and A431 cells and that P⁺ Opa⁻ GC trigger clustering of at least one heparan sulphate proteoglycan (Fig. 1C).

Second, increased local receptor concentration should allow bacterial adhesins to bind more avidly to host cell receptors. Increased avidity could (i) impart greater resistance to detachment by shear stresses, such as occur in the male urethra during urination; and (ii) allow low-affinity adhesin–receptor pairs to bind under conditions in which otherwise they would not. Data supporting the latter prediction were presented recently. Kallstrom *et al.* (1998) reported that neisserial pili trigger an intracellular [Ca²⁺] signal in ME-180 epithelial cells and that agents that elevate intracellular [Ca²⁺] permit ME-180 cells to bind *Neisseriae* even in the absence of Opa, Opc or pilus expression. In addition, receptor clustering might drive the coupling of bacterial adhesins with their host receptors despite the presence of antiadhesins such as MC capsule or sialylated lipopolysaccharide (Virji *et al.*, 1993; 1995; van Putten *et al.*, 1995).

A key prediction of our model is that additional host receptors for gonococcal adhesins, such as CD46, CD66 and asialoglycoprotein receptor, will also be enriched in pilus-induced cortical plaques. Experiments are under way to test this prediction. Both empirical studies (Yamada and Geiger, 1997; Adams and Nelson, 1998) and theoretical analyses (Ward *et al.*, 1994; 1995) show that receptor clustering and plaque formation are critical aspects of mammalian cell–cell and cell–substrate adhesion. Receptor clustering and plaque formation may also prove to be fundamental features of microbe–host interactions.

Experimental procedures

Cell lines, bacterial strains and infections

A431 and Chang cells were maintained in RPMI-1640 (Life Technologies) with 10% heat-inactivated fetal bovine serum (FBS) at 37°C (Hyclone or Life Technologies) and 5% CO₂. HEC-1-B cells were grown as described previously (Waldbeser *et al.*, 1994). For microscopy experiments, cells were usually plated at about 15% confluence onto ethanol-washed, autoclaved glass coverslips (no. 1.5; Fisher Scientific) 48 h before infection experiments. GC and MC strains (Table 1) were grown overnight on GC agar with Kellogg's supplements; *E. coli* (Table 1) were grown on Luria–Bertani (LB) agar. Piliation and Opa phenotypes were monitored by colony morphology and with Western blots. For infection experiments, bacteria were resuspended and diluted into unsupplemented Dulbecco's modified Eagle medium (DMEM; Life Technologies), then added to epithelial cells at a ratio of ≈10 colony-forming

units (cfu) per cell or as specified. Treatment with the microfilament-disrupting agent cytochalasin D (Sigma) was carried out either before or during infection with GC MS11A as described. Dimethyl sulphoxide (DMSO), used to dissolve CCD, was added to control cells (Merz and So, 1997).

Gentamicin protection assays

Gentamicin protection assays were performed as described previously (Waldbeser *et al.*, 1994), except that serum was omitted during infections, heparin (≈6000 molecular weight; Sigma) was added to the concentrations specified, and GC broth containing 0.5% saponin was used to lyse the epithelial cells. After gentamicin treatment, wash supernatants were monitored for viable counts to ensure that killing of extracellular organisms was complete.

Antibodies and sera

Rabbit sera 11507 and 8547 (Nassif *et al.*, 1994; Merz and So, 1997) react against whole GC and MC, respectively, and were diluted 1:1500. Monoclonal antibody (mAb) 4B12 recognizes a conserved epitope on Opa and was a gift from Milan Blake, North American Vaccine. Human EGFR was detected using monoclonal antibody EGF(R)-(528), diluted 1:500, and affinity-purified polyclonal antibody EGF(R)-(1005), diluted 1:500, both from Santa Cruz Biotechnology. panCD44 was detected using mAbs H4C4 and Hermes-1. H4C4 and Hermes-1 supernatants were diluted 1:100 and were obtained from the Developmental Studies Hybridoma Bank (maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, USA, under contract N01-HD-6–2915 from the National Institute of Child Health and Human Development). Human CD44v3 was detected using mAb 3C5 at 10 µg ml⁻¹. 3C5 was obtained from R and D Systems. Monoclonal antibody OKT9 reacts against the human transferrin receptor (TfR). OKT9 supernatants were diluted 1:40. Monoclonal antibody P2A4 recognizes human ICAM-1 and was obtained from the Developmental Studies Hybridoma Bank. P2A4 supernatants were diluted 1:100. Rabbit antiserum B22 recognizes human ezrin and was a kind gift from Anthony Bretscher. B22 was used at 1:500. Affinity-purified secondary goat antisera against rabbit or mouse immunoglobulins were conjugated to either BODIPY FL or Texas red X, and BODIPY 581/591-phalloidin, was obtained from Molecular Probes. These fluorescent reagents were diluted 1:500.

Fluorescence staining

After infection, samples were fixed for 20 min at room temperature in picric acid paraformaldehyde (PAPF; Zamboni and Martino, 1967), then blocked and detergent extracted for 30 min in isotonic PBS containing 3% (v/v) normal goat serum (Gibco BRL), 0.02% (w/v) saponin (Aldrich) and 0.02% (w/v) NaN₃. Primary antibodies were diluted as specified above in blocking buffer, added to samples and incubated overnight at 4°C in a moist chamber. After rinsing in PBS and reblocking, secondary antibodies and BODIPY 581/591-phalloidin (Molecular Probes) were diluted 1:250 and added to

samples for 1–2 h at 25°C. Samples were rinsed extensively in PBS before mounting in 50 mM Tris, pH 8.0, diluted 1:9 in glycerol, with *n*-propyl gallate (Sigma) added to a final concentration of 20 mg ml⁻¹ (NPG-TBG) (Longin *et al.*, 1993). Staining and imaging controls included the substitution of primary mAb with isotype-matched antibodies of different specificity, incubation of uninfected cells with anti-GC and -MC sera and omission of primary antibodies to exclude artifacts caused by immunological cross-reactivity or optical bleedthrough.

Microscopy

Photographs were made using a Nikon Microphot FX microscope and Kodak TMAX 400 Professional film used at an exposure index of 800 or 1600 and developed in XTOL (Kodak). A Leica laser-scanning confocal microscope equipped with 40× and 63× Plan Apochromat objectives was used to obtain sets of optical sections. For some of the images shown, sets of optical sections were assembled into 'extended focus' projections using the brightest point algorithm in NIH Image software (version 1.62 running under MacOS version 8.1). Colour composites, in which two fluorescence channels were merged, were made in Adobe Photoshop version 4.0. For the evaluation of cortical plaque staining, coverslips were examined on the Nikon microscope using both epifluorescence and phase-contrast optics. Representative fields are depicted in Figs 1B and 4. At least 50 colonies were examined per treatment (except with strains that were completely non-adherent, e.g. MC8013 *pilE::Km*) in at least two independent experiments performed on different days. Scoring of protein clustering (Table 1) was conservative and was performed by examining a field of cells at 400× or 600× magnification and noting the positions of membrane protein clusters. Only then were phase and/or fluorescence used to determine the position of adherent GC. Colonies that were associated with the patches of fluorescent staining identified in the first step were scored as positive, and colonies not associated with distinct patches of staining were scored as negative. Isolated organisms were seldom found associated with clusters of membrane proteins, regardless of the bacterial phenotype. For this reason, only colonies of 10 or more bacteria were scored.

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