INFECTION AND IMMUNITY, Oct. 1997, p. 4341–4349 Vol. 65, No. 10
0019-9567/97/$04.00+0

Attachment of Piliated, Opa$^-$ and Opc$^-$ Gonococci and Meningococci to Epithelial Cells Elicits Cortical Actin Rearrangements and Clustering of Tyrosine-Phosphorylated Proteins

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Received 6 May 1997/Returned for modification 18 June 1997/Accepted 5 August 1997

Attachment of piliated *Neisseria gonorrhoeae* or *Neisseria meningitidis* cells to A431, Chang, HEC-1-B, or polarized T_{84} cells triggers rearrangements of cortical microfilaments and the accumulation of phosphotyrosine-containing proteins at sites of bacterial contact. Actin stress fibers and the microtubule network remain unaltered in infected cells. The rearrangements reported here are triggered by piliated, Opa$^-$ and Opc$^-$ strains and also by nonpiliated gonococci (GC) that produce the invasion-associated OpaA protein. Thus, neisserial adhesion via either of at least two different adhesins can trigger cortical rearrangements. In contrast, these rearrangements are not triggered by nonadherent GC or meningococcal strains, by heat-killed or chloramphenicol-treated GC strains, or by *Escherichia coli* recombinants that adhere to cells via GC OpaA or OpaI fusion proteins, suggesting that additional neisserial components are involved. Immunoblotting experiments did not detect consistent increases in the phosphorylation of specific proteins. Possible biological implications of these *Neisseria*-induced cortical rearrangements are discussed.

It is now widely accepted that microbial attachment to host cells is not simply a passive process by which microorganisms initiate colonization. Rather, it can be a necessary prelude to further interactions between the newly associated partners. Attachment can potentiate or directly stimulate host cell responses, including cytokine and chemokine release, toxicity, and microbial uptake (7–9, 15–17). Results from several systems show that adhesion to host cells can occur as an ordered process in which multiple microbial adhesins sequentially engage different receptors on the host cell surface (e.g., reference 27). Such processes might be comparable to cell-cell and cell-substrate adhesion cascades which occur during normal metazoan growth and immune function (6). If this is the case, similar events, including cytoskeletal rearrangements and the activation of host signal transducers, should occur soon after microbes adhere to host cells. In several cases, this prediction appears to be valid. For example, attachment of enteropathogenic *Escherichia coli* cells to host cells results in accumulation of filamentous actin and tyrosine phosphorylation of host proteins (10, 27). Similar phenomena occur when bacteria producing the *Yersinia enterocolitica* Inv protein adhere to and invade epithelial cells via β_1 integrin receptors and upon colonization of enterocytes by *Helicobacter pylori* (30, 37).

Cells of the pathogenic *Neisseria* species *N. gonorrhoeae* (gonococci [GC]) and *N. meningitidis* (meningococci [MC]) possess multiple components that promote bacterium-host cell interactions (19, 23). The type IV pilus may be an essential virulence factor in GC and appears to be absolutely required for adhesion to host cells by encapsulated MC (19). Opa and Opc constitute a large family of structurally related outer membrane proteins. Opa and Opc variants bind to different receptors on host cells, as well as to serum factors and neisserial lipooligosaccharides. In the absence of pili and capsule, Opa or Opc variants are sufficient to confer adhesive and, under some conditions, invasive phenotypes (19, 23). The presence of pili can either inhibit or promote invasion, depending on the cell line assayed (14, 18). Functional interactions between pili and Opa or Opc may occur but have not yet been thoroughly dissected.

Recently, Grassmé and coworkers observed microfilament rearrangements in epithelial cells after attachment of nonpiliated GC that express certain invasion-associated Opa proteins (4). In this report, we show that the attachment of MC as well as GC to cultured epithelial cells results in localized rearrangements of the cortical actin cytoskeleton at the sites of bacterial adhesion. We show that actin rearrangements occur not only with a very invasive GC strain (nonpiliated, OpaA$^+$), as reported (4), but also with much less invasive GC and MC strains (Opa$^-$ and Opc$^-$) that adhere to cells by using type IV pili. We show that these strains trigger dramatic accumulations of phosphotyrosine-containing proteins at contact sites. Finally, we show that these cortical rearrangements occur not only at the dorsal cortex of cells cultured on coverslips, but also at the apical surface of polarized epithelial cells grown on permeable supports. *Neisseria*-induced cortical rearrangements are therefore likely to occur in vivo and are probably not an artifact of culture conditions. Our results indicate that extensive cortical rearrangements occur in several different cell types after GC or MC attachment via either pili or an invasion-associated Opa protein.

Infection experiments and bacterial strains. A431 and Chang cells were maintained in RPMI 1640 (Life Technologies) with 10% heat-inactivated (at 37°C) fetal bovine serum (Hyclone or Life Technologies) in 5% CO_2. HEC-1-B cells were grown as described previously (34). For microscopy experiments, cells were plated at about 15% confluence onto ethanol-washed, autoclaved glass coverslips (1.5; Fisher Scien-

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TABLE 1. Summary of results of fluorescence experiments with A431 cells

Strain	Genotype and/or phenotype[a]	Fluorescence result for:		Source or reference
		f-Actin	Phosphotyrosine	
MC				
8013.6	P^+ Opc^- *opc*	+	+	21
8013 *pilC1*::Km	P^+ Opc^- *opc* (low adherence)	−	−	22
GC				
MS11A	P^+ Opa^-	+	+	29
MS11A (+ chloramphenicol)		−	−	
MS11A (heat killed)		−	−	
MS11 Cm23	P^- (*pilE1 pilE2*) Opa^- (nonadherent)	−	−	30a
15063G	P^+ Opa^+	+	+	34
MS11 AM13	P^- ($PilC^-$) Opa^- (nonadherent)	−	−	This study
MS11 AM13.1	P^- ($PilC^-$) $OpaA^+$	+	+	This study
MS11 AM13.1R[b]	P^- ($PilC^-$) Opa^- (nonadherent)			This study
MOI of 10/host cell		−	−	
MOI of 200/host cell		−/+	−/+	
E. coli				
HB101(pEXA)	$OpaA^+$	−	−/+	B. Belland
HB101(pEXI)	$OpaI^+$	−	−	B. Belland
HB101(pGEM)	Opa^- (nonadherent)	−	−	B. Belland

[a] P^- Opa^- organisms adhered poorly; the few adherent colonies were seldom associated with clusters of actin or phosphotyrosine.
[b] At an extremely high MOI, at least some of the adherent organisms were associated with actin or phosphotyrosine clusters; these may represent spontaneous Opa^+ phase variants.

tific) 48 h prior to infection experiments. T_{84} cells were grown, polarized, and prepared for microscopy as described previously (18). GC and MC strains were grown overnight on GC agar with Kellog's supplements, and *E. coli* cells were grown on Luria-Bertani agar with carbenicillin added to 100 μg/ml. For infection experiments, bacteria were resuspended and diluted into unsupplemented Dulbecco's modified Eagle's medium (Life Technologies) and then were added to epithelial cells at a ratio of ~10 CFU per cell.

MC *opc* genotypes (Table 1) were evaluated by Southern hybridization of chromosomal DNA with a probe made from pEB501, a gift from M. Achtman. GC and MC Opa and Opc phenotypes (Table 1) were assayed by immunoblotting with monoclonal antibody (MAb) 4B12 (a gift from M. Blake) and MAb B306 (a gift from M. Achtman). Opa^+ or Opc^+ strains were included in the blots as positive controls. GC strain MS11 AM13 and its derivatives (Table 1) contain deletions in the *pilC2* locus generated by transformation and allelic exchange with mutated *pilC2* DNA, in which a 544-bp *Eco*RV fragment is replaced by a chloramphenicol resistance cassette. MS11 AM13 produces pilin as assayed by immunoblotting with a MAb against the conserved SM1 epitope, and piliated colonies arise spontaneously at a frequency of $\sim 10^{-3}$ to 10^{-4}. Transformation of the piliated revertants with DNA encoding a disrupted *pilC1* locus gives rise exclusively to nonpiliated colonies. These experiments indicate that MS11 AM13 and its derivatives have intact *pilC1* loci that are "off" phase variants. For a description of analogous MC strains, see reference 22.

Adhesion via pili triggers cortical actin rearrangements. A previous report indicated that nonpiliated GC which produce invasion-associated Opa proteins could elicit actin rearrangements. We wished to determine whether similar rearrangements are elicited when GC or MC attach to cells via type IV pili. Cells cultured on glass coverslips were infected with piliated GC or MC for various times and then fixed and stained for actin microfilaments with BODIPY 581/591 or rhodamine-phalloidin as described previously (18). Samples were rinsed in phosphate-buffered saline (PBS) and mounted 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 (NPG-TBG) (12). Photographs were made with a Nikon microscope and Kodak TMAX 400 film used at an exposure index of 800 or 1600.

In A431 (Fig. 1), HEC-1-B, and Chang cells (not shown), distinct microfilament accumulations were found at the periphery of adherent, piliated diplococci. Tightly packed organisms (microcolonies) triggered the assembly of a microfilament meshwork resembling structures previously observed beneath adherent enteropathogenic *E. coli*, *Y. enterocolitica*, or *H. pylori* cells or fibronectin-coated latex beads (5, 26, 27, 30, 37). Isolated diplococci were associated with actin accumulations less frequently than small microcolonies. These actin rearrangements appeared highly localized and were confined to the cell cortex. The overall distributions of actin stress fibers and perijunctional actin were unchanged in infected cells. Actin rearrangements were visible in A431 cells within 1 h after infection. Similar experiments suggested that the microtubule network was not altered in infected A431 cells (11).

Bacterial components required for actin rearrangements. Several different GC, MC, and *E. coli* strains were assayed for the ability to cause actin rearrangements in A431 cells (Fig. 1 and Table 1). Only strongly adherent strains triggered actin accumulation. Piliated, Opa^- GC adhered strongly and caused microfilament accumulation, as did encapsulated, piliated, Opc^- 8013.6 MC. Together, these results indicate that GC or MC attachment via type IV pili triggers actin rearrangements, that these rearrangements can occur in the absence of Opa or Opc, and that they are not inhibited by the presence of the MC polysialic acid capsule.

In contrast, nonpiliated (MS11 AM13, AM13.1R, or Cm23 GC) or piliated but poorly adhesive (8013 *pilC1*::Km MC) derivatives of the same strain did not trigger actin accumulation (Table 1). Additional experiments were done with piliated, Opa^- GC that were heat killed (50°C for 30 min) or chloramphenicol treated (10 μg/ml added 20 min prior to inoculation onto cells). Under each of these conditions, large numbers of filamentous structures, presumed to be type IV

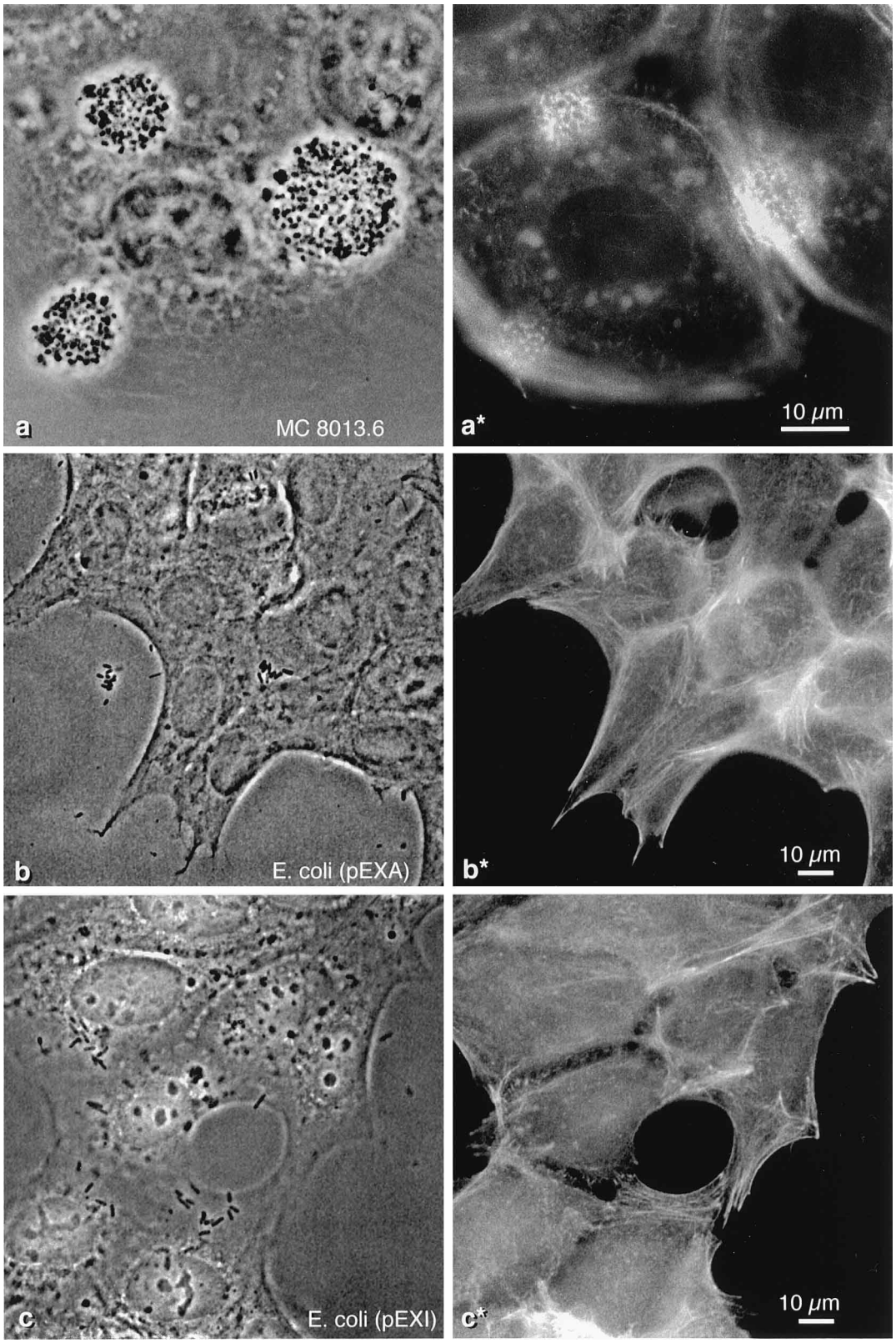

FIG. 1. Staining of actin microfilaments in infected A431 cells at 4 h postinfection. Cells were infected with MC8013.6 (piliated, Opc$^-$) (a and a*) or with *E. coli* cells producing OpaA (b and b*) or OpaI (c and c*) fusion proteins. Panels show phase-contrast images (a to c) or the same cells stained for filamentous actin (a* to c*).

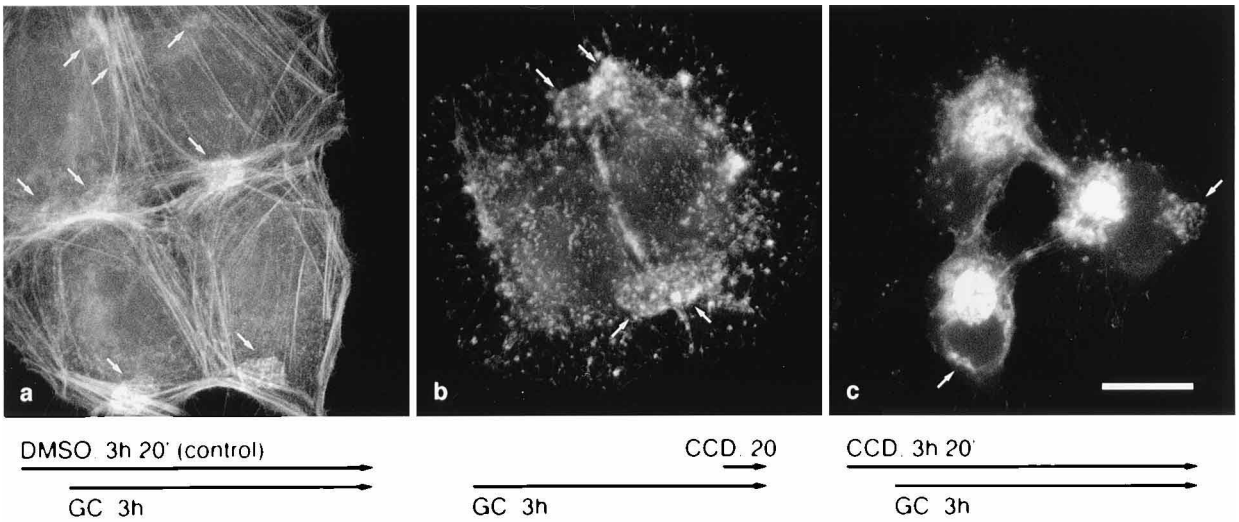

FIG. 2. Effects of the microfilament-disrupting agent CCD (5-μg/ml final concentration) on GC-induced actin accumulation. A431 cells were infected with MS11A GC (piliated, Opa^-) for 3 h and then were fixed and stained for actin polymer as described for Fig. 1. (a) Infection carried out in the presence of DMSO vehicle. (b) CCD present during the final 20 min of infection. (c) CCD added 20 min prior to infection. These images are extended-focus projections. Each image is derived from nine confocal optical sections taken at 1.0-μm intervals. Arrows indicate the locations of GC microcolonies as determined by phase-contrast microscopy. Scale bar, 15 μm.

pili, were visible by immunofluorescence with a rabbit antiserum raised against whole, piliated GC. At longer times after chloramphenicol treatment, fewer and fewer piliated GC were observed, but shed pili remained visible in the cultures, and appeared to form large, rigid bundles that ranged from 2 to 25 μm in length. These treatments therefore did not appear to cause pilus disassembly per se, but they did potently inhibit (>100-fold) pilus-based GC attachment to A431 cells. Even when many treated bacteria were added to the cultures, only a few diplococci were observed to be associated with host cells, and microcolonies did not form. The few adherent diplococci were almost never found associated with actin accumulations (Table 1). These experiments suggest that pilus-mediated adhesion requires ongoing bacterial protein synthesis and indicate that actin rearrangements may only occur under conditions that promote efficient bacterial attachment.

Experiments with Opa-mediated adhesion buttress this interpretation. Consistent with previous results (4), we found that nonpiliated (*pilC1* Δ*pilC2*), $OpaA^+$ MS11 AM13.1 GC caused actin accumulation (Table 1). Both the Opa^- parent and an Opa^- revertant from this strain were poorly adhesive and failed to cause actin accumulation. When extremely large numbers of the Opa^- revertant were used to infect A431 cells, a small fraction of the organisms that did adhere triggered microfilament accumulation (Table 1). These adherent GC may have been spontaneous Opa^+ phase variants in the culture, which arise at a frequency of $\sim 10^{-2}$ (19).

Further experiments indicated that adhesion per se was not sufficient to trigger actin accumulation (Fig. 1b and c and Table 1). A431 cells were infected with *E. coli* recombinants that expressed either OpaA or OpaI fusion proteins. Consistent with results reported for OpaA fusions (4), actin accumulations were found to be associated with fewer than 1% of adherent *E. coli* cells producing either the OpaA or OpaI fusion proteins (n = 200 colonies in two independent experiments). Similarly, sheared GC pili either alone or coating polystyrene beads under either aggregating or nonaggregating conditions failed to elicit phosphotyrosine or actin accumulation (unpublished data). Together, these results suggest that additional GC and MC components besides the primary Opa and pilus adhesins may be required to elicit cortical actin rearrangements in host cells.

Pilus-mediated actin accumulation occurs in the presence of CCD. To further characterize the actin-containing structures beneath piliated, Opa^- GC, cells were treated with the microfilament-disrupting agent cytochalasin D (CCD) either before or during infection with MS11A GC (Fig. 2). The concentration of CCD used, 5 μg/ml, potently inhibits internalization of GC by epithelial cells (1, 2, 31). Dimethyl sulfoxide (DMSO), used to dissolve CCD, was added to control cells. Infected cells were then fixed and stained to detect microfilaments as before. A Leica laser-scanning confocal microscope was used to obtain sets of optical sections, which were then assembled into "extended-focus" projections by using the brightest point algorithm in NIH Image software (version 1.60, running under MacOS version 7.5).

Infected cells treated with DMSO alone beginning at 20 min prior to infection were indistinguishable from untreated infected cells (Fig. 2a). Treatment with CCD during the final 20 min of a 3-h infection (Fig. 2b) completely disrupted stress fibers and partially disrupted the actin band associated with the *adherens* junctional complex. However, the accumulation of microfilaments beneath adherent GC microcolonies was not diminished by this treatment. Longer treatment with CCD, beginning 20 min prior to the 3-h GC infection (Fig. 2c), resulted in the nearly complete disassembly of *adherens*-associated microfilaments and the loss of cell-cell adhesion. This treatment also caused the movement of the bulk of polymeric actin to the perinuclear region. Surprisingly, microfilaments still accumulated beneath adherent GC. It should be noted that CCD treatment disrupts many actin-containing structures, but it does not change the ratio of polymeric to monomeric actin in cultured cells (20). Together, these experiments suggested that the mechanism responsible for microfilament accretion at sites of pilus-mediated GC adhesion differs from the mechanism responsible for the formation of stress fibers and *adherens*-associated microfilament bands.

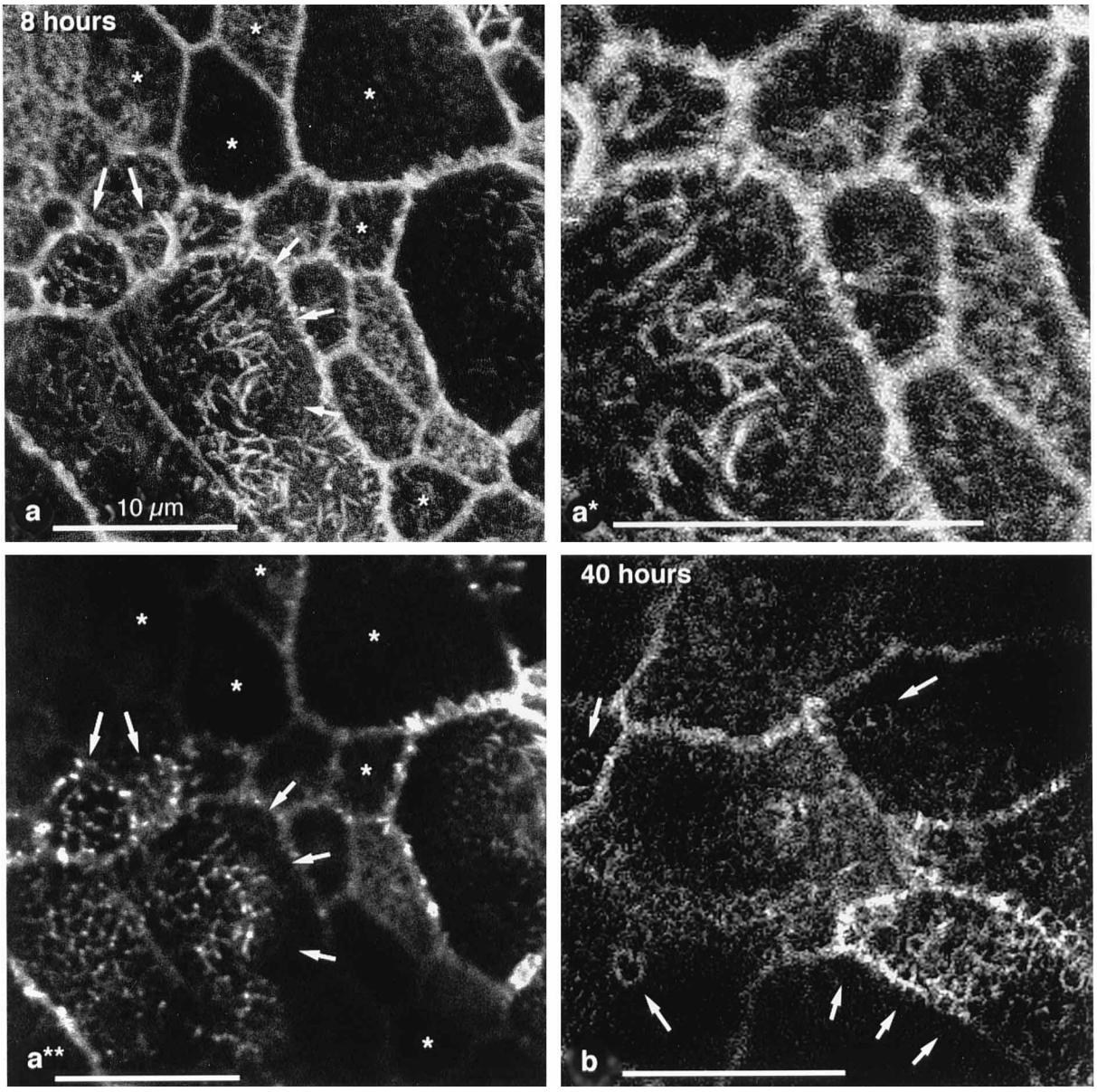

FIG. 3. Staining of microfilaments at the apical surface of polarized T_{84} cells infected with 8013.6 MC for 8 h (a) or 40 h (b). For the 8-h time point, three views of the same area are shown. In panel a, an extended-focus projection is shown. This image is derived from nine optical sections taken at 0.5-μm intervals. In panel a*, an enlarged area of the same projection is shown. In panel a**, a single optical section from the stack used to compute panel a is shown. In this section, taken at the level of the cell surface, the outlines of adherent diplococci are visible. (b) Microfilament distribution at 40 h postinfection. This is an extended-focus projection of three optical sections taken at 0.5-μm intervals. Arrows point to regions of *Neisseria*-induced microfilament rearrangements. Asterisks in the figure panels indicate regions where rearrangements are not visible.

GC and MC cause actin rearrangements at the apical surface of polarized epithelial cells. Cells cultured on glass or plastic acquire a relatively nonpolarized architecture, because they are unable to feed from their basolateral surfaces as they would in vivo. These conditions also promote the formation of cytoskeletal structures (stress fibers) not found in epithelial cells in vivo. Consequently, it was possible that the microfilament rearrangements observed above were artifacts arising from the culture conditions used. To address this possibility, infection experiments were performed with T_{84} cells grown on permeable supports. This system yields highly polarized epithelial sheets that have many characteristics of native epithelia, including a robust barrier function, and which support GC and MC adhesion, invasion, and epithelial traversal (18). In infections of polarized T_{84} cells, piliated GC or MC interacted initially with microvilli, which in many cases appeared elongated after bacterial contact (Fig. 3a to a**). At later times after infection, the infected cells were relatively denuded of microvilli, but many diplococci were still observed to associate with actin-containing structures. *Neisseria*-associated microfilament structures at the cell surface became less punctate and more continuous at longer times after infection (compare Fig. 3a** to b). These observations are consistent with the hypothesis that cortical actin rearrangements occur after GC or MC adhere to native epithelial cells and are not morphological artifacts arising from standard culture conditions.

GC or MC attachment triggers accumulation of phosphotyrosine-containing proteins. Phosphotyrosine-containing proteins concentrate at sites of cell-cell and cell-substrate attachment, in microvilli, and at sites of cytoskeletal remodeling (13). To determine whether tyrosine phosphorylation might be involved in the assembly of actin-containing structures beneath adherent GC, A431 cells were infected as before, and indirect immunofluorescence microscopy was used to examine the distribution of phosphotyrosine. After infection, samples were fixed for 20 min at room temperature in picric acid paraformaldehyde (38) and then blocked and detergent extracted for 30 min in isotonic PBS containing 3% normal goat serum (Gibco BRL), 10 μg of saponin per ml (Aldrich), and 100 μg of sodium azide per ml. Primary antibodies were diluted in blocking buffer, added to samples, and incubated overnight at 4°C in a moist chamber. Polyclonal rabbit sera 11507 and 8547 (22) react against whole GC and MC, respectively, and were diluted 1:1,500. Phosphotyrosine was labeled with MAb 4G10 at 2.5 μg/ml (generously provided by B. Druker). After samples were rinsed in PBS and reblocked, secondary antibodies (Texas red-conjugated goat anti-rabbit and BODIPY FL-conjugated anti-mouse; Molecular Probes) were diluted 1:250 and added to samples for 1 to 2 h at 25°C. Samples were again rinsed extensively prior to mounting as described above. Staining and imaging controls included the substitution of primary MAbs with isotype-matched antibodies of different specificities, 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 bleed through.

As expected, in uninfected cells phosphotyrosine-containing proteins were concentrated at focal adhesions, at cell-cell junctions, and in microvilli (Fig. 4a) (13). The attachment of either MS11A GC or 8013.6 MC triggered the formation of dense foci of phosphotyrosine labeling in A431 and HEC-1-B cells (Fig. 4b and c). Similar results were obtained with Chang cells and polarized T_{84} cells (data not shown). By 4 h postinfection, >90% of adherent MS11A GC were associated with phosphotyrosine accumulations in A431 cells (n = 200 colonies, two independent experiments). No antiphosphotyrosine labeling of GC or MC was observed in the absence of epithelial cells. Digital analysis of images obtained by conventional (nonconfocal) microscopy indicated that areas of A431 plasma membrane with attached GC were often >20-fold more intensely fluorescent than adjacent areas devoid of bacteria. As in the case of actin accumulation, phosphotyrosine accumulation was elicited by GC adhering via either type IV pili (Fig. 4b) or via OpaA (Table 1). However, phosphotyrosine accumulations were seldom found beneath *E. coli* cells producing the OpaA fusion protein and were virtually never found beneath *E. coli* cells producing the OpaI fusion protein (Table 1 and Fig. 4d and e). Thus, as in the case of actin rearrangements, bacterial adhesion appears to be necessary but not sufficient to trigger cortical phosphotyrosine accumulation.

Analysis of tyrosine phosphorylation after attachment of piliated GC or MC. The foci of phosphotyrosine accumulation at sites of bacterial adhesion could have formed through de novo phosphorylation of one or more proteins, through aggregation of existing phosphotyrosine-containing proteins, or both. To address whether de novo phosphorylation is triggered by neisserial attachment, immunoblotting experiments were performed with the MAb against phosphotyrosine (Fig. 5).

A431 cells were grown as described above to ~85% confluence on 100-mm-diameter dishes and left uninfected or inoculated with piliated, Opa^- MS11A GC (multiplicity of infection [MOI] of 100). At 4 h postinfection, the cells were rinsed twice with ice-cold PBS and once with ice-cold Tris-saline-azide buffer (TSA; 10 mM Tris [pH 7.3], 140 mM NaCl, 0.025% [wt/vol] NaN_3). Cells were lysed in 0.4 ml of ice cold radioimmunoprecipitation assay (RIPA-V) buffer (TSA with 1% [vol/vol] Triton X-100, 1% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS], 1 mM sodium vanadate, 100 μM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 1 μg of pepstatin per ml, and 1 μg of leupeptin per ml. Lysates were scraped loose, the dishes were rinsed with an additional 0.4 ml of RIPA-V buffer, and the combined lysates were cleared by centrifugation (16,000 × g, 20 min, 4°C). Protein contents of the lysates were determined with the bicinchoninic acid assay (Pierce). Samples were diluted into 1.2× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, boiled for 5 min, and loaded onto linear 5 to 20% gradient SDS-PAGE gels. Approximately 10% of the total protein in infected cells was bacterial. For Fig. 4, 25 μg of protein was loaded onto each lane except for the GC-only lane, which contained 3 μg. Proteins were electroblotted onto polyvinylidene difluoride membranes (Boehringer-Mannheim), blocked with 3% (wt/vol) gelatin in TSAT (TSA with 0.02% [vol/vol] Tween 20), and incubated with MAb 4G10 in TSAT with 1% bovine serum albumin. MAb 4G10 was detected with alkaline phosphatase-conjugated goat anti-mouse antibody followed by nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium (BCIP) chromogen (Boehringer).

In these experiments, no consistent changes were observed in the phosphorylation of particular proteins after infection with MS11A GC or 8013.6 MC. Similar results were obtained by several different protocols for making cell lysates, with cells infected at different MOIs (from 20 to 200), at times ranging from 30 min to 4.5 h postinfection, and with a different MAb against phosphotyrosine (data not shown). In addition, Triton X-100-soluble and -insoluble fractions were immunoblotted to determine if any tyrosine-phosphorylated species migrated to the detergent-insoluble (i.e., cytoskeletal) fraction as a result of MC attachment. No consistent differences were observed between infected and uninfected cells in these experiments (data not shown). In two experiments, increases were noted in the intensity of Triton X-100-insoluble proteins migrating at 105 to 120 kDa. However, these differences were not consistent from one experiment to the next. In the blot shown in Fig. 4, two or three faint bands are visible in the lane with GC lysates alone; these bands may be due to weak cross-reactivity of the secondary reagent with GC components. These bands appeared only after relatively long periods of chromogen development.

These experiments suggested that the phosphotyrosine-containing structures observed by microscopy might have arisen largely or completely through the aggregation of proteins that were already phosphorylated. It should be noted, however, that de novo phosphorylation may have contributed only a small fraction to the total cellular pool of phosphotyrosine residues and therefore might have been difficult to detect in immunoblots of crude lysates.

Significance of GC- and MC-induced cortical rearrangements. Our observations indicate that attachment of piliated GC or MC elicits substantial rearrangements in the host cell cortex directly beneath sites of bacterial contact. These rearrangements include the accumulation of filamentous actin and proteins containing phosphotyrosine and occur in A431, HEC-1-B, Chang, and polarized T_{84} cells. In the case of polarized T_{84} cells, the rearrangements seem to occur in at least two steps. First, GC or MC interact with microvilli, which appear to elongate upon contact with the bacteria. Subsequently, the T_{84} cell surface becomes relatively denuded of microvilli, while

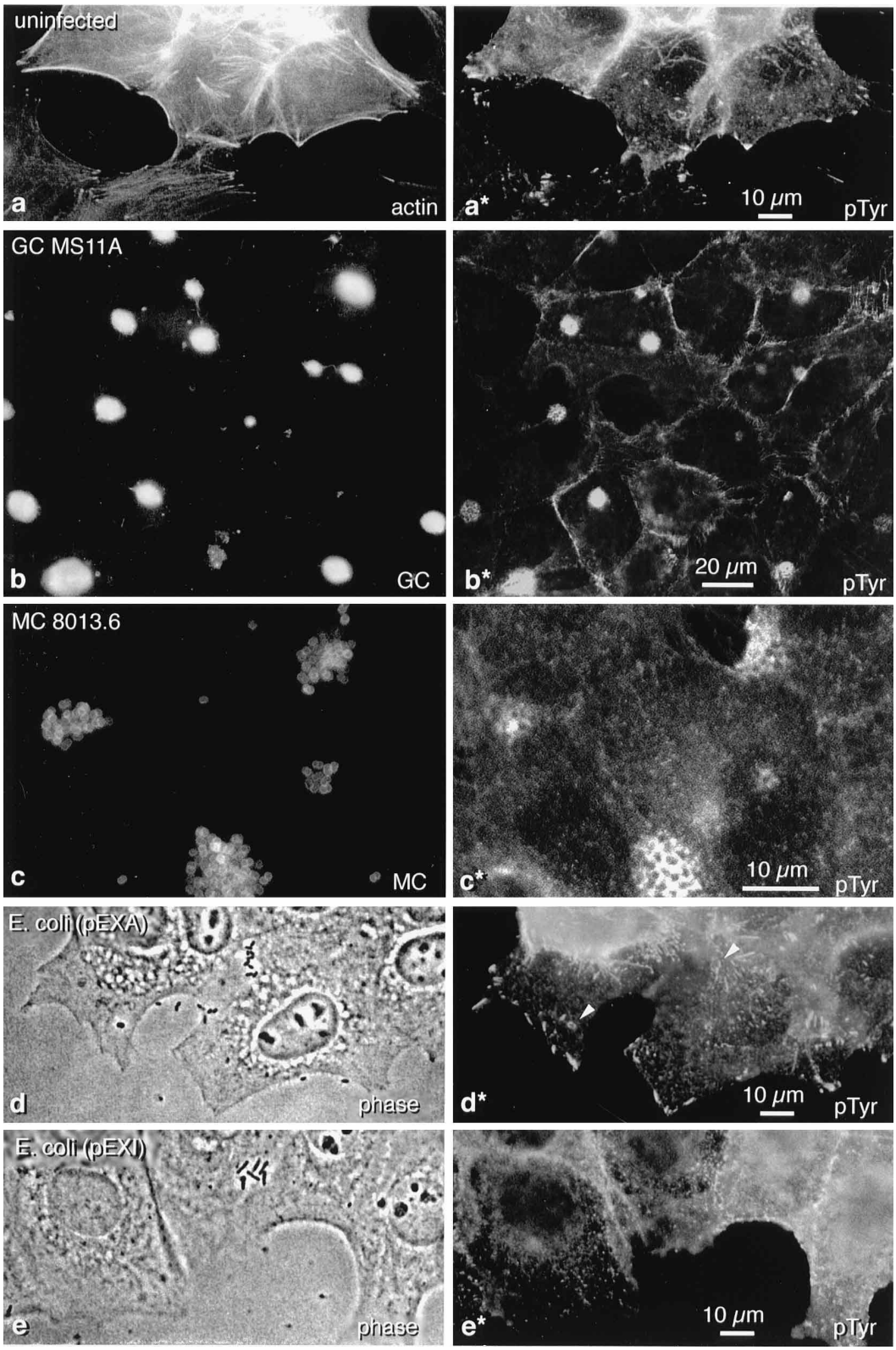

FIG. 4. Localization of phosphotyrosine (pTyr) at 4 h postinfection. (a and a*) Uninfected cells. Note that phosphotyrosine is concentrated at cell-cell junctions and at focal adhesions at the termini of actin stress fibers. (b and b*) A431 cells infected with MS11A GC (piliated, Opa^-) for 4 h. In this low-magnification view, phosphotyrosine accumulations are visible beneath almost all adherent GC microcolonies. (c and c*) HEC-1-B cells infected with 8013.6 MC (piliated, Opc^-). (d and d* and e and e*) A431 cells infected with *E. coli* cells producing either OpaA (d) or OpaI (e) fusion proteins. Arrowheads in panel d indicate possible sites of phosphotyrosine accumulation beneath adherent bacteria. Such accumulations were present beneath <5% of the *E. coli* cells that produced the OpaA fusion. The images presented above were acquired by conventional fluorescence microscopy except for panels c and c*, which are extended-focus projections of five confocal optical sections taken at 0.5-μm intervals.

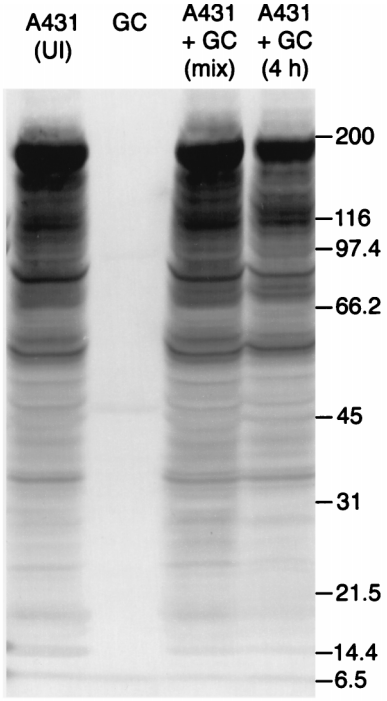

FIG. 5. Immunoblot analysis of tyrosine phosphorylation. From left to right, lanes represent uninfected A431 lysates (UI), MS11A GC lysates (GC), uninfected A431 lysates mixed with lysates of MS11A GC (mix), and A431 cells infected with MS11A GC for 4 h (4 h). Molecular mass markers (kilodaltons) are shown on the right.

adherent organisms remain associated with actin accumulations. Similar structural changes are observed by electron microscopy of GC- and MC-infected T_{84} cells (17a) in infected organ cultures (32, 33) and in urethral epithelial cells from GC-infected patients (24, 35). Together, these data strongly suggest that cortical rearrangements are a general feature of GC and MC adhesion to epithelial cells.

We also observe that dramatic accumulations of phosphotyrosine form beneath adherent GC and MC. In preliminary experiments, however, changes in the tyrosine phosphorylation of specific host cell proteins are not detected by immunoblotting. These data are consistent with the interpretation that the phosphotyrosine accumulations beneath adherent GC and MC arise largely or entirely through the aggregation of proteins that are already phosphorylated, rather than through changes in tyrosine kinase or phosphatase activity. Alternatively, it is distinctly possible that such changes do occur but are difficult to detect against the background of total cellular phosphotyrosine in immunoblots of crude lysates.

Regardless of whether de novo tyrosine phosphorylation occurs after neisserial attachment, rearrangements at the cell cortex have the potential to alter or modulate the activity of several signal transduction systems in the host cell. Tyrosine phosphorylation appears to promote the assembly of modular signaling complexes that include components such as protein kinases and phosphatases, lipid kinases, and lipid hydrolases (25). It will be interesting to determine whether other signaling pathways are modulated following neisserial attachment.

In agreement with another report (4), we observed that nonpiliated GC which produce an invasion-associated Opa protein also trigger actin rearrangements in epithelial cells. In addition, our results indicate that both actin rearrangements and phosphotyrosine accumulation are triggered by GC or MC that adhere by using type IV pili but that lack Opa or Opc. Therefore, neisserial attachment via either of at least two different adhesins elicits apparently similar changes in the host cell cortex. In control experiments, however, *E. coli* cells that adhered via either of two GC Opa variants elicited either very minor cortical rearrangements or none at all. Thus, additional neisserial components may be required to trigger the formation of actin and phosphotyrosine clusters.

Cortical rearrangements also occur after attachment of *Y. enterocolitica*, enteropathogenic *E. coli*, or *H. pylori* cells or fibronectin-coated beads. In these cases, both microfilaments and tyrosine-phosphorylated proteins accumulate at the contact region, while the microtubule distribution remains more or less unaltered (5, 26, 27, 30, 37). In contrast, *Salmonella typhimurium* causes the accumulation of microtubules as well as microfilaments at contact sites (3).

Invasion of epithelial cells by GC is strongly inhibited by CCD (1, 2, 31), implicating actin microfilaments in the uptake process. Indeed, it has been suggested that actin accumulation is an early signal for GC invasion (4). However, our data show that both actin and phosphotyrosine accumulation can occur in the absence of invasion. Invasion of A431, Chang, or HEC-1-B cells by the Opa^- strain MS11A is minimal until several hours postinfection, and yet this strain triggers rapid (<1 h postinfection [data not shown]) changes in microfilament and phosphotyrosine distribution. Although actin rearrangement is probably necessary for GC invasion, it is evidently not sufficient. This conclusion is further supported by the observation that both piliated, Opa^- GC (Fig. 2) and nonpiliated, $OpaA^+$ GC (4) trigger actin accumulation even in the presence of CCD at concentrations that potently inhibit invasion.

The host cell responses reported here might also be involved in triggering the inflammatory response. Attachment of enteropathogenic *E. coli* or *S. typhimurium* cells to the apical surface of polarized T_{84} epithelial monolayers elicits the basolateral release of neutrophil chemoattractants, including interleukin-8 (15, 16, 28). Similarly, very recent data indicate that the attachment of MS11 GC to HeLa cells elicits cytokine expression and activation of the host transcription factor NF-κB (36). It is conceivable that such events could occur generally upon attachment of pathogenic microbes to apical epithelial surfaces and that the phosphotyrosine accumulations observed upon attachment of GC and other microbes represent part of the signal which elicits this putative response.

We gratefully acknowledge Jody Engstrom (OHSU Molecular Microbiology and Immunology Core Facility) for assistance with confocal microscopy; M. Hoffman for help in characterizing the *N. meningitidis* strains; B. Belland for providing Opa expression vectors; X. Nassif for sharing results prior to publication and for helpful discussions; K. Heldwein for comments on the manuscript; and B. Druker for reagents, advice, and encouragement.

This work was supported by NIH grant AI34560 to M.S. A.M. receives support through an NIH Doctoral Training Fellowship and an N. L. Tartar Research Fellowship.

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Editor: P. E. Orndorff