Host Cell Signaling by *Neisseria gonorrhoeae* Type IV Pili

by

Shaun Wook Lee

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

Shaun Wook Lee

has been approved

[redacted]

Dr. Richard Maurer, Professor in charge of thesis

[redacted]

Dr. Magdalene So, Member

[redacted]

Dr. Fred Heffron, Member

[redacted]

Dr. Klaus Früh, Member

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The most beautiful experience we can have is the mysterious. It is the fundamental emotion which stands at the cradle of true art and true science.

-Albert Einstein

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Abstract

Neisseria gonorrhoeae is a gram-negative bacterial pathogen that causes gonorrhea exclusively in the human host. *Neisseria* infection is a multistep process; initial adherence to epithelial cells is mediated by type IV pili- retractile fibers that extend from the outer membrane. Type IV pili promote bacterial adhesion, though the mechanism by which this occurs is not understood. In this dissertation I demonstrate that *Neisseria gonorrhoeae* type IV pili trigger specific host cell signaling cascades, through receptor-based activation, and through the generation of host cell membrane tension using retractile force, that are important for the infection process. Furthermore, I show that *Neisseria gonorrhoeae* exploit host cell signaling to generate a eukaryotic chemical that crosses species to act directly on bacteria. Identification of the cellular signaling pathways involved in *Neisseria* infection may reveal novel targets for pharmacologic intervention of disease. Type IV pili are produced in many other pathogens, some of which have been shown to be retractile. These studies may therefore also shed light on how Type IV pili may function in other bacterial-host interactions.

Chapter 1

Introduction

1.0 Introduction

The two closely related species of pathogenic Neisseria, *Neisseria gonorrhoeae* (GC) and *Neisseria meningitidis* (MC) have exclusive tropism for humans. GC causes gonorrhea, while MC causes meningitis and bacterial septicemia. GC and MC share many similarities although they cause different diseases. The work presented in this thesis uses GC as a model system to examine the role of type IV pili in host-pathogen interplay; however, type IV pili and other virulence factors are shared in both species and insights into the infection process of GC will be important for both pathogens.

1.1 Gonococcal pathogenesis

Neisseria gonorrhoeae (GC) is a gram-negative diplococcus that causes gonorrhea exclusively in the human host. This tropism is due in part to its

utilization of receptors unique to human cells, and requirements for human transferrin (Cornelissen et al., 1998). The primary mode of transmission of *Neisseria gonorrhoeae* is through direct sexual contact. Gonococcal infection in both women and men initiates through colonization of the mucosal epithelia. Infection in men clinically presents as an acute form of urethritis characterized by an intense focal inflammatory response and shedding of infected cells, two to five days following initial infection. GC infection in women may have multiple sequelae. Often following initial colonization, women may not present with any symptoms. This carrier state, maintained by GC, can result in further transmission (Turner et al., 2002). The mechanism by which this carrier state is achieved is not known, but strains capable of a quiescent state likely possess selection factors that limit the activation of the host immune response. Some strains of GC are capable of ascending the urogential tract to the oviducts, ultimately inducing an inflammatory response that can result in pelvic inflammatory disease (PID) and salpingitis (Rice and Schachter, 1991). Pelvic inflammatory disease (PID) is the leading cause of infertility among women in the United States; GC and *Chlamydia trachomatis* are the leading causes of PID (See reviews, (Britigan et al., 1985; Cohen and Sparling, 1992; Hook and Holmes, 1985;

Kazmierczak et al., 2001; Merz and So, 2000; Meyer et al., 1994; Turner et al., 2002).

Disseminated gonococcal infection (DGI) can cause acute arthritis and lead to myocarditis in some individuals (Cucurull and Espinoza, 1998; Masi and Eisenstein, 1981). In gonococcal keratoconjunctivitis, *Neisseria* infection of the eye can result in visual loss through rapid, severe, ulcerative keratitis (Lee et al., 2002), and gonococcal ophthalmia neonatorum (GCON) is an important cause of infant blindness (Laga et al., 1989). Finally, GC infection has been shown to facilitate HIV transmission (Ghys et al., 1997; Laga et al., 1993). Thus, although gonococcal infections are not life threatening *per se*, complications arising from GC infection continue to make GC disease a serious concern. There is no current vaccine candidate available for GC. A better understanding of how *Neisseria gonorrhoeae* initiates infection will therefore be of benefit to human health.

1.2 Steps in GC infection of host cells

Neisseria gonorrhoeae infection is a multistep process characterized by the following general stages (Figure 1). (1). The bacteria first adhere loosely to the surface of mucosal epithelial cells. This process is mediated by type IV

pili- long, fibrous structures protruding from the surface of the bacteria (Buchanan et al., 1978; Cannon et al., 1996; Kellogg et al., 1963; McGee et al., 1981; Merz et al., 1996; Mosleh et al., 1997; Nassif et al., 1993; Pujol et al., 1997; Seifert et al., 1994; Stephens and McGee, 1981; Swanson, 1973). GC aggregate into microlonies as they adhere to the host cell apical membrane, with subsequent microvilli elongation around the adhered bacteria (Pujol et al., 1999; Shaw and Falkow, 1988; Stephens et al., 1983; Ward and Watt, 1972; Ward et al., 1974). Microcolonies contain 10-100 diplococci and aggregation requires type IV pili. At this stage, host cell signaling proteins accumulate directly beneath the sites of attachment into structrures termed cortical plaques (Hoffmann et al., 2001; Merz et al., 1999; Merz and So, 1997). (2). 6-18 hours after infection, the bacteria shed their pili, associate tightly with the plasma membrane in monolayers, and enter through the apical membrane through a process resembling macropinocytosis (Apicella et al., 1996; Pujol et al., 1997; Zenni et al., 2000). (3). After cell entry, GC transcytose the cell layer and exit the basal membrane to reach subepithelial stromal tissues without perturbing host cell integrity (Hopper et al., 2000a; Hopper et al., 2000b; Ilver et al., 1998; Merz et al., 1996; Pujol et al., 1997; Stephens and Farley, 1991; Wang et al., 1998). It is speculated that intracellular bacteria can take an alternate route

whereby they recycle to the apical layer and reinfect at the mucosal surface, though there is at present no direct evidence to support this claim.

Internalized bacteria can continue to survive and replicate inside cells (Hopper et al., 2000b; Ilver et al., 1998; Lin et al., 1997; Merz et al., 1996; Pujol et al., 1997; Stephens and Farley, 1991; Wang et al., 1998). Typically, transcytosis of GC occurs in 24-36h, while MC transcytose epithelial cells in 6-8h (Hopper et al., 2000b; McGee et al., 1981; Merz and So, 2000; Stephens et al., 1983; Wang et al., 1998).

Cell death and cytotoxity occur later in infection, and has been documented in both tissue culture and organ models of infection (Apicella et al., 1996; McGee et al., 1992; Mosleh et al., 1997; Ward and Watt, 1972). Neisserial components responsible for inducing cytotoxicity include lipooligosaccharides (LOS- endotoxin), peptidoglycan, the porin ion channel, and type IV pili (Dunn et al., 1995; Gregg et al., 1981; Melly et al., 1981; Melly et al., 1984; Muller et al., 1999; Stephens et al., 1986; Virji et al., 1982).

1.3 Type IV Pili

GC initiate contact on host cells using type IV pili (Figure 2). Type IV pili are long, fibrous protein polymers 6nm in diameter and several μm in length.

Type IV pili are produced by many gram-negative bacteria, including *Neisseria meningitidis, Vibrio cholerae,* enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *E. coli, Moraxella bovis, Eikenelle corrodens,* the cyanobacterium *Synechocystis sp., Pseudomonas aeruginosa,* and *Myxococcus xanthus* (Strom and Lory, 1993).The GC type IV pilus has an array of biological functions, including cell adhesion, DNA transformation, and twitching-based motility (Brossay et al., 1994; Maier et al., 2002; Merz et al., 2000; Nassif et al., 1993; Swanson, 1973). Type IV pili in other bacteria serve functions such as 'social gliding' motility, bacteriophage susceptibility, conjugation, and secretion of virulence factors (Bradley, 1974; Comolli et al., 1999; Wu et al., 1997; Yoshida et al., 1999).

1.3.1 Architecture of Type IV pili

Type IV pili are composed of identical protein subunits called pilin, whose three-dimensional structure has been determined (Parge et al., 1995). Pilin subunits are 18-22kDa polypeptides that contain a distinct amino-terminal secretion signal that distinguishes it from other types of pilins (Frost et al., 1978; Hermodson et al., 1978). These pilin subunits are posttranslationally modified through O-glycosylation and phosphorylation (Forest et al., 1999; Marceau et al., 1998; Marceau and Nassif, 1999; Parge et al., 1995; Stimson

et al., 1995; Virji et al., 1993) that confer antigenic variation. The pilin subunit is encoded by the *pilE* gene in GC(Meyer et al., 1984).

The pilin monomer is composed of a C-terminal globular "head" domain and an N-terminal hydrophobic tail that folds into a long alpha-helix. The monomers polymerize into a right-handed helical cylinder with fivefold symmetry about the helix axis (Forest and Tainer, 1997; Parge et al., 1995). The hydrophobic tail domains are directed into the center of the cylinder to form a helical coiled-coiled bundle. The globular domains are exposed to the cylinder surface. Each domain contains a loop domain that is produced by an invariant pair of cysteines at the ends that close the loop with disulfide bonds. This loop has been identified as the "hypervariable" region of pilin that displays the greatest amount of antigentic diversity (Seifert, 1996). In this way the pilus fiber can produce enormous antigenic diversity while the overall assembly and structure retains structural integrity. Overall, the type IV pilus structure and mode of assembly are conserved in other bacterial systems that express type IV pili (Dalrymple and Mattick, 1987; Hoyne et al., 1992; Patel et al., 1991).

1.3.2 Assembly and dissassembly of type IV pili

Pilus assembly requires more than ten specialized gene products as well as the function of the general secretory pathway in prokaryotes (Duong and Wickner, 1997; Pugsley, 1993; Pugsley et al., 1997). Type IV pilus assembly is thought to occur in the outer face of the cytoplasmic membrane or periplasm (Fussenegger et al., 1997; Hultgren et al., 1993; Pugsley, 1993). Assembly occurs after pilin subunits have translocated from the cytoplasm via a membrane preprotein translocase (Pugsley, 1993). PilD, a polytopic inner membrane protein, is required for type IV pilus assembly. PilD methylates and cleaves prepilin subunits through recognition of a secretion signal at the amino terminus of prepilin, resulting in mature pilin subunits with the characteristic alpha- methylated phenylalanine at its N- terminus (N-Met-Phe) (Freitag et al., 1995; Nunn and Lory, 1991; Strom et al., 1993)(Freitag et al.1995; Nunn and Lory 1991; Strom et al 1993). Mature pilin subunits are assembled into pilus fibers and the growing fiber is thought to traverse the outer membrane through a large pore-like complex composed of the neisserial PilQ protein (Drake and Koomey, 1995). PilQ belongs to the protein family known as secretins (Bitter et al., 1998). Secretins are polytopic proteins that exist as homooligomers within the outer membrane. In some cases, secretins have been demonstrated to form

conductance pores through which assembled polymers are able to be extruded (Marciano et al., 1999; Nouwen et al., 1999). When *pilQ* expression was abrogated by removing IPTG from an IPTG-inducible *pilQ* mutant, the periplasm became distended and filled with pilin polymers, supporting the notion that pilus fibers may traverse the outer membrane through PilQ pores (Dunham et al., 1998).

1.3.3 PilF and PilT ATPases in type IV pili

Many gene products in GC that are involved in pilus assembly or function contain conserved ATP-binding motifs, and are found localized in the bacterial cytoplasm or associated with the bacterial inner membrane. The Neisseria gene products of *pilF* and *pilT* contain Walker boxes (conserved ATPase domains) and are located in the bacterial cytoplasm. PilF has been shown to be necessary for pilus fiber assembly in GC (Freitag et al., 1995) as well as in *Pseudomonas aeruginosa* (Watson et al., 1996). Mutations in *pilF* were found to result in a nonpiliated phenotype. Although little is known about the exact function of the PilF ATPase in bacteria, a gene responsible for the human disorder Bardet-Biedl syndrome (BBS) was recently cloned, and amazingly, found to contain a prokaryotic *pilF* domain (Ansley et al., 2003). It was further demonstrated that the human *pilF*

homologue, BBS8, localized to centrosomes and basal bodies and interacted with another protein involved in ciliogenesis. This opens the possibility that PilF may contain a universal ATPase motor conserved in eukaryotic and prokaryotic systems that is involved in assembly of helical fiber proteins such as type IV pili or the axoneme of ciliary bodies.

1.3.4 The PilT protein

PilT has been shown to be necessary for pilus fiber retraction (Merz et al., 2000; Wolfgang et al., 2000). The PilT protein is a member of a large family of NTPases from type II and type IV secretion systems (Herdendorf et al., 2002; Okamoto and Ohmori, 2002; Wolfgang et al., 1998). The Nesserial PilT and its homologues have defects in virulence (Bieber et al., 1998; Merz et al., 1999), suggesting that retractile processes are involved in the ability of GC to efficiently infect host cells.

Type IV pili allow bacteria to crawl along two-phase interfaces, such as liquid/solid or solid/air. This is achieved through twitching motility, which has been shown to be dependent on the ability of the pili to undergo retraction through the activity of PilT (Merz et al., 2000; Okamoto and Ohmori, 2002; Skerker and Berg, 2001).

Twitching motility was first described in *P. aeruginosa* by David Bradley in 1970. Bradley noted that wt *P.aeruginosa* had many phage attached to the bacterial cell body and concentrated at the bases of pili, while a nonsusceptible but piliated mutant had pili uniformly covered with phage but few phage on the cell body. Interestingly, the nonsusceptible mutants did not twitch. These results supported the hypothesis that pili retract into the cell body (Bradley, 1972a; Bradley, 1972b; Bradley, 1974). The lesion in one of these mutants was later mapped to the *pilT* locus (He, 1998; Whitchurch et al., 1991).

A homologue to the Pseudomonas *pilT* gene was identified in *Neisseria gonorrhoeae* by sequence homology (Brossay et al., 1994; Wolfgang et al., 1998). GC *pilT* mutants were found to be hyperpiliated and lacked twitching motility, consistent with the hypothesis that *pilT* mutants are not able to retract pili (Wolfgang et al., 1998).

1.3.5 Recent evidence for pilus retraction

Recently, studies in three model bacterial systems provide conclusive evidence that type IV pilus retraction is responsible for generating gliding or

twitching motility (Merz et al., 2000; Skerker and Berg, 2001; Sun et al., 2000).

Using *M. xanthus*, Sun et al. used a tethering assay to observe the motion of tethered bacteria on a highly viscous medium. They observed that *M. xanthus* moved by attaching themselves endwise to a surface, then proceeded to lay down flat on the surface, before proceeding to move over the surface from their initial positions. *PilT* mutants were able to tether to the surface but could not lay down nor move from their initial positions, indicating that PilT was required for generating retractile force.

Skerker and Berg used *P. aeruginosa* labeled with an amino-specific Cy3 fluorescent dye and visualized retraction in real-time using a total internal reflection microscope. In this way, they were able to directly observe pilus fibers retracting into the bacterial body.

Merz et al. employed laser tweezers trapping to demonstrate pilus retraction in GC. When a latex bead coated with anti-pilin antibodies was placed near a bacterium immobilized with another larger bead, the smaller coated bead was seen repeatedly being pulled toward the immobilized cells. These retraction events were sporadic, occuring as long as 20 seconds apart. The force exerted on the smaller trapped bead averaged 80 pN. This retractile force was absent in a *pilT* mutant.

1.4 How does GC type IV pili promote infectivity?

Despite long standing evidence of an adhesion role for type IV pili (Buchanan and Pearce, 1976; Gubish et al., 1982; James-Holmquest et al., 1974; Stephens and McGee, 1981; Virji et al., 1991), the first receptor for neisserial pili, CD46 was identified only recently (Kallstrom et al., 1997). It was found that crudely purified GC or MC pili bound native CD46 in an overlay assay. Purified ectodomains of CD46 were found to block adhesion of GC or MC to epithelial cells. Finally, expression of particular CD46 splice variants allowed otherwise nonpermissive cells to support adhesion by piliated GC or MC.

CD46 or Membrane Cofactor Protein (MCP), is a complement-regulatory membrane glycoprotein that prevents cell damage by autologous complement components (Liszewski and Atkinson, 1992; Liszewski et al., 1991; Seya et al., 1995) (Figure 3). CD46 consists of an ectodomain with four short consensus repeats (SCR1-4) followed by a segment of serine-threonine-proline rich domains (STP-A, -B, -C), a short region of unknown function, a transmembrane anchor, a juxtamembrane cytoplasmic segment (JxM), and one of two cytoplasmic tails (Cyt1 or Cyt2). CD46 isoforms, the result of differential RNA splicing, differ in the number of SCR domains and

their cytoplasmic tails (Liszewski et al., 1994). Most cell types express all CD46 isoforms, though in different ratios.

Efficient adherence of *N. gonorrhoeae* (GC) was found to require SCR-3 and the STP domain (Kallstrom et al., 2001). CD46 serves as a receptor for numerous pathogens, including group A strains of Measles virus (Dorig et al., 1993), *Streptococcus pyogenes* (Okada et al., 1995), human Herpesvirus 6 (Santoro et al., 1999), and group B Adenovirus (Gaggar et al., 2003).

The CD46 cytoplasmic tails are potential substrates for cellular kinases and therefore are likely to have signaling functions. In the RAW264.7 mouse macrophage cell line, the CD46 tail interacts with multiple kinases, and this interaction correlates with tyrosine phosphorylation of the CD46 cytoplasmic domains (Wong et al., 1997). In Jurkat cells, the CD46 Cyt2 isoform is tyrosine phosphorylated by the Src kinase Lck following antibody ligation (Wang et al., 2000).

Cells expressing CD46 with truncated tails fail to support bacterial adhesion (Kallstrom et al., 2001), suggesting that the tail fulfills an important function in this process. Furthermore, GC adhesion is inversely proportional to the level of CD46 expression (Tobiason and Seifert, 2001), indicating that pilus-

mediated adhesion does not require high levels of CD46. Taken together, these observations suggest that pilus-mediated cellular adhesion may occur through signaling cascade(s) generated through the CD46 cytoplasmic tail.

1.4.1 Pilus-mediated signaling

Several lines of evidence suggest that type IV pili induce multiple signaling cascades. It has been shown that piliated GC and MC and purified pili trigger a cytosolic Ca^{2+} flux in human epithelial cells (Kallstrom et al., 1998). This event occured minutes after the addition of bacteria or semipurified pili. Importantly, depletion of intracellular Ca^{2+} stores or treatment with particular kinase inhibitors resulted in diminished bacterial adherence. Finally, the calcium signal was shown to be blocked in the presence of antibodies to CD46. These results suggest that pili may induce an early host signaling response that involves CD46.

The pilus triggered Ca^{2+} flux was subsequently observed to cause an exocytic event in the host cell, resulting in the recruitment of lysosomal membrane proteins such as Lamp-1 and Lamp-2 at the plasma membrane and the release of lysosomal contents into the extracellular medium (Ayala et al., 2001).

1.4.2 Cortical plaques

Piliated *Neisseria* induce a series of rearrangements on the host cell during infection, resulting in the formation of cortical plaques (Figure 4). These structures contain tyrosine-phosphorylated proteins, actin, ezrin, and a subset of transmembrane glycoproteins directly beneath sites of GC or MC attachment in epithelial cells (Hoffmann et al., 2001; Merz et al., 1999; Merz and So, 1997). Fulminant plaques require the expression of both Type IV pili and the function of PilT (Merz et al., 1999), suggesting that protein clustering is affected by twitching or mechanical tension exerted by pilus retraction.

1.4.3 Mechanical signals

One important question in addressing the role of signaling by type IV pili is whether pilus retraction can generate host cell membrane tension that would directly activate signaling cascades. It has been established that mechanical forces applied to the plasma membrane of eukaryotic cells enhance the formation of plaque-like structures and cytoskeletal rearrangements in both cell-cell and cell-substrate adhesion (Sheetz et al., 1998; Wang et al., 1993). Forces required to generate signaling changes and elongate microvilli are well within range of those measured for pilus retraction (Miao et al., 2002;

Raucher and Sheetz, 2000; Raucher et al., 2000; Shao et al., 1998; Sheetz et al., 1998; Wang et al., 1993). Thus, pilus retraction by bacteria adhered to a host cell could generate sufficient tension to activate signaling events.

1.5 Present work

When I began my thesis work, CD46 had just been identified as a type IV pilus receptor by Jonsson's group. Furthermore, Wong reported that CD46 recruits kinases to its tail domains, and Aktkinson's group demonstrated that antibody ligation of CD46 induced tyrosine phosphoryation of the CD46 cyt2 isoform. It was likely, given this evidence, that CD46 could also be activated by piliated GC adhering to host cells. Manuscript I describes the first report of CD46 phosphorylation by GC infection.

Merz et al. had previously described the appearance of cortical plaques—accumulation of host cell signaling proteins and cytoskeletal components at the sites of GC infection. We drew on this insight to try to identify a host cell kinase that would be involved in CD46 phosphorylation by GC. *In vitro* evidence showed that CD46 was able to be tyrosine phosphorylated by src-kinase. We found that c-yes, a src-family kinase member enriched in epithelial cells, had accumulated in GC-induced cortical

plaques. Biochemical studies confirmed that c-yes associated with CD46 *in vivo* and that c-yes was involved in CD46 phosphorylation. Importantly, a src-kinase inhibitor reduced the ability of GC to attach to host cells by 50%, demonstrating that the activation of c-yes kinase and CD46 phosphorylation by GC type IV pili were important events in GC infection.

As mentioned earlier, only very recently were biophysical measurements of the force of pilus retraction revealed. Using lazer tweezers trapping, forces sometimes exceeding 100 pN were measured for pilus retraction (Maier et al., 2002; Merz et al., 2000). This was a considerable amount of force given the dynamics of pilus structure and function, and it was reasoned that a typical microcolony of 10-100 piliated bacteria could exert more than enough force to generate signals through tension on membranes. It had not yet been investigated whether membrane tension due to pilus binding and subsequent retraction would generate signaling cascades. However, biophysical experiments had demonstrated that mechanical tension could increase the efficiency of cellular adhesion plaque formation in mammalian cells, and reorganize cytoskeletal anchoring during cell-cell and cell-substrate adhesion (Sheetz et al., 1998; Wang et al., 1993). Thus, if pili could exert sufficient tension upon adhesion to host cell membranes, it

became likely that signals would be generated by this event, and that these signals would perhaps influence the course of infection.

Membrane tension has been shown to activate the phosphoinositide-3 kinase pathway (Miao et al., 2002; Raucher et al., 2000). PI-3 kinases (PI3K) orchestrate many cellular events including cell growth, motility, differentiation, survival, and intracellular trafficking (Cantley, 2002; Foster et al., 2003). Importantly, PI-3 kinases have been implicated in bacterial invasion processes. *Listeria monocytogenes*, *Helicobacter pylori*, and *Chlamydia pneumoniae,* all require PI-3 kinase activation for efficient invasion of host cells (Coombes et al., 2002; Ireton and Cossart, 1997; Kwok et al., 2002).

In manuscript II, I investigated the hypothesis that GC uses pilus retraction to activate the PI-3 kinase pathway through host cell membrane tension. I demonstrate that the PI3K/Akt pathway is activated in host epithelial cells infected with piliated GC. Furthermore, I show that this activation is dependent on the ability of GC pili to retract, as retraction-defective *pilT* mutants were impaired in their ability to activate this cascade. Importantly, I show that one of the host cell lipid products generated by this signaling cascade, [PI(3,4,5)P3], has a direct effect on GC aggregative behavior in

culture, and that this lipid directly increases transcription of the *pilT* gene. This important finding supported the idea that bacteria and host could engage in interspecies signaling.

Manuscript III continues the analysis of the role of CD46 in GC pathogenesis. It describes the production of monoclonal antibodies specifically directed against the cytoplasmic domains of CD46. These antibodies were used to analyze the contributions of each cytoplasmic variant in the overall process of GC infection. I show that the CD46 cytoplasmic tail 1 and tail 2 isoforms exhibit different levels of clustering in GC-induced cortical plaques. Furthermore, the appearance of each CD46 cytoplasmic variant in GC-induced cortical plaques differed depending on the cell type infected.

In the final chapter, I discuss the results presented here in light of very recent data and describe some emerging themes in bacterial pathogenesis that will pave the way for directions in future work.

Figure 1. Steps in GC infection of host epithelium. 1. The bacteria adhere loosely to the apical surface of mucosal epithelial using type IV pili. Microcolonies begin to form about 30min post infection and robust cortical plaques are evident at 3h with host cell microvilli elongation. 2. Bacteria shed pili, and associate tightly with the plasma membrane as individual bacteria. The bacteria begin to enter cells 6h post infection. 3. GC transcytose the cell layer and exit the basal membrane to reach subepithelial stromal tissues.

Images of scanning electron microscopy of GC infection on Fallopian tube organ culture epithelium (A,C) from *(Stephens, D.S. Clin Microbiol Rev.2:104-11, 1989.)* Images of scanning and transmission electron microscopy of GC infection on HeLa cells (B,D) from *(Bilker et al. EMBO:21(4), 560-71, 2002).*

FIGURE 1: Steps in *Neisseria gonorrhoeae* infection

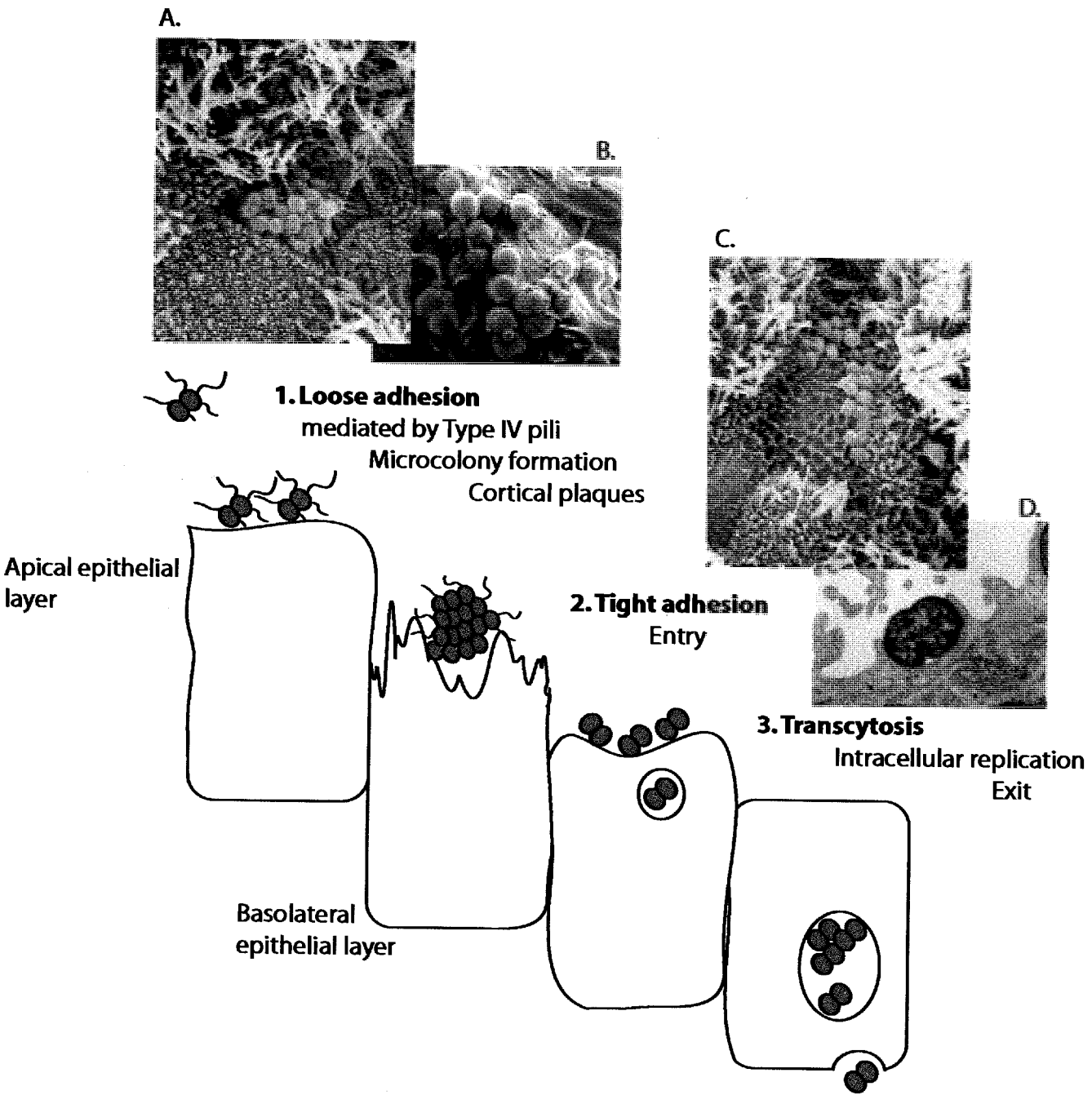


Figure 2. Structure of Type IV pili. A. Growing pilin subunits are processed by PilD and assembled at the inner membrane-periplasm interface by PilF and dissassembled through the action of PilT, generating dynamic pili. PilT drives pilus retraction. Pilus fibers are extruded through the PilQ pore. B. Mutants lacking the PilQ pore, indicating pilin polymers trapped in the periplasm. C. Mutants lacking PilT and PilQ.

[Figure from *Wolfgang et al., EMBO 19(23): 6408-64118, 2000*]

FIGURE 2: Structure of Type IV Pili

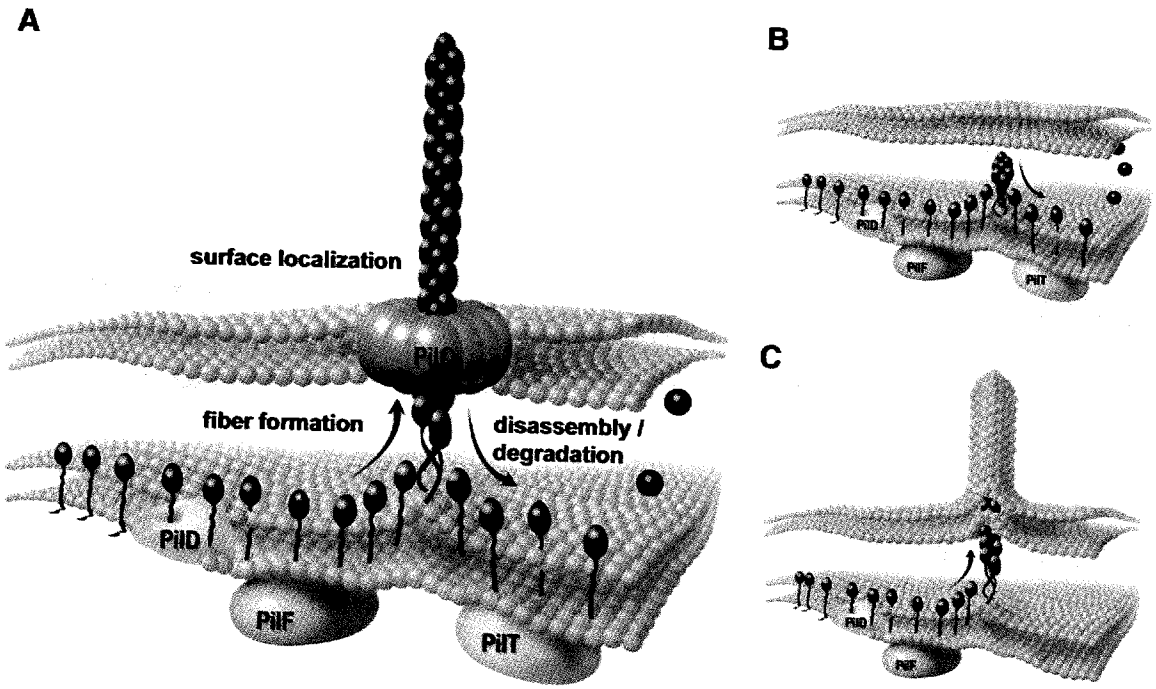
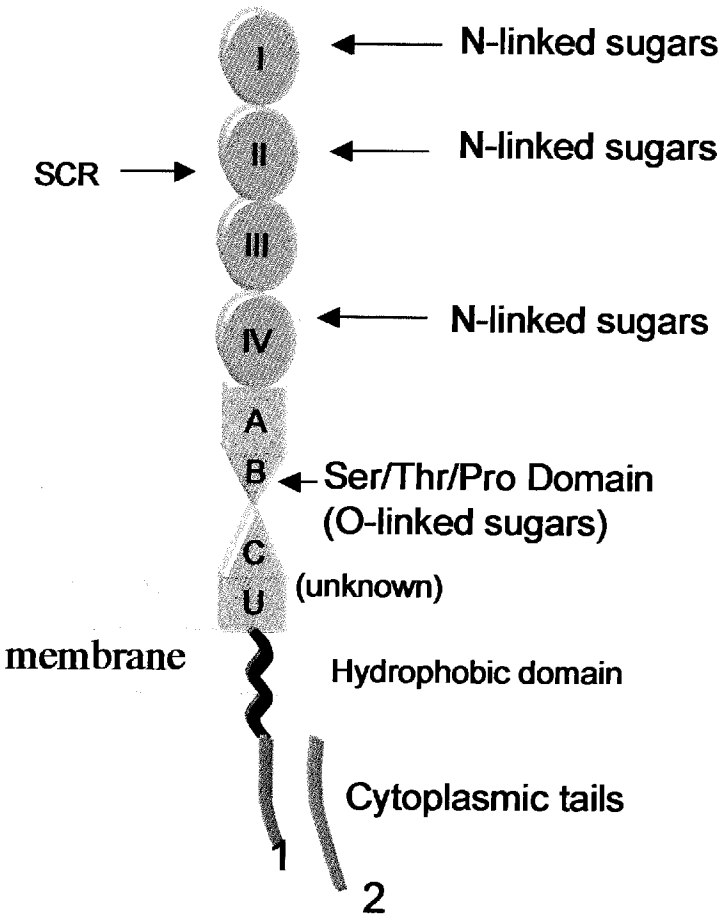


Figure 3. CD46 structure. Ectodomain consists of four short consensus repeats (SCR1-4), followed by a segment of serine-threonine-proline rich domains (STP-A,B,C), a short region of unknown function, a transmembrane domain, and one of two cytoplasmic tails. Many CD46 isoforms result from alternative splicing. Sequences of cytoplasmic tail 1 and tail 2 are depicted below.

FIGURE 3: CD46 structure



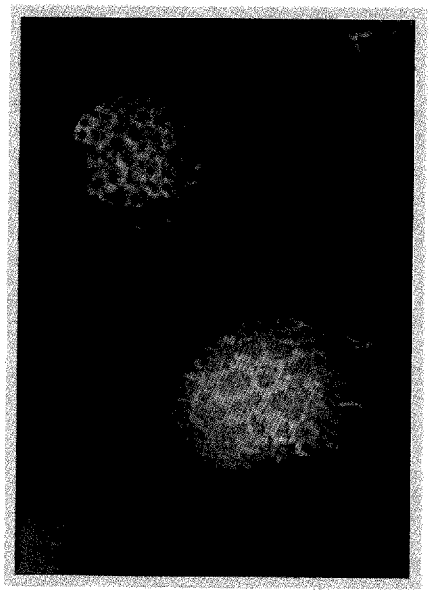
CD46 Isoforms
SCR1-4 +
ABC1
ABC2
BC1
BC2
B1
B2
C1
C2

CD46 cytoplasmic Isoforms

Cyt1 tail	PYRYLQRRKKKGKADGGAEYATYQTKSTTPAEQRG
Cyt2 tail	PYRYLQRRKKKGTYLTDETHREVKFTSL

Figure 4. *Neisseria gonorrhoeae* trigger cortical plaque formation. A431 cells infected with GC for 3h were stained for a plaque component ezrin, in red, and anti-GC antibodies to visualize type IV pili (green).

FIGURE 4: *Neisseria gonorrhoeae* induced cortical plaques



(Lee, S., Potter, L. and So, M. unpublished data)

References

Ansley, S. J., Badano, J. L., Blacque, O. E., Hill, J., Hoskins, B. E., Leitch, C. C., Kim, J. C., Ross, A. J., Eichers, E. R., Teslovich, T. M., *et al.* (2003). Basal body dysfunction is a likely cause of pleiotropic Bardet-Biedl syndrome. Nature *425*, 628-633.

Apicella, M. A., Ketterer, M., Lee, F. K., Zhou, D., Rice, P. A., and Blake, M. S. (1996). The pathogenesis of gonococcal urethritis in men: confocal and immunoelectron microscopic analysis of urethral exudates from men infected with Neisseria gonorrhoeae. J Infect Dis *173*, 636-646.

Ayala, B. P., Vasquez, B., Clary, S., Tainer, J. A., Rodland, K., and So, M. (2001). The pilus-induced Ca2+ flux triggers lysosome exocytosis and increases the amount of Lamp1 accessible to Neisseria IgA1 protease. Cell Microbiol *3*, 265-275.

Bieber, D., Ramer, S. W., Wu, C. Y., Murray, W. J., Tobe, T., Fernandez, R., and Schoolnik, G. K. (1998). Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic Escherichia coli [see comments]. Science *280*, 2114-2118.

Bitter, W., Koster, M., Latijnhouwers, M., de Cock, H., and Tommassen, J. (1998). Formation of oligomeric rings by XcpQ and PilQ, which are involved in protein transport across the outer membrane of Pseudomonas aeruginosa. Mol Microbiol *27*, 209-219.

Bradley, D. E. (1972a). Evidence for the retraction of Pseudomonas aeruginosa RNA phage pili. Biochem Biophys Res Commun *47*, 142-149.

Bradley, D. E. (1972b). Shortening of Pseudomonas aeruginosa pili after RNA-phage adsorption. J Gen Microbiol *72*, 303-319.

Bradley, D. E. (1974). The adsorption of Pseudomonas aeruginosa pilus-dependent bacteriophages to a host mutant with nonretractile pili. Virology *58*, 149-163.

Britigan, B., Cohen, M., and Sparling, P. (1985). Gonococcal Infection: A Model of Molecular Pathogenesis. New Eng J Med *312*, 1683-1693.

Brossay, L., Paradis, G., Fox, R., Koomey, M., and Hebert, J. (1994). Identification, localization, and distribution of the PilT protein in Neisseria gonorrhoeae. Infect Immun *62*, 2302-2308.

Buchanan, T., Chen, K., Jones, R., Hildebrandt, J., Pearce, W., Hermodson, M., Newland, J., and Luchtel, D. (1978). Pili and Principal Outer Membrane Protein of *Neisseria gonorrhoeae*: Immunochemical,

Structural, and Pathogenic Aspects. In Immunobiology of *Neisseria gonorrhoeae*, G. Brooks, E. Gotschlich, K. Holmes, W. Sawyer, and F. Young, eds. (Washington, D.C., American society for Microbiology), pp. 145-154.

Buchanan, T. M., and Pearce, W. A. (1976). Pili as a mediator of the attachment of gonococci to human erythrocytes. Infect Immun *13*, 1483-1489.

Cannon, J. G., Cohen, M. S., Isbey, S. F., Snodgrass, T. L., Wallace, A., Dempsey, J. A. F., Apicella, M., and Zhou, D. (1996). Infectivity of gonococcal mutants in the human challenge model. In Abstracts of the Tenth International Pathogenic Neisseria Conference, W. D. Zollinger, C. E. Frasch, and C. D. Deal, eds. (Baltimore), pp. 11-12.

Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. Science *296*, 1655-1657.

Cohen, M. S., and Sparling, P. F. (1992). Mucosal infection with *Neisseria gonorrhoeae*: bacterial adaptation and mucosal defenses. *89*, 1699-1705.

Comolli, J. C., Hauser, A. R., Waite, L., Whitchurch, C. B., Mattick, J. S., and Engel, J. N. (1999). Pseudomonas aeruginosa gene products PilT

and PilU are required for cytotoxicity in vitro and virulence in a mouse model of acute pneumonia. Infect Immun *67*, 3625-3630.

Coombes, B. K., Johnson, D. L., and Mahony, J. B. (2002). Strategic targeting of essential host-pathogen interactions in chlamydial disease. Curr Drug Targets Infect Disord *2*, 201-216.

Cornelissen, C. N., Kelley, M., Hobbs, M. M., Anderson, J. E., Cannon, J. G., Cohen, M. S., and Sparling, P. F. (1998). The transferrin receptor expressed by gonococcal strain FA1090 is required for the experimental infection of human male volunteers. Mol Microbiol *27*, 611-616.

Cucurull, E., and Espinoza, L. R. (1998). Gonococcal arthritis. Rheum Dis Clin North Am *24*, 305-322.

Dalrymple, B., and Mattick, J. S. (1987). An analysis of the organization and evolution of type 4 fimbrial (MePhe) subunit proteins. J Mol Evol *25*, 261-269.

Dorig, R. E., Marcil, A., Chopra, A., and Richardson, C. D. (1993). The human CD46 molecule is a receptor for measles virus (Edmonston strain). Cell *75*, 295-305.

Drake, S. L., and Koomey, M. (1995). The product of the pilQ gene is essential for the biogenesis of type IV pili in Neisseria gonorrhoeae. Mol Microbiol *18*, 975-986.

Dunham, S. A., Bjøras, M., Koomey, M., and Tønjum, T. (1998). Structure and function of a meningococcal secretin required for pilus biogenesis. In Abstracts of the Tenth International Pathogenic *Neisseria* Conference, X. Nassif, M. Quentin-Millet, and M. Taha, eds. (Paris, Éditions E.D.K.), pp. 280.

Dunn, K. L., Virji, M., and Moxon, E. R. (1995). Investigations into the molecular basis of meningococcal toxicity for human endothelial and epithelial cells: the synergistic effect of LPS and pili. Microb Pathog *18*, 81-96.

Duong, F., and Wickner, W. (1997). Distinct catalytic roles of the SecYE, SecG and SecDFyajC subunits of preprotein translocase holoenzyme. Embo J *16*, 2756-2768.

Forest, K. T., Dunham, S. A., Koomey, M., and Tainer, J. A. (1999). Crystallographic structure reveals phosphorylated pilin from Neisseria: phosphoserine sites modify type IV pilus surface chemistry and fibre morphology. Mol Microbiol *31*, 743-752.

Forest, K. T., and Tainer, J. A. (1997). Type-4 pilus-structure: outside to inside and top to bottom--a minireview. Gene *192*, 165-169.

Foster, F. M., Traer, C. J., Abraham, S. M., and Fry, M. J. (2003). The phosphoinositide (PI) 3-kinase family. J Cell Sci *116*, 3037-3040.

Freitag, N. E., Seifert, H. S., and Koomey, M. (1995). Characterization of the pilF-pilD pilus-assembly locus of Neisseria gonorrhoeae. Mol Microbiol *16*, 575-586.

Frost, L. S., Carpenter, M., and Paranchych, W. (1978). N-methylphenylalanine at the N-terminus of pilin isolated from Pseudomonas aeruginosa K. Nature *271*, 87-89.

Fussenegger, M., Rudel, T., Barten, R., Ryll, R., and Meyer, T. F. (1997). Transformation competence and type-4 pilus biogenesis in Neisseria gonorrhoeae--a review. Gene *192*, 125-134.

Gaggar, A., Shayakhmetov, D. M., and Lieber, A. (2003). CD46 is a cellular receptor for group B adenoviruses. Nat Med *9*, 1408-1412.

Ghys, P. D., Fransen, K., Diallo, M. O., Ettiegne-Traore, V., Coulibaly, I. M., Yeboue, K. M., Kalish, M. L., Maurice, C., Whitaker, J. P., Greenberg, A. E., and Laga, M. (1997). The associations between cervicovaginal HIV shedding, sexually transmitted diseases and

immunosuppression in female sex workers in Abidjan, Cote d'Ivoire. Aids *11*, F85-93.

Gregg, C., Melly, M., Hellerqvist, C., Coniglio, J., and McGee, Z. (1981). Toxic Activity of Purified Lipopolysaccharide of *Neisseria gonorrhoeae* for Human Fallopian Tube Mucosa. J Infect Dis *143*, 432-439.

Gubish, E. R., Jr., Chen, K. C., and Buchanan, T. M. (1982). Attachment of gonococcal pili to lectin-resistant clones of Chinese hamster ovary cells. Infect Immun *37*, 189-194.

He, S. Y. (1998). Type III protein secretion systems in plant and animal pathogenic bacteria. Ann Rev Phytopathol *36*, 363-392.

Herdendorf, T. J., McCaslin, D. R., and Forest, K. T. (2002). Aquifex aeolicus PilT, homologue of a surface motility protein, is a thermostable oligomeric NTPase. J Bacteriol *184*, 6465-6471.

Hermodson, M. A., Chen, K. C., and Buchanan, T. M. (1978). *Neisseria* pili proteins: amino-terminal amino acid sequences and Identification of an unusual amino acid. Biochemistry *17*, 442-445.

Hoffmann, I., Eugene, E., Nassif, X., Couraud, P. O., and Bourdoulous, S. (2001). Activation of ErbB2 receptor tyrosine kinase supports invasion

of endothelial cells by Neisseria meningitidis. J Cell Biol *155*, 133-143.

Hook, E. W. I., and Holmes, K. K. (1985). Gonocuccal infections. Ann Intern Med *102*, 229-243.

Hopper, S., Vasquez, B., Merz, A., Clary, S., Wilbur, J. S., and So, M. (2000a). Effects of the immunoglobulin A1 protease on Neisseria gonorrhoeae trafficking across polarized T84 epithelial monolayers. Infect Immun *68*, 906-911.

Hopper, S., Wilbur, J. S., Vasquez, B. L., Larson, J., Clary, S., Mehr, I. J., Seifert, H. S., and So, M. (2000b). Isolation of Neisseria gonorrhoeae mutants that show enhanced trafficking across polarized T84 epithelial monolayers. Infect Immun *68*, 896-905.

Hoyne, P. A., Haas, R., Meyer, T. F., Davies, J. K., and Elleman, T. C. (1992). Production of Neisseria gonorrhoeae pili (fimbriae) in Pseudomonas aeruginosa. J Bacteriol *174*, 7321-7327.

Hultgren, S., Abraham, S., Caparon, M., Falk, P., St Geme III, J., and Normark, S. (1993). Pilus and nonpilus bacterial adhesins: assembly and function in cell recognition. Cell *73*, 887-901.

Ilver, D., Kallstrom, H., Normark, S., and Jonsson, A. B. (1998). Transcellular passage of Neisseria gonorrhoeae involves pilus phase variation. Infect Immun *66*, 469-473.

Ireton, K., and Cossart, P. (1997). Host-pathogen interactions during entry and actin-based movement of Listeria monocytogenes. Annu Rev Genet *31*, 113-138.

James-Holmquest, A. N., Swanson, J., Buchanan, T. M., Wende, R. D., and Williams, R. P. (1974). Differential attachment by piliated and nonpiliated Neisseria gonorrhoeae to human sperm. Infect Immun *9*, 897-902.

Kallstrom, H., Blackmer Gill, D., Albiger, B., Liszewski, M. K., Atkinson, J. P., and Jonsson, A. B. (2001). Attachment of Neisseria gonorrhoeae to the cellular pilus receptor CD46: identification of domains important for bacterial adherence. Cell Microbiol *3*, 133-143.

Kallstrom, H., Islam, M. S., Berggren, P. O., and Jonsson, A. B. (1998). Cell signaling by the type IV pili of pathogenic Neisseria. J Biol Chem *273*, 21777-21782.

Kallstrom, H., Liszewski, M. K., Atkinson, J. P., and Jonsson, A. B. (1997). Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic Neisseria. Mol Microbiol *25*, 639-647.

Kazmierczak, B. I., Mostov, K., and Engel, J. N. (2001). Interaction of bacterial pathogens with polarized epithelium. Annu Rev Microbiol *55*, 407-435.

Kellogg, D. S., Peacock, W. L., Deacon, W. E., Brown, L., and Pirkle, C. I. (1963). *Neisseria gonorrhoeae.* I. Virulence Genetically linked to Clonal Varation. J Bacteriol *85*, 1274-1279.

Kwok, T., Backert, S., Schwarz, H., Berger, J., and Meyer, T. F. (2002). Specific entry of Helicobacter pylori into cultured gastric epithelial cells via a zipper-like mechanism. Infect Immun *70*, 2108-2120.

Laga, M., Manoka, A., Kivuvu, M., Malele, B., Tuliza, M., Nzila, N., Goeman, J., Behets, F., Batter, V., Alary, M., and et al. (1993). Non-ulcerative sexually transmitted diseases as risk factors for HIV-1 transmission in women: results from a cohort study [see comments]. Aids *7*, 95-102.

Laga, M., Meheus, A., and Piot, P. (1989). Epidemiology and control of gonococcal ophthalmia neonatorum. Bull World Health Organ *67*, 471-477.

Lee, J. S., Choi, H. Y., Lee, J. E., Lee, S. H., and Oum, B. S. (2002). Gonococcal keratoconjunctivitis in adults. Eye *16*, 646-649.

Lin, L., Ayala, P., Larson, J., Mulks, M., Fukuda, M., Carlsson, S. R., Enns, C., and So, M. (1997). The Neisseria type 2 IgA1 protease cleaves LAMP1 and promotes survival of bacteria within epithelial cells. Mol Microbiol *24*, 1083-1094.

Liszewski, M. K., and Atkinson, J. P. (1992). Membrane cofactor protein. Curr Top Microbiol Immunol *178*, 45-60.

Liszewski, M. K., Post, T. W., and Atkinson, J. P. (1991). Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. Annu Rev Immunol *9*, 431-455.

Liszewski, M. K., Tedja, I., and Atkinson, J. P. (1994). Membrane cofactor protein (CD46) of complement. Processing differences related to alternatively spliced cytoplasmic domains. J Biol Chem *269*, 10776-10779.

Maier, B., Potter, L., So, M., Long, C. D., Seifert, H. S., and Sheetz, M. P. (2002). Single pilus motor forces exceed 100 pN. Proc Natl Acad Sci U S A *99*, 16012-16017.

Marceau, M., Forest, K., Beretti, J. L., Tainer, J., and Nassif, X. (1998). Consequences of the loss of O-linked glycosylation of meningococcal type IV pilin on piliation and pilus-mediated adhesion. Mol Microbiol *27*, 705-715.

Marceau, M., and Nassif, X. (1999). Role of glycosylation at Ser63 in production of soluble pilin in pathogenic Neisseria. J Bacteriol *181*, 656-661.

Marciano, D. K., Russel, M., and Simon, S. M. (1999). An aqueous channel for filamentous phage export [see comments]. Science *284*, 1516-1519.

Masi, A. T., and Eisenstein, B. I. (1981). Disseminated gonococcal infection (DGI) and gonococcal arthritis (GCA): II. Clinical manifestations, diagnosis, complications, treatment, and prevention. Semin Arthritis Rheum *10*, 173-197.

McGee, Z. A., Clemens, C. M., Jensen, R. L., Klein, J. J., Barley, L. R., and Gorby, G. L. (1992). Local induction of tumor necrosis factor as a molecular mechanism of mucosal damage by gonococci. *12*, 333-341.

McGee, Z. A., Johnson, A. P., and Taylor-Robinson, D. (1981). Pathogenic mechanisms of *Neisseria gonorrhoeae*: observations on damage to human Fallopian tubes in organ culture by gonococci of colony type 1 or type 4. *143*, 413-422.

Melly, M. A., Gregg, C. R., and McGee, Z. A. (1981). Studies of toxicity of *Neisseria gonorrhoeae* for human fallopian tube mucosa. *143*, 423-431.

Melly, M. A., McGee, Z. A., and Rosenthal, R. S. (1984). Ability of monomeric peptidoglycon fragments from *Neisseria gonorrhoeae* to damage human fallopian-tube mucosa. J Infec Dis *149*, 378-386.

Merz, A. J., Enns, C. A., and So, M. (1999). Type IV pili of pathogenic neisseriae elicit cortical plaque formation in epithelial cells. Mol Microbiol *32*, 1316-1332.

Merz, A. J., Rifenbery, D. B., Arvidson, C. G., and So, M. (1996). Traversal of a polarized epithelium by pathogenic Neisseriae: facilitation by type IV pili and maintenance of epithelial barrier function. Mol Med *2*, 745-754.

Merz, A. J., and So, M. (1997). Attachment of piliated, Opa- and Opc- gonococci and meningococci to epithelial cells elicits cortical actin rearrangements and clustering of tyrosine-phosphorylated proteins. Infect Immun *65*, 4341-4349.

Merz, A. J., and So, M. (2000). Interactions of pathogenic neisseriae with epithelial cell membranes. Annu Rev Cell Dev Biol *16*, 423-457.

Merz, A. J., So, M., and Sheetz, M. P. (2000). Pilus retraction powers bacterial twitching motility. Nature *407*, 98-102.

Meyer, T. F., Billyard, E., Haas, R., Storzbach, S., and So, M. (1984). Pilus genes of Neisseria gonorrheae: chromosomal organization and DNA sequence. Proc Natl Acad Sci U S A *81*, 6110-6114.

Meyer, T. F., Pohlner, J., and van Putten, J. P. (1994). Biology of the pathogenic Neisseriae. Curr Top Microbiol Immunol *192*, 283-317.

Miao, H., Yuan, S., Wang, Y., Tsygankov, A., and Chien, S. (2002). Role of Cbl in shear-activation of PI 3-kinase and JNK in endothelial cells. Biochem Biophys Res Commun *292*, 892-899.

Mosleh, I. M., Boxberger, H. J., Sessler, M. J., and Meyer, T. F. (1997). Experimental infection of native human ureteral tissue with Neisseria gonorrhoeae: adhesion, invasion, intracellular fate, exocytosis, and passage through a stratified epithelium. Infect Immun *65*, 3391-3398.

Muller, A., Gunther, D., Dux, F., Naumann, M., Meyer, T. F., and Rudel, T. (1999). Neisserial porin (PorB) causes rapid calcium influx in target cells and induces apoptosis by the activation of cysteine proteases. Embo J *18*, 339-352.

Nassif, X., Lowy, J., Stenberg, P., O'Gaora, P., Ganji, A., and So, M. (1993). Antigenic variation of pilin regulates adhesion of Neisseria meningitidis to human epithelial cells. Mol Microbiol *8*, 719-725.

Nouwen, N., Ranson, N., Saibil, H., Wolpensinger, B., Engel, A., Ghazi, A., and Pugsley, A. P. (1999). Secretin PulD: Association with pilot PulS, structure, and ion- conducting channel formation. Proc Natl Acad Sci U S A *96*, 8173-8177.

Nunn, D. N., and Lory, S. (1991). Product of the Pseudomonas aeruginosa gene pilD is a prepilin leader peptidase. Proc Natl Acad Sci U S A *88*, 3281-3285.

Okamoto, S., and Ohmori, M. (2002). The cyanobacterial PilT protein responsible for cell motility and transformation hydrolyzes ATP. Plant Cell Physiol *43*, 1127-1136.

Parge, H. E., Forest, K. T., Hickey, M. J., Christensen, D. A., Getzoff, E. D., and Tainer, J. A. (1995). Structure of the fibre-forming protein pilin at 2.6 A resolution. Nature *378*, 32-38.

Patel, P., Marrs, C. F., Mattick, J. S., Ruehl, W. W., Taylor, R. K., and Koomey, M. (1991). Shared antigenicity and immunogenicity of type 4 pilins expressed by Pseudomonas aeruginosa, Moraxella bovis, Neisseria gonorrhoaea, Dichelobacter nodosus, and Vibrio cholerae. Infect Immun *59*, 4674-4676.

Pugsley, A. P. (1993). The complete general secretory pathway in gram-negative bacteria. Microbiol Rev *57*, 50-108.

Pugsley, A. P., Francetic, O., Possot, O. M., Sauvonnet, N., and Hardie, K. R. (1997). Recent progress and future directions in studies of the main terminal branch of the general secretory pathway in Gram-negative bacteria--a review. Gene *192*, 13-19.

Pujol, C., Eugene, E., de Saint Martin, L., and Nassif, X. (1997). Interaction of Neisseria meningitidis with a polarized monolayer of epithelial cells. Infect Immun *65*, 4836-4842.

Pujol, C., Eugene, E., Marceau, M., and Nassif, X. (1999). The meningococcal PilT protein is required for induction of intimate attachment to epithelial cells following pilus-mediated adhesion. Proc Natl Acad Sci U S A *96*, 4017-4022.

Raucher, D., and Sheetz, M. P. (2000). Cell spreading and lamellipodial extension rate is regulated by membrane tension. J Cell Biol *148*, 127-136.

Raucher, D., Stauffer, T., Chen, W., Shen, K., Guo, S., York, J. D., Sheetz, M. P., and Meyer, T. (2000). Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion [In Process Citation]. Cell *100*, 221-228.

Rice, P. A., and Schachter, J. (1991). Pathogenesis of pelvic inflammatory disease. What are the questions? Jama *266*, 2587-2593.

Santoro, F., Kennedy, P. E., Locatelli, G., Malnati, M. S., Berger, E. A., and Lusso, P. (1999). CD46 is a cellular receptor for human herpesvirus 6. Cell *99*, 817-827.

Seifert, H. S. (1996). Questions about gonococcal pilus phase- and antigenic variation. Mol Microbiol *21*, 433-440.

Seifert, H. S., Wright, C. J., Jerse, A. E., Cohen, M. S., and Cannon, J. G. (1994). Multiple gonococcal pilin antigenic variants are produced during experimental human infections. J Clin Invest *93*, 2744-2749.

Seya, T., Nakamura, K., Masaki, T., Ichihara-Itoh, C., Matsumoto, M., and Nagasawa, S. (1995). Human factor H and C4b-binding protein serve as factor I-cofactors both encompassing inactivation of C3b and C4b. Mol Immunol *32*, 355-360.

Shao, J. Y., Ting-Beall, H. P., and Hochmuth, R. M. (1998). Static and dynamic lengths of neutrophil microvilli. Proc Natl Acad Sci U S A *95*, 6797-6802.

Shaw, J. H., and Falkow, S. (1988). Model for invasion of human tissue culture cells by *Neisseria gonnorrhoeae*. *56*, 1625-1632.

Sheetz, M. P., Felsenfeld, D. P., and Galbraith, C. G. (1998). Cell migration: regulation of force on extracellular-matrix-integrin complexes. Trends Cell Biol *8*, 51-54.

Skerker, J. M., and Berg, H. C. (2001). Direct observation of extension and retraction of type IV pili. Proc Natl Acad Sci U S A *98*, 6901-6904.

Stephens, D. S., and Farley, M. M. (1991). Pathogenic events during infection of the human nasopharynx with *Neisseria meningitids* and *Haemophilus influenzae*. *13*, 22-33.

Stephens, D. S., Hoffman, L. H., and McGee, Z. A. (1983). Interaction of *Neisseria meningitidis* with human nasopharyngeal mucosa: attachment and entry into columnar epithelial cells. *148*, 369-376.

Stephens, D. S., and McGee, Z. A. (1981). Attachment of Neisseria meningitidis to human mucosal surface: influence of pili and type of receptor cell. J Infect Dis *143*, 525-532.

Stephens, D. S., Whitney, A. M., Melly, M. A., Hoffman, L. H., Farley, M. M., and Frasch, C. E. (1986). Analysis of damage to human ciliated nasopharyngeal epithelium by Neisseria meningitidis. Infect Immun *51*, 579-585.

Stimson, E., Virji, M., Makepeace, K., Dell, A., Morris, H. R., Payne, G., Saunders, J. R., Jennings, M. P., Barker, S., Panico, M., and et al. (1995). Meningococcal pilin: a glycoprotein substituted with digalactosyl 2,4- diacetamido-2,4,6-trideoxyhexose. Mol Microbiol *17*, 1201-1214.

Strom, M. S., and Lory, S. (1993). Structure-function and biogenesis of the type IV pili. Annu Rev Microbiol *47*, 565-596.

Strom, M. S., Nunn, D. N., and Lory, S. (1993). A single bifunctional enzyme, PilD, catalyzes cleavage and N- methylation of proteins belonging to the type IV pilin family. Proc Natl Acad Sci U S A *90*, 2404-2408.

Sun, H., Zusman, D. R., and Shi, W. (2000). Type IV pilus of Myxococcus xanthus is a motility apparatus controlled by the frz chemosensory system. Curr Biol *10*, 1143-1146.

Swanson, J. (1973). Studies on gonococcus infection. IV. Pili: their role in attachment of gonococci to tissue culture cells. J Exp Med *137*, 571-589.

Turner, C. F., Rogers, S. M., Miller, H. G., Miller, W. C., Gribble, J. N., Chromy, J. R., Leone, P. A., Cooley, P. C., Quinn, T. C., and Zenilman, J. M. (2002). Untreated gonococcal and chlamydial infection in a probability sample of adults. Jama *287*, 726-733.

Virji, M., Everson, J. S., and Lambden, P. R. (1982). Effect of anti-pilus antisera on virulence of variants of Neisseria gonorrhoeae for cultured epithelial cells. J Gen Microbiol *128*, 1095-1100.

Virji, M., Kayhty, H., Ferguson, D. J., Alexandrescu, C., Heckels, J. E., and Moxon, E. R. (1991). The role of pili in the interactions of pathogenic Neisseria with cultured human endothelial cells. Mol Microbiol *5*, 1831-1841.

Virji, M., Saunders, J. R., Sims, G., Makepeace, K., Maskell, D., and Ferguson, D. J. (1993). Pilus-facilitated adherence of Neisseria meningitidis to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin. Mol Microbiol *10*, 1013-1028.

Wang, J., Gray-Owen, S. D., Knorre, A., Meyer, T. F., and Dehio, C. (1998). Opa binding to cellular CD66 receptors mediates the transcellular traversal of Neisseria gonorrhoeae across polarized T84 epithelial cell monolayers [In Process Citation]. Mol Microbiol *30*, 657-671.

Wang, N., Butler, J. P., and Ingber, D. E. (1993). Mechanotransduction across the cell surface and through the cytoskeleton [see comments]. Science *260*, 1124-1127.

Ward, M. E., and Watt, P. J. (1972). Adherence of *Neisseria gonorrhoeae* to urethral mucosal cells: an electron-microscopic study of human gonorrhea. *126*, 601-605.

Ward, M. E., Watt, P. J., and Robertson, J. N. (1974). The human fallopian tube: a laboratory model for gonococcal infection. J Infect Dis *129*.

Watson, A. A., Alm, R. A., and Mattick, J. S. (1996). Identification of a gene, pilF, required for type 4 fimbrial biogenesis and twitching motility in Pseudomonas aeruginosa. Gene *180*, 49-56.

Whitchurch, C. B., Hobbs, M., Livingston, S. P., Krishnapillai, V., and Mattick, J. S. (1991). Characterisation of a Pseudomonas aeruginosa twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. Gene *101*, 33-44.

Wolfgang, M., Lauer, P., Park, H. S., Brossay, L., Hebert, J., and Koomey, M. (1998). PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated Neisseria gonorrhoeae. Mol Microbiol *29*, 321-330.

Wolfgang, M., van Putten, J. P., Hayes, S. F., Dorward, D., and Koomey, M. (2000). Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. Embo J *19*, 6408-6418.

Wong, T. C., Yant, S., Harder, B. J., Korte-Sarfaty, J., and Hirano, A. (1997). The cytoplasmic domains of complement regulatory protein

CD46 interact with multiple kinases in macrophages. J Leukoc Biol *62*, 892-900.

Wu, S. S., Wu, J., and Kaiser, D. (1997). The Myxococcus xanthus pilT locus is required for social gliding motility although pili are still produced. Mol Microbiol *23*, 109-121.

Yoshida, T., Kim, S. R., and Komano, T. (1999). Twelve pil genes are required for biogenesis of the R64 thin pilus. J Bacteriol *181*, 2038-2043.

Zenni, M. K., Giardina, P. C., Harvey, H. A., Shao, J., Ketterer, M. R., Lubaroff, D. M., Williams, R. D., and Apicella, M. A. (2000). Macropinocytosis as a mechanism of entry into primary human urethral epithelial cells by Neisseria gonorrhoeae. Infect Immun *68*, 1696-1699.

Chapter 2: Manuscript I

CD46 is phosphorylated at tyrosine 354 upon infection of epithelial cells by *Neisseria gonorrhoeae*

Shaun W. Lee, Robert A. Bonnah, Dustin L. Higashi, John P. Atkinson,

Sharon L. Milgram & Magdalene So

Department of Molecular Microbiology & Immunology,

Oregon Health Sciences University

Portland, Oregon, 97201-3098

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Summary

The Neisseria type IV pilus promotes bacterial adhesion to host cells. The pilus binds CD46, a complement-regulatory glycoprotein present on nucleated human cells (Kallstrom et al., 1997). CD46 mutants with truncated cytoplasmic tails fail to support bacterial adhesion (Kallstrom et al., 2001), suggesting that this region of the molecule also plays an important role in infection. Here, we report that infection of human epithelial cells by piliated *N. gonorrhoeae* (GC) leads to rapid tyrosine phosphorylation of CD46. Studies with wt and mutant tail fusion constructs demonstrate that Src kinase phosphorylates tyrosine 354 in the Cyt2 isoform of the CD46 cytoplasmic tail. Consistent with these findings, infection studies show that PP2, a specific Src-family kinase inhibitor, but not PP3, an inactive variant of this drug, reduces the ability of epithelial cells to support bacterial adhesion. Several lines of evidence point to the role of c-Yes, a member of the Src family of non-receptor tyrosine kinases, in CD46 phosphorylation. GC infection causes c-Yes to aggregate in the host cell cortex beneath adherent bacteria, increases binding of c-Yes to CD46, and stimulates c-Yes kinase activity. Finally, c-Yes immunoprecipitated from epithelial cells is able to phosphorylate the wt Cyt2 tail but not the mutant derivative in which

tyrosine 354 has been substituted with alanine. We conclude that GC infection leads to rapid tyrosine phosphorylation of the CD46 Cyt2 tail, and that the Src kinase c-Yes is involved in this reaction. Taken together, the findings reported here and elsewhere strongly suggest that pilus binding to CD46 is not a simple static process. Rather, they support a model in which pilus interaction with CD46 promotes signaling cascades important for Neisseria infectivity.

Introduction

The type IV pilus expressed by pathogenic Neisseria is a retractile structure that mediates bacterial motility on solid substrates (Brossay et al., 1994; Merz et al., 2000), DNA uptake (Wolfgang et al., 1998) and adhesion to host cells (Nassif et al., 1993; Swanson, 1973). Pili on live bacteria induce the formation of adhesion-promoting cortical plaques directly beneath the site of attachment. These plaques contain high concentrations of cortical actin and ezrin, membrane glycoproteins ICAM1, EGFR and CD44, and unidentified tyrosine-phosphorylated proteins (Merz et al., 1999; Merz and So, 1997). Purified pili trigger the release of Ca^{2+} from intracellular stores (Kallstrom et al., 1998) and a subsequent redistribution of Lamp1 compartments in target cells (Ayala et al., 1998). These activities of the type IV pilus suggest that the structure may promote attachment by stimulating signaling pathways in the host cell.

The Neisseria type IV pilus binds CD46, or Membrane Cofactor Protein (Kallstrom et al., 1997), a complement-regulatory membrane glycoprotein that prevents cell damage by autologous complement components (Liszewski et al. 1991; Seya et al. 1995). CD46 consists of an ectodomain

with four short consensus repeats (SCR1-4) followed by a segment of serine-threonine-proline rich domains (STP-A, -B, -C), a short region of unknown function, a transmembrane anchor, a juxtamembrane cytoplasmic segment (JxM), and one of two cytoplasmic tails (Cyt1 or Cyt2). CD46 isoforms, the result of differential RNA splicing, differ in the number of SCR domains and their cytoplasmic tail. Most cell types express all CD46 isoforms, though in different ratios. Efficient adherence of *N. gonorrhoeae* (GC) requires SCR-3 and the STP domain (Kallstrom et al., 2001). CD46 serves as a receptor for numerous pathogens, including group A strains of measles virus (Dorig et al., 1993), *Streptococcus pyogenes* (Okada et al., 1995), and human herpesvirus 6 (Santoro et al., 1999).

The CD46 cytoplasmic tails are potential substrates for cellular kinases and therefore are likely to have signaling functions. In the RAW264.7 mouse macrophage cell line, the CD46 tail interacts with multiple kinases, and this interaction correlates with tyrosine phosphorylation of the CD46 cytoplasmic domains (Wong et al., 1997). In Jurkat cells, the CD46 Cyt2 isoform is tyrosine phosphorylated by the Src kinase Lck following antibody ligation (Wang et al., 2000).

Cells expressing CD46 with truncated tails fail to support bacterial adhesion (Kallstrom et al., 2001), suggesting that the tail fulfills an important function in this process. Furthermore, GC adhesion is inversely proportional to the level of expression of CD46 (Tobiason and Seifert, 2001), indicating that pilus-mediated adhesion does not require high levels of CD46. These observations strongly suggest that pilus-mediated cellular adhesion may occur through signaling cascade(s) generated through the CD46 cytoplasmic tail.

To better understand the role of CD46 in Neisseria adhesion, we studied the fate of its cytoplasmic COOH-terminus upon infection. In particular, we tested the hypothesis that *N. gonorrhoeae* infection would trigger tyrosine phosphorylation of the CD46 COOH-terminus. We report that GC infection of human epithelial cells triggers rapid tyrosine phosphorylation of CD46, and that exogenous Src kinase phosphorylates the the Cyt2 tail isoform at tyrosine 354 *in vitro*. In support of these findings, treatment of epithelial cells with PP2, a specific Src-family kinase inhibitor, but not PP3, an inactive variant of this drug, reduces GC adherence. Several lines of evidence strongly suggest that the Src kinase c-Yes is involved in this reaction. GC infection rapidly leads to the clustering of c-Yes in the cell

cortex beneath adherent bacteria, increases binding of c-Yes to the CD46 tail and results in c-Yes activation. Finally, c-Yes isolated from epithelial cells preferentially phosphorylates the CD46 Cyt2 tail at tyrosine 354. We conclude that GC infection triggers rapid phosphorylation of tyrosine residue 354 of the Cyt2 tail of CD46, and that c-Yes participates in these reactions. The results reported here and elsewhere support the notion that the GC type IV pilus promotes bacterial adherence through signaling cascade(s) via the CD46 tail.

Results and discussion

CD46 is rapidly tyrosine phosphorylated upon GC infection

We tested the hypothesis that GC infection triggers tyrosine phosphorylation of CD46. A431 human endocervical epithelial cells were infected with GC strain MS11 N400 (Wolfgang et al., 1998) (P^+, Opa^-) and CD46 was immunoprecipitated from cell lysates with a monoclonal antibody that recognizes the SCR repeats (Mohler et al., 1999). The presence of phosphorylated tyrosine residues in the precipitate was determined by immunoblotting with a monoclonal antibody that recognizes phosphotyrosine (Fig. 1A, upper panel). Infected cells had noticeably higher levels of tyrosine phosphorylated CD46 than uninfected cells (0 time point). The level of tyrosine phosphoryated CD46 peaked within 5 min after infection and was undetectable by 30 min post-infection. Total levels of CD46 in the precipitate were determined by reprobing the same blot with polyclonal anti-CD46 antibodies recognizing all isoforms of the protein (Kallstrom et al., 2001; Wang et al., 2000). These controls show that the same amount of CD46 had been immunoprecipitated from each sample (Fig. 1A, lower panel). Thus, the increase in tyrosine phosphoryated CD46 occurred specifically in response to bacterial infection. Tyrosine

phosphorylated CD46 was not detectable in infected cultures treated with Src kinase inhibitor PP2 (data not shown). These and all other experiments have been repeated at least twice, with identical results. Finally, the same results were obtained with GC-infected T84 epithelial cells (data not shown). Thus, GC infection induces CD46 tyrosine phosphorylation in more than one cell line.

The migration of immunoprecipitated CD46 as two major bands in SDS-PAGE (lower panel), reflects the polymorphic nature of the receptor. It is not clear how many CD46 isoforms are in these bands, or how the proteins in these bands differ with regard to their SCR repeats, STP regions and cytoplasmic tails. At the moment, there is no reagent that can distinguish between isoforms containing Cyt1 and Cyt2 tails.

Src kinase phosphorylates CD46 at Y354 of the Cyt2 isoform *in vitro*

The CD46 Cyt1 and Cyt2 COOH-termini contain tyrosine residues (Y348 in Cyt1 and Y354 in Cyt2) that are potential substrates for tyrosine kinases (Wang et al., 2000) (Fig. 1B). We determined whether Src kinase is able to phosphorylate these tails *in vitro*. Wild type and mutant Cyt1 and Cyt2 tails (Fig. 1B) were fused to GST (Glutathione S-transferase) and purified from

E. coli. Fusion proteins were incubated with Src and [γ-^{32}P] ATP, and analyzed by SDS-PAGE and autoradiography. As expected, Src phosphorylated itself and enolase, a commonly used substrate for Src family kinases. Src also robustly phosphorylated wt GST-Cyt2 (Fig. 1C). In contrast, it phosphorylated the GST-Cyt2Y354A mutant and the wt and mutant GST-Cyt1 tails at very low levels.

The low level Src activity on the latter three GST-tail fusions is unlikely to be due to contaminants copurifying with the fusions, as these constructs were purified from *E. coli*, which does not make tyrosine kinase. The background activity is most likely to be due to low level phosphorylation of tyrosine residue 336 at the N-terminal end of the tail fusions (Fig. 1B). This residue is in the transmembrane domain of CD46 (Liszewski et al. 1991) and would therefore be inaccessible to kinases *in vivo*. However, because our purified fusion proteins are free of membrane, Src may be able to phosphorylate Y336 at low levels, even though the flanking sequences are not consensus tyrosine kinase sites. The difference in phosphorylation levels between CD46 wtCyt1 and CD46 Y348ACyt1 is most likely due to background phosphorylation on the tyrosine at 348. However, this difference is slight compared to that seen between the wt Cyt2 tails. When we compare

phosphorylation levels of CD46 Cyt2wt and Cyt1wt, it is clear that the tyrosine residue at 348 is not phosphorylated above background.

Taken together, results from this experiment indicate that the Cyt2 tail of CD46 is a substrate for Src kinase *in vitro,* and phosphorylation occurs at Y354. These data lend additional support to our previous findings of the presence of tyrosine phosphorylated CD46 in GC infected cells.

Src family tyrosine kinases play a role in bacterial adherence

We next examined the importance of Src family tyrosine kinases in GC adherence. A431 cultures were infected with MS11 N400 in the presence of PP2, an inhibitor specific for Src-family kinases, or PP3, an inactive variant of this drug, and bacterial adhesion to these cells were determined. PP2 inhibits Src kinase activity by competing with ATP for binding at the catalytic domain of the target enzyme. PP3, a chemical variant of PP2, does not inhibit Src kinases. Bacterial adhesion to PP2-treated cultures was reduced by 47.5 % compared to adhesion to untreated cultures (Adhesion index for PP2-treated cultures: 16.3% +/- 5.0 % S.D. Adhesion index for untreated cultures: 31% +/- 5.1% S.D.). In contrast, adhesion to PP3-cultures

treated was unaffected (31.5% +/- 6.7% S.D. adhesion index). These results strongly suggest that GC adhesion to epithelial cells requires the participation of Src-family tyrosine kinases. They are consistent with a recent report demonstrating that *N. meningitidis* infection of endothelial cells is reduced in the presence of Src kinase inhibitors (Hoffmann et al., 2001). It is likely that the activation of other kinases by Neisserial adhesins contribute to the overall infection process (Hauck et al., 1998; Chen et al., 1997; Hoffman et al., 2001). Other GC adhesins trigger signaling cascades and cross-talk is likely to occur, making the complete abrogation of adhesion by a Src-family specific inhibitor unlikely. However, the significant reduction in adhesion by PP2 indicates that Src-family kinases play an important role in infection. Indeed, these findings are consistent with our hypothesis that signaling through CD46 activation contributes signaling cascades that are important for Neisseria pathogenesis.

c-Yes aggregates beneath adherent GC microcolonies

Piliated Neisseria triggers the formation of cortical plaques, structures beneath sites of bacterial attachment that are enriched in membrane proteins, signaling proteins, and cytoskeletal components (Merz et al., 1999; Merz and So, 1997). One constituent of these plaques is ezrin,

whose function is to tether proteins, directly or indirectly, to the actin cytoskeleton (Bretscher et al., 1997). The Src kinase p62c-Yes associates with ezrin in MDCK cells (Crepaldi et al., 1997) and is enriched in many epithelial cell types (Mohler et al., 1999; Zhao et al., 1990). Furthermore, c-Yes and Src have overlapping substrate specificities. Therefore, c-Yes is a candidate kinase for CD46 activation.

We used a number of approaches to investigate the participation of c-Yes in CD46 phosphorylation. First, the location of c-Yes in infected and uninfected cells was compared by immunofluorescence deconvolution microscopy. c-Yes visibly accumulated beneath GC microcolonies in infected cells (Fig. 2). In contrast, the kinase was diffusely distributed throughout the cytosol of uninfected cells. c-Yes was also observed to cluster beneath GC in infected T84 cells (data not shown).

c-Yes is activated and associates with CD46 following infection

Using a co-immunoprecipitation approach, we next examined the ability of c-Yes to associate with the CD46 tail. CD46 was immunoprecipitated from GC-infected A431 cells and immunoblotted with c-Yes antisera to determine

the presence of the kinase in the precipitate (Fig. 3A). A large amount of c-Yes co-immunoprecipitated with CD46 within 5 minutes of infection. The amount of co-immunoprecipitated c-Yes decreased with longer periods of infection. Very little of the kinase was found in association with CD46 in uninfected cells (0 min). Control immunoblots of c-Yes in total cell lysates showed that equal amounts of sample had been loaded into the gel. Densitometric analyses of the c-Yes signal from the 0 and 5 min time points indicate that the amount of c-Yes associated with CD46 tail increased four-fold upon infection. EGFR, a membrane protein present in the cortical plaques, did not co-immunoprecipitate with CD46 from infected or uninfected cells (data not shown). These results strongly suggest that GC infection of epithelial cells results in a rapid but brief physical association of c-Yes with CD46.

We also determined whether GC infection stimulates c-Yes kinase activity. c-Yes was immunoprecipitated from GC-infected A431 cells and the precipitate was incubated with enolase and [γ-^{32}P] ATP. Phosphorylation of enolase was detected by SDS-PAGE and autoradiography (Fig. 3B). As expected, c-Yes auto-phosphorylated. c-Yes from infected cells phosphorylated enolase to a greater degree than c-Yes from uninfected cells

(0 time point). Cells infected for 5 min had the highest c-Yes activity. A parallel control experiment in which total cell lysates were immunoblotted for c-Yes demonstrated that equal amounts of the kinase were present in each sample. These results show that c-Yes is rapidly but briefly activated upon GC infection.

c-Yes isolated from epithelial cells phosphorylates CD46 Cyt2 at Tyr354

Finally, we tested the ability of c-Yes to phosphorylate the CD46 tail. c-Yes was immunoprecipitated from uninfected A431 cells and incubated with CD46 GST-tail fusion proteins (GST-Cyt1, GST-Cyt2, GST-Cyt1-Y348A and GST-Cyt2 Y354A) in the presence of [γ-^{32}P] ATP. The majority of c-Yes in uninfected cells was expected to be in the inactive form. Therefore, the immunoprecipitates were incubated with phosphoSrc peptide 521-533 (Calbiochem) prior to the kinase assay. This peptide binds to the SH2 domain of c-Yes and relieves inhibitory intra- or intermolecular interactions with a phosphotyrosine in the COOH- terminus of the enzyme. Activated c-Yes robustly phosphorylated GST-Cyt2 (Fig. 3, lane 2). As expected, cYes phosphorylated itself (upper 62 kDa band) and a protein migrating immediately below all the GST-tail fusions. The identity of this lower band in unknown but is likely to be a protein that co-precipitated with cYes, as it

is absent from samples lacking c-Yes (data not shown). That c-Yes phosphorylated GST-Cyt2 but not GST-Cyt2Y354A demonstrates the specificity of the kinase for the tyrosine residue at position 354 of the Cyt2 tail. That c-Yes did not phosphorylate the Cyt1 tails demonstrates its specificity for Cyt2.

In this report, we presented evidence that GC infection results in rapid tyrosine phosphorylation of CD46 (Fig. 1A). Exogenous Src robustly phosphorylates wt GST-Cyt2 fusion protein, in contrast to its low level activity on the mutant GST-Cyt2Y354A fusion, the wt GST-Cyt1 fusion and the mutant GST-Cyt1Y348A fusion (Fig. 1C). PP2 inhibitor studies are consistent with these findings, and indicate the importance of Src family kinases in promoting GC adherence to epithelial cells. Many bacterial pathogens trigger cellular phosphorylation events through Src-family tyrosine kinases (Isberg and Leong, 1990; Van Putten et al., 1994). Inhibiting Src kinases reduces the infectivity of *Pseudomonas aeruginosa* for epithelial cells (Esen et al., 2001) and *N. meningitidis* for endothelial cells (Hoffmann et al., 2001). Our inhibitor studies are also consistent with these findings.

Several lines of evidence strongly suggest that c-Yes participates in the phosphorylation of the CD46 tail. c-Yes is abundant in the apical regions of human epithelial cells, the site of Neisseria infection. c-Yes is recruited to the cell cortex beneath adherent GC (Fig. 2). Infection rapidly activates c-Yes and increases its association with CD46 (Fig. 3). The timing of CD46 phosphorylation upon GC infection is exactly coincident with the timing of c-Yes activation and binding to CD46. Finally, c-Yes isolated from epithelial cells is able to phosphorylate the wt GST-Cyt2 tail fusion, but has little activity on the mutant GST-Cyt2Y354A tail or the GST-Cyt1 tails (Fig. 3).

The Cyt2 tail is found in association with many CD46 isoforms. As there is no reagent that will distinguish the two tail isoforms from each other, we are unable to determine which CD46 ectodomain and STP region(s) are associated with the phosphorylated Cyt2 tail. The cytoplasmic tails of many signaling proteins are often associated with complexes of kinases, phosphatases and anchoring proteins; in some cases, receptor activation requires the serial activation of these enzymes. Indeed, the CD46 tail has been shown to interact with several unidentified kinases from a macrophage cell line (Wong et al., 1997). Moreover, recent studies demonstrate a

functional interaction between CD46 and Dlg4- an important scaffolding protein containing protein-protein interaction domains as well as an SH3 (Src-homology 3) domain (Ludford-Menting et al., 2001). It is unclear whether c-Yes directly phosphorylates Cyt2 in GC infected cells. Nevertheless, our results indicate that c-Yes has the capability of phosphorylating the Cyt2 tail, either directly or through an intermediary. Further experiments will clarify this issue.

CD46 is a substrate for Src kinase in Jurkat cells (Wang et al., 2000). Upon antibody ligation of CD46, Cyt2 is rapidly phosphorylated by Lck, a Src kinase family member enriched in immune cells. As in GC infected epithelial cells, CD46 phosphorylation occurs rapidly in Jurkat cells, reaching a peak within minutes after ligation and declining thereafter. The rise and fall of phosphorylated protein levels is a common theme in signal transduction, where cycles of phosphorylation are rapidly followed by cycles of dephosphorylation.

How does CD46 phosphorylation promote GC infectivity? One hypothesis is that CD46 activation triggers the formation of cortical plaques. These plaques, which promote Neisseria adhesion, contain high concentrations of

cytoskeletal components, tyrosine-phosphorylated proteins, signaling molecules and Opa receptors (Merz et al., 1999; Merz and So, 1997; Hoffmann et al., 2001). Both CD46 phosphorylation and cortical plaque formation are triggered by infection with piliated organisms. It is reasonable then, to speculate that the rapid association of activated c-Yes with the CD46 tail upon infection may serve to localize signaling machinery to the sites of bacterial attachment, and that these reactions in turn trigger plaque formation. A similar process occurs in the formation of focal adhesion complexes, where attachment to substrates or other cells triggers signal transduction cascades that result the clustering of cytoskeletal components, tyrosine-phosphorylated proteins and signaling molecules to the cell cortex at sites of adhesion. Thus, bacteria may engage signaling pathways that are normally used for cell-cell or cell-substrate adhesion.

Neisseria pili also trigger the release Ca^{2+} from intracellular stores and lysosome exocytosis (Ayala et al., 1998; Kallstrom et al., 1998). Taken together, the activities of the Neisseria type IV pilus argue against the traditional model for pilus function in which the structure serves to bind bacteria passively to host cells. Rather, they support a model in which pilus

interaction with CD46 promotes signaling cascades important for Neisseria infectivity.

Materials and Methods

Cell lines, bacterial strains and infections

A431 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) at 37°C (Life Technologies) and 5% CO_2. *N. gonorrhoeae* strain MS11 N400 (Wolfgang et al., 1998) was used for all infection experiments. Piliation and Opa phenotypes were monitored by colony morphology. For infection experiments, bacteria were resuspended and diluted into unsupplemented DMEM, then added to epithelial cells at a ratio of 500 colony forming units (CFU) per cell or as specified. *Eschericia coli* were grown on Luria-Bertani (LB) agar.

Construction and purification of GST-CD46 tail fusion proteins

Sequences of the Cyt1 and Cyt2 isoforms of the CD46 tails have been published (Lublin et al., 1988; Wong et al., 1997). CD46 Cyt1 and Cyt2 isoforms encoding amino acids 335-362 of the Cyt1 tail or 335-369 of the Cyt2 tail were amplified by PCR using CD46 BC-Cyt1 or CD46 BC-Cyt2 cDNA as template and the following primers- Cyt2 wt: upstream, 5'-AGGATCCCGTACAGATATCTTCAAAGG-3', downstream

5'-TCTCGAGTCAGCCTCTCTGCTCTGCTGGAGT-3'; Cyt2 Y354A: downstream

5'- GCCTCTCTGCTCTGCTGGAGTGGTTGATTTAGTCTGGT AAGTGGCAGCTTTCAGCTCC; Cyt1 wt: upstream-5'-TAGCAATTTGGAGCGGTAAGCCCCCAATAT-3', downstream, 5'-TCTCGAGTCAGAGAGAAGTAAAATTTTACTTC-3' Cyt1 Y348A: TCTCGAGTCAGAGAGAAGTAAATTTTACTTCTCTGTGGGTCTCAT CAGTTAGGGCTGTGCCTTTC-3'. Primers were used to create a BamH1 site proximal to the tail and an Xho1 site immediately after the stop codon. Y to A point mutations in the cytoplasmic tails were introduced by primer-directed PCR mutagenesis as described (Hirano et al., 1996; Yant et al., 1997). PCR products were cloned into the pGEX-5x-2 vector (Amersham Pharmacia) to generate the GST-CD46 tail constructs encoding amino acids 335-362 of the Cyt1 tail or 335-369 of the Cyt2 tail fused at the beginning of the juxtamembrane region to GST. The sequences of all the fusion proteins were confirmed by DNA sequencing.

The BL-21 strain of *E. coli* harboring the various GST-CD46 DNA recombinant plasmids were induced with 100mM IPTG at 30°C for 2h. The GST fusion proteins were affinity-purified with Glutathione-Sepharose as directed by the manufacturer (Amersham Pharmacia). The purified proteins

were quantitated using a Bicinchoninic (BCA) assay (PIERCE Biochemicals) and visualized by Coomasie Blue staining of the gel bands.

Adhesion assay

Confluent A431 cells on six well plates were serum starved for 18h before infection to lower basal levels of tyrosine phosphorylation. Cells were treated for 2h with Src-family kinase inhibitor PP2 (Calbiochem) or the inactive analogue PP3 (Calbiochem) at a concentration of 5μM prior to infection. Adhesion was measured as described previously (Waldbeser et al., 1994; Shaw and Falkow, 1988). Cells were inoculated with MS11 N400 at an MOI of 50 for 2h. After washing 5X in PBS to remove nonadherent bacteria, cells were lysed and collected in GCB +0.5% (w/v) saponin, serially diluted and spread onto GCB plates, grown for 48h and colony forming units were counted. Adhesion index was measured as the number of cell-associated bacteria divided by the total number of bacteria in the well at the end of the infection.

***In vitro* kinase assay**

Purified GST fusion proteins containing the CD46 cytoplasmic tails were incubated in kinase buffer, [100mM Tris-HCl, pH 7.2, 125mM $MgCl_2$,

25mM $MnCl_2$, 2mM EGTA, 0.25 mM sodium orthovavadate, 2mM dithiothreitol, and a protease inhibitor cocktail (2 μg/ml leupeptin, 1.6 μg/ml benzamidine, 0.3 μg/ml PMSF)] (Trotter et al., 1999). 2μCi of [γ-^{32}P] ATP and 1 μl of purified c-Src kinase (Upstate Biotech) were added and the mixture incubated for 30 min at 30°C. All reactions were terminated by the addition of 6X Laemmli buffer containing 0.6% β-mercaptoethanol, and proteins were resolved by electrophoresis in 10% SDS polyacrylamide gels under reducing conditions. Gels were dried at -80°C for 45 minutes and exposed to Kodak X-ray film.

Immunoprecipitation

A431 cells were infected with MS11 N400 for different periods, washed 3X with phosphate-buffered saline (PBS), and incubated for 20 min at 4°C in lysis buffer containing a 3:1 ratio of RIPA[20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholic acid, 5mM EDTA) to TEE (50 mM Tris-HCl, pH 7.4, 1mM EDTA, 1mM EGTA, 150 mM NaCl, 1% Triton X-100) containing 0.25 mM sodium orthophosphate and a protease inhibitor cocktail (2 μg/ml leupeptin, 1.6 μg/ml benzamidine, 0.3 μg/ml PMSF)] (Mohler et al., 1999). Nuclei and insoluble materials were removed by centrifugation at 13,000g for 20 min at 4°C, and cell

homogenates were incubated overnight in lysis buffer with either 1μg of mouse monoclonal antibody directed against CD46, clone J4-48 Lot #4 (Immunotech) or c-Yes kinase (BD Transduction Labs). Protein G-agarose beads (Oncogene) were added to the cell preparations for 1h. The beads were then washed carefully 5X with lysis buffer (TEE) and pelleted at 7,000 rpm for 60s. The pellets were then resuspended in 40μl Laemmli buffer containing 0.6% β-Mercaptoethanol and boiled for 5 min at 100°C. Proteins were separated by SDS-10% polyacrylamide gels and transferred onto nitrocellulose sheets. Membranes were probed, stripped and reprobed as necessary (Ausubel et al., 1990). For kinase assays using immunoisolated c-Yes, the immunoprecipitates were resuspended in kinase buffer after the last wash, and kinase assays were performed as described earlier. Western blot analysis was performed as described (Ausubel et al., 1990), using rabbit anti-CD46 (gift from J. Atkinson) at a dilution of 1:3000. Peroxidase-conjugated monoclonal antibody against phosphotyrosine (Ab-4) was used per manufacturer's recommendations (Oncogene Labs). Monoclonal antibody against c-Yes was used at a concentration of 1:3000 for immunoblotting, and 1:500 for immunofluorescence microscopy.

Immunofluorescence microscopy

A431 cells were grown on coverslips to 30-50% confluence and infected for 3h with MS11 N400 (Wolfgang et al., 1998) at an MOI of 100. Coverslips were washed 3X in PBS, fixed for 20 min at room temperature in 4% paraformaldehyde and blocked for 1h in isotonic PBS containing 3%(v/v) normal goat serum (Gibco BRL) and 0.02% saponin (Sigma). Primary antibody was diluted as specified above in blocking buffer, added to samples and incubated overnight at 4°C in a moist chamber. The coverslips were rinsed in PBS and incubated with an Alexa-488 conjugated secondary antibody (Molecular Probes) diluted 1:250 in blocking buffer for 1h at 25°C. The cells were also incubated with DAPI at 1:1000 for 10 min at 25°C to visualize the bacteria and nuclei. Samples were rinsed extensively in PBS before mounting in Fluromount-G (Fisher Scientific). Omission of primary antibodies was used as a negative control. 0.2 μm optical sections in the Z-axis plane were obtained with a Nikon 60X oil immersion objective and the images were processed using a Deltavision Restoration Microscope (Applied Precision Instruments, Inc., Issaquah, WA) and Silicon graphics workstation with accompanying API software. The images were subsequently exported to Adobe Photoshop and Adobe Illustrator for manuscript preparation.

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References

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1990. Current Protocols in Molecular Biology. Publishing Associates and Wiley Interscience, New York.

Ayala, P., L. Lin, S. Hopper, M. Fukuda, and M. So. 1998. Infection of epithelial cells by pathogenic neisseriae reduces the levels of multiple lysosomal constituents. *Infect Immun.* 66:5001-5007.

Bliska, J.B., J.E. Galán, and S. Falkow. 1993. Signal transduction in the mammalian cell during bacterial attachment and entry. *Cell.* 73:903-920.

Bretscher, A., D. Reczek, and M. Berryman. 1997. Ezrin: a protein requiring conformational activation to link microfilaments to the plasma membrane in the assembly of cell surface structures. *J Cell Sci.* 110:3011-3018.

Brossay, L., G. Paradis, R. Fox, M. Koomey, and J. Hebert. 1994. Identification, localization, and distribution of the PilT protein in Neisseria gonorrhoeae. *Infect Immun.* 62:2302-2308.

Chen, T., Grunert, F., Medina-Marino, A., E.C. Gotschlich. 1997. Several carcinoembryonic antigens (CD66) serve as receptors for gonococcal opacity proteins. *J Exp Med.* 185:1557-1564.

Crepaldi, T., A. Gautreau, P.M. Comoglio, D. Louvard, and M. Arpin. 1997. Ezrin is an effector of hepatocyte growth factor-mediated migration and morphogenesis in epithelial cells. *Journal of Cell Biology.* 138:423-434.

Dehio, C., M.C. Prevost, and P.J. Sansonetti. 1995. Invasion of epithelial cells by Shigella flexneri induces tyrosine phosphorylation of cortactin by a pp60c-src-mediated signalling pathway. *Embo J.* 14:2471-2482.

Dorig, R.E., A. Marcil, A. Chopra, and C.D. Richardson. 1993. The human CD46 molecule is a receptor for measles virus (Edmonston strain). *Cell.* 75:295-305.

Esen, M., H. Grassmé, J. Riethmüller, A. Riehle, K. Fassbender, and E. Gulbins. 2001. Invasion of human epithelial cells by Pseudomonas aeruginosa involves src-like tyrosine kinases p60Src and p59Fyn. *Infect Immun.* 69:281-287.

Finlay, B.B., and P. Cossart. 1997. Exploitation of mammalian host cell functions by bacterial pathogens. *Science.* 276:718-725.

Galán, J.E. 2000. Alternative strategies for becoming an insider: lessons from the bacterial world. *Cell*. 103:363-366.

Galán, J.E., J. Pace, and M.J. Hayman. 1992. Involvement of the epidermal growth factor receptor in the invasion of cultured mammalian cells by Salmonella typhimurium. *Nature*. 357:588-589.

Hanke, J.H., J.P. Gardner, R.L. Dow, P.S. Changelian, W.H. Brisette, E.J. Weringer, B.A. Pollok, and P.A. Connelly. 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and Fyn-T dependent T cell activation. J Biol Chem. 271:695-701.

Haucke, C.R., T.F. Meyer, F. Lang, and E. Gulbins. 1998. CD66-mediated phagocytosis of Opà52 Neisseria gonorrhoeae requires a Src-like tyrosine kinase- and Rac1-dependent signalling pathway. *EMBO J.* 17:443-454.

Hirano, A., S. Yant, K. Iwata, J. Korte-Sarfaty, T. Seya, S. Nagasawa, and T.C. Wong. 1996. Human cell receptor CD46 is down regulated through recognition of a membrane-proximal region of the cytoplasmic domain in persistent measles virus infection. *J Virol.* 70:6929-6936.

Hoffmann, I., E. Eugene, X. Nassif, P.O. Courad, and S. Bourdolous S. 2001. Activation of ErbB2 receptor tyrosine kinase supports invasion of endothelial cells by *Neisseria meningitidis. Journal of Cell Biology*.155:133-143.

Isberg, R.R., and J.M. Leong. 1990. Multiple beta 1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. *Cell*. 60:861-871.

Källström, H., D. Blackmer-Gill, B. Albiger, M.L. Liszewski, J.P. Atkinson, and J. A-B. 2001. Attachment of Neisseria gonorrhoeae to the cellular pilus receptor CD46: identification of domains important for bacterial adherence. *Cellular Microbiology*. 3:133-143.

Källström, H., M.S. Islam, P.O. Berggren, and A.B. Jonsson. 1998. Cell signaling by the type IV pili of pathogenic Neisseria. *J Biol Chem*. 273:21777-21782.

Källström, H., M.K. Liszewski, J.P. Atkinson, and A.B. Jonsson. 1997. Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic Neisseria. *Mol Microbiol*. 25:639-647.

Liszewski, M.K., T.W. Post, and J.P. Atkinson. 1991. Membrane cofactor protein (MCP or CD46): Newest member of the regulators of

complement activation gene cluster. *Annual Reviews in Immunology*. 9:431-455.

Liu, Y., A. Bishop, L. Witucki, B. Kraybill., E. Shimizu, J. Tsien, J. Ubersax, J. Blethrow, D.O. Morgan, and K.M. Shokat. 1999. Structural basis for selective inhibition of Src family kinases by PP1. Chem Biol. 6:671-678.

Lublin, D.M., M.K. Liszewski, T.W. Post, M.A. Arce, M.M. Le Beau, M.B. Rebentisch, L.S. Lemons, T. Seya, and J.P. Atkinson. 1988. Molecular cloning and chromosomal localization of human membrane cofactor protein (MCP). Evidence for inclusion in the multigene family of complement-regulatory proteins. *J Exp Med.* 168:181-194.

Ludford-Menting, M.J., Thomas, S.J., Crimeen, B., Harris, L.J., Loveland, B.E., Bills, M., Ellis, S., and S. M. Russel. 2001. A functional interaction between CD46 and Dlg4: a role for Dlg4 in epithelial polarization. *J Biol Chem.* [epub M108479200].

Merz, A.J., C.A. Enns, and M. So. 1999. Type IV pili of pathogenic Neisseriae elicit cortical plaque formation in epithelial cells. *Mol Microbiol.* 32:1316-1332.

Merz, A.J., and M. So. 1997. Attachment of piliated, Opa- and Opc- gonococci and meningococci to epithelial cells elicits cortical actin

rearrangements and clustering of tyrosine-phosphorylated proteins. *Infect Immun.* 65:4341-4349.

Merz, A.J., and M. So. 2000. Interactions of pathogenic neisseriae with epithelial cell membranes. *Annu Rev Cell Dev Biol.* 16:423-457.

Merz, A.J., M. So, and M.P. Sheetz. 2000. Pilus retraction powers bacterial twitching motility. *Nature.* 407:98-102.

Mohler, P.J., S.M. Kreda, R.C. Boucher, M. Sudol, M.J. Stutts, and S.L. Milgram. 1999. Yes-associated protein 65 localizes p62c-Yes to the apical compartment of airway epithelia by association with EBP50. *Journal of Cell Biology.* 147:879-890.

Nassif, X., J. Lowy, P. Stenberg, P. O'Gaora, A. Ganji, and M. So. 1993. Antigenic variation of pilin regulates adhesion of Neisseria meningitidis to human epithelial cells. *Mol Microbiol.* 8:719-725.

Okada, N., M.K. Liszweski, J.P. Atkinson, and M. Caparon. 1995. Membrane cofactor protein (CD46) is a keratinocyte receptor for the M protein of the group A streptococcus. *Proceedings of the National Academy of Science USA.* 92:2489-2493.

Pujol, C., E. Eugene, P. Morand, and X. Nassif. 2000. Do pathogenic neisseriae need several ways to modify the host cell cytoskeleton? *Microbes Infect.* 2:821-827.

Rosenshine, I., S. Ruschkowski, V. Foubister, and B.B. Finlay. 1994. Salmonella typhimurium invasion of epithelial cells: role of induced host cell tyrosine protein phosphorylation. *Infect Immun.* 62:4969-4974.

Santoro, F., P.E. Kennedy, G. Locatelli, M.S. Malnati, E.A. Berger, and P. Lusso. 1999. CD46 is a cellular receptor for human herpesvirus 6. *Cell.* 99:817-827.

Seya, T. 1995. Human regulator of complement activation (RCA) gene family proteins and their relationship to microbial infection. *Microbiology and Immunology.* 39:295-305.

Shaw, J.H., and S. Falkow. 1988. Model for invasion of human tissue culture cells by Neisseria gonnorhoeae. *Infect Immun.* 56:1625-1632.

Singer, S.J. 1992. Intercellular communication and cell-cell adhesion. *Science.* 13:1671-1677.

Swanson, J. 1973. Studies on gonococcus infection. IV. Pili: their role in attachment of gonococci to tissue culture cells. *J Exp Med.* 137:571-589.

Tobiason, D.M., and H.S. Seifert. 2001. Inverse relationship between pilus-mediated gonococcal adherence and surface expression of the pilus receptor, CD46. *Microbiology.* 147:2333-2340.

Trotter, K.W., I.D. Fraser, G.K. Scott, M.J. Stutts, J.D. Scott, and S.L. Milgram. 1999. Alternative splicing regulates the subcellular localization of A-kinase anchoring protein 18 isoforms. *J Cell Biol.* 147:1481-1492.

Van Langendonck, N., P. Velge, and E. Bottreau. 1998. Host cell protein tyrosine kinases are activated during the entry of Listeria monocytogenes. Possible role of pp60c-src family protein kinases. *FEMS Microbiol Lett.* 162:169-176.

Van Putten, J.P., J.F. Weel, and H.U. Grassme. 1994. Measurements of invasion by antibody labeling and electron microscopy. *Methods Enzymol.* 236:420-437.

Waldbeser, L.S., R.S. Ajioka, A.J. Merz, D. Puaoi, L. Lin, M. Thomas, and M. So. 1994. The *opaH* locus of *Neisseria gonnorhoeae* MS11A is involved in epithelial cell invasion. *Mol Microbiol.* 13: 919-928.

Wall, D., and D. Kaiser. 1999. Type IV pili and cell motility. *Mol Microbiol.* 32:1-10.

Wang, G., M.K. Liszweski, A.C. Chan, and J.P. Atkinson. 2000. Membrane cofactor protein(MCP; CD46): Isoform-specific Tyrosine phosphorylation. *Journal of Immunology.* 164:1839-1846.

Wolfgang, M., P. Lauer, H.S. Park, L. Brossay, J. Hebert, and M. Koomey. 1998. PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated Neisseria gonorrhoeae. *Mol Microbiol.* 29:321-330.

Wong, T.C., S. Yant, B.J. Harder, J. Korte-Sarfaty, and A. Hirano. 1997. The cytoplasmic domains of complement regulatory protein CD46 interact with multiple kinases in macrophages. *J Leukoc Biol.* 62:892-900.

Yamada, K.M., and B. Geiger. 1997. Molecular interactions in cell adhesion complexes. *Curr Opin Cell Biol.* 9:76-85.

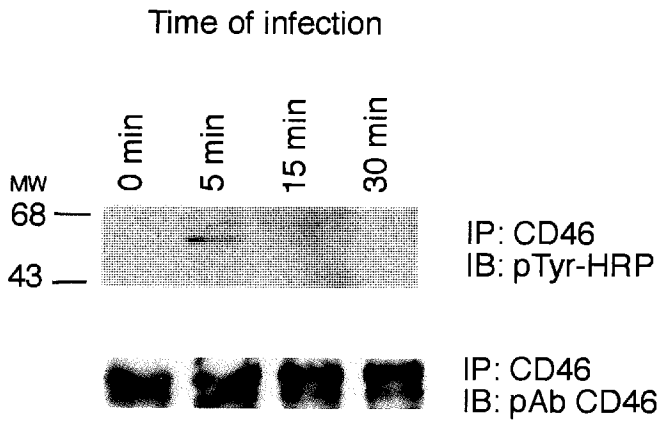
Yant, S., A. Hirano, and T.C. Wong. 1997. Identification of a cytoplasmic Tyr-X-X-Leu motif essential for down regulation of the human cell receptor CD46 in persistent measles virus infection. *J Virol.* 71:766-770.

Zhao, Y.H., J.G. Krueger, and M. Sudol. 1990. Expression of cellular-yes protein in mammalian tissues. *Oncogene.* 5:1629-1635.

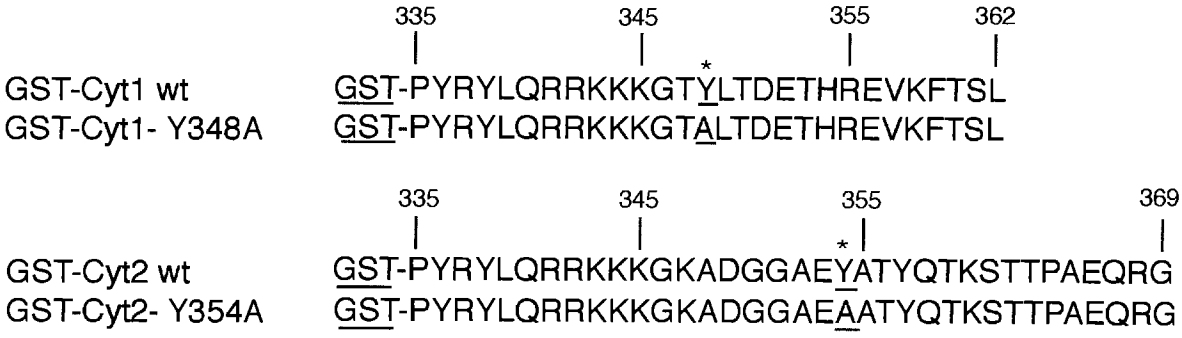
Figure 1. Identification of the site of CD46 tyrosine phosphorylation. (A) Presence of phosphotyrosine in CD46 immunoprecipitated from GC-infected cells. CD46 was immunoprecipated from A431 cells infected with MS11 N400 for various lengths of time, using an anti-CD46 monoclonal antibody (Immunotech). The presence of phosphorylated tyrosine residues in the precipitate (upper panel) was determined by immunoblotting with horseradish peroxidase-labeled monoclonal antibodies to phospho-tyrosine (pTyr-HRP). Total levels of CD46 in the precipitates (lower panel) were determined by reprobing the same blot with polyclonal antibodies (pAb) to CD46 (gift from J. Atkinson). (B) Sequences of the CD46 tails fused to GST. The Cyt1 and Cyt2 isoforms of the CD46 tail were fused to GST at Pro-335. In the GST-Cyt1 Y348A fusion protein, tyrosine at position 348 was changed to alanine. In the GST-Cyt2 Y354A fusion, tyrosine at position 354 was changed to alanine. (C) Phosphorylation of GST-Cyt2 by c-Src. GST-fusion proteins were incubated with exogenous c-Src (Upstate Biotech) and [γ-^{32}P] ATP in an *in vitro* kinase assay with enolase (Sigma) as the control substrate for Src activity.

A.

FIGURE 1


Time of infection
0 min
5 min
15 min
30 min
MW
68
43
IP: CD46
IB: pTyr-HRP
IP: CD46
IB: pAb CD46

B.


335
345
355
362
GST-Cyt1 wt
GST-PYRYLQRRKKKGTYLTDETHREVKFTSL
GST-Cyt1- Y348A
GST-PYRYLQRRKKKGTALTDETHREVKFTSL
335
345
355
369
GST-Cyt2 wt
GST-PYRYLQRRKKKGKADGGAEYATYQTKSTTPAEQRG
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C.

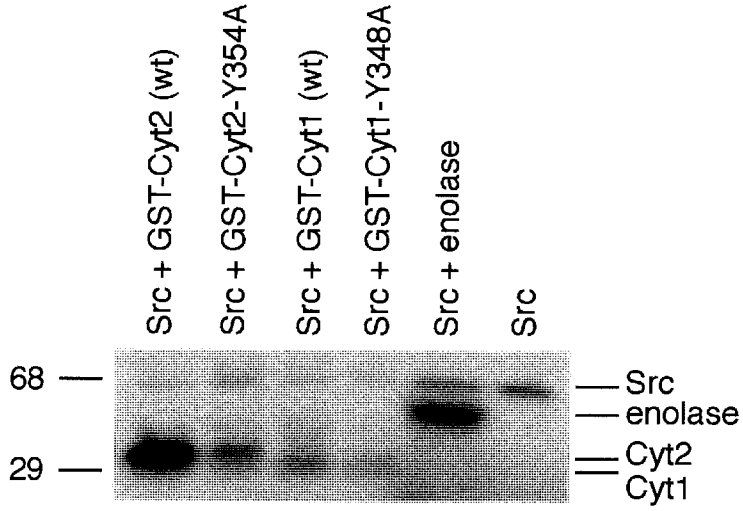

Src + GST-Cyt2 (wt)
Src + GST-Cyt2-Y354A
Src + GST-Cyt1 (wt)
Src + GST-Cyt1-Y348A
Src + enolase
Src
68
29
Src
enolase
Cyt2
Cyt1

Figure 2. c-Yes clustering beneath GC microcolonies in infected epithelial cells. A431 endocervical epithelial cells were infected with MS11 N400 for 3h (upper panels) or left uninfected (lower panels), then fixed and processed for immunofluorescence deconvolution microscopy using anti-c-Yes antibodies to visualize the kinase (center panels), and DAPI to visualize nuclei and GC microcolonies (leftmost panels). The clustering of c-Yes beneath the microcolonies can be seen in the composite image (rightmost panels). Arrows denote the location of some microcolonies. Scale bars are shown in the leftmost panels. Images are 0.2 μm thick optical sections of the apical surfaces of cells obtained using a Deltavision Restoration Microscope (API) with a 60X oil objective.

FIGURE 2

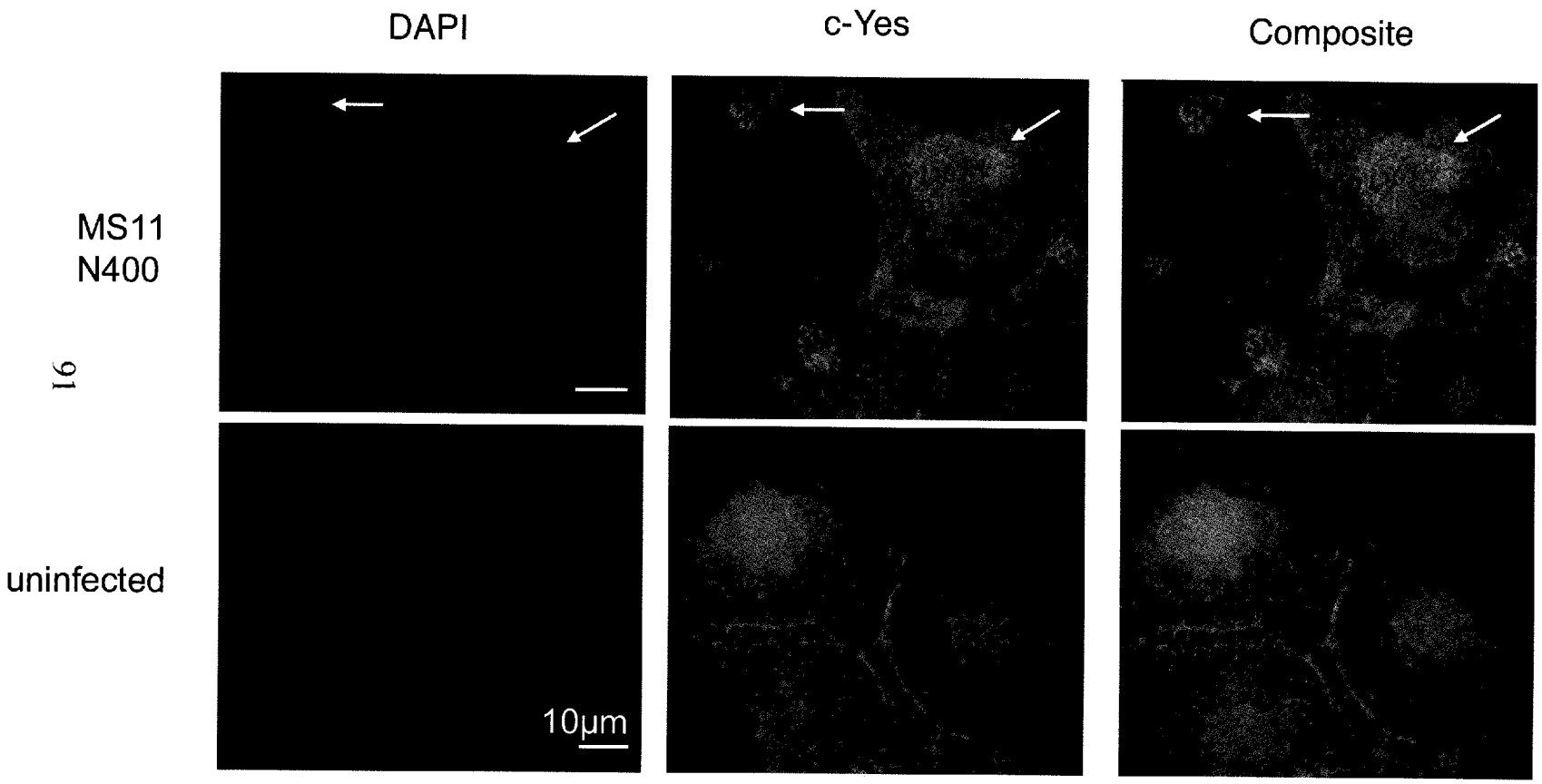
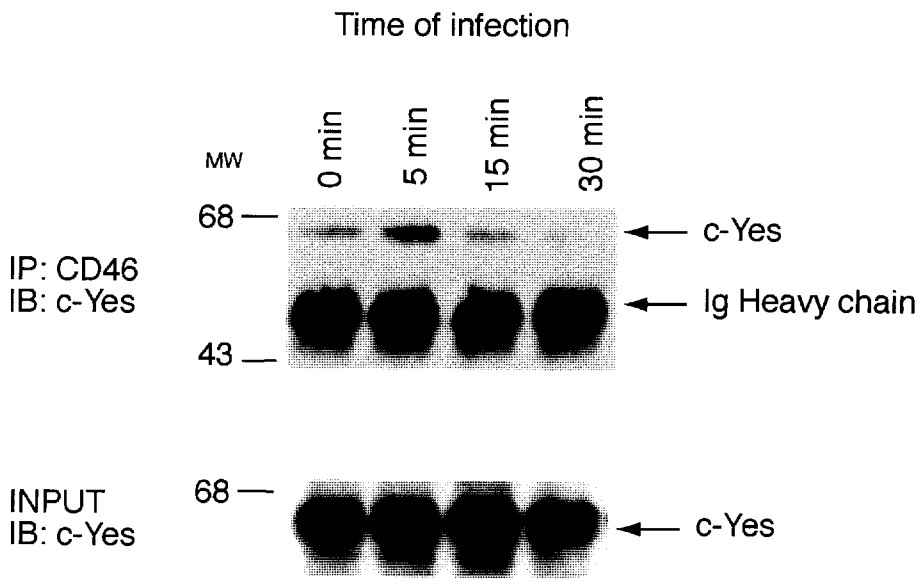


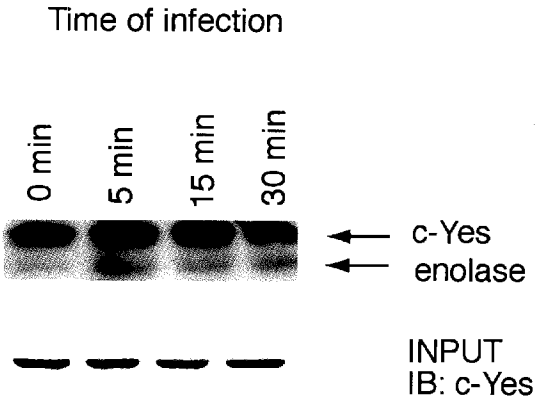
Figure 3. Association of c-Yes tyrosine kinase with CD46 and its activation upon infection. (A) Co-immunoprecipitation of c-Yes with CD46. CD46 was immunoprecipitated from A431 cells infected with MS11 N400. The presence of c-Yes in the precipitates was determined by immunoblotting with monoclonal anti-c-Yes antibodies (upper panel). Inputs (total cell lysates from each time point) were immunoblotted with anti-c-Yes antibodies to confirm uniform levels of the kinase in the samples (lower panel). (B) Activation of c-Yes upon GC infection. c-Yes was immunoprecipitated from MS11 N400-infected A431 cells and used in an *in vitro* kinase assay with enolase as the substrate (upper panel). Inputs (total cell lysates from each time point) were immunoblotted with anti-c-Yes antibodies to confirm uniform levels of the kinase in the samples (lower panel). (C). c-Yes phosphorylation of CD46 Cyt2 Tyr354. c-Yes was immunoprecipitated from uninfected A431 cells and activated with Src peptide 521-533 before incubation with GST-CD46 tail fusion proteins in the presence of [γ-^{32}P] ATP. Lane 1: GST-Cyt2 wt incubated with Src. Lanes 2-5: Other GST-CD46 tail isoforms, as indicated, incubated with activated c-Yes. Lane 6: GST- Cyt2 wt incubated with c-Yes in the absence of activating peptide.

FIGURE 3

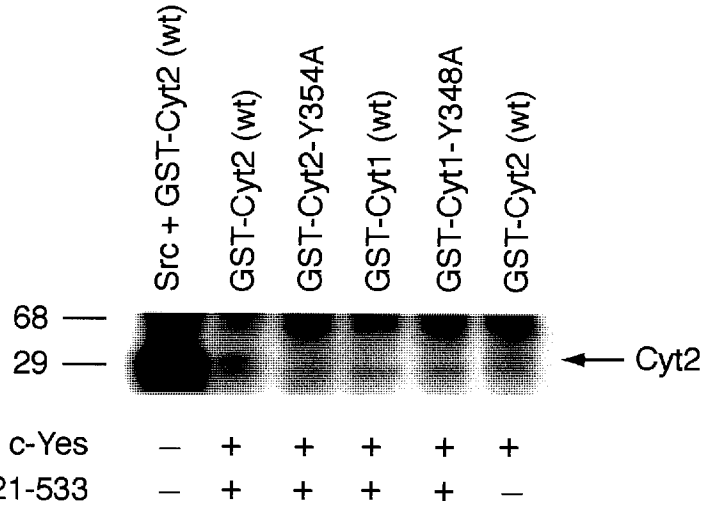
A.


Time of infection
MW
0 min
5 min
15 min
30 min
68
43
IP: CD46
IB: c-Yes
c-Yes
Ig Heavy chain
INPUT
IB: c-Yes
68
c-Yes

B.


Time of infection
0 min
5 min
15 min
30 min
c-Yes
enolase
INPUT
IB: c-Yes

C.


Src + GST-Cyt2 (wt)
GST-Cyt2 (wt)
GST-Cyt2-Y354A
GST-Cyt1 (wt)
GST-Cyt1-Y348A
GST-Cyt2 (wt)
68
29
Cyt2
IP: c-Yes – + + + + +
521-533 – + + + + –

Chapter 3: Manuscript II

Pilus retraction by *Neisseria gonorrhoeae* activates PI-3 kinase and triggers the production of a novel bacterial effector by epithelial cells

Shaun W. Lee, Dustin L. Higashi, George S. Niemann, Laura Potter,

Aurelie Snyder, & Magdalene So

Department of Molecular Microbiology & Immunology

Oregon Health and Science University

Portland, Oregon, 97201-3098

To be submitted

Summary

The retractile type IV pilus of *Neisseria gonorrhoeae* (GC) participates in a number of biological processes, including bacterial motility, DNA uptake/transformation and interactions with host cells. The importance of pilus retraction to bacterial virulence is illustrated by defects of the nonretractile *pilT* mutants in invasion and cortical plaque formation. We provide evidence that GC infection of human epithelial cells in culture activates the PI-3 kinase/Akt (PKB) pathway, and that pilus retraction greatly enhances this activation. PI-3 kinase plays a major role in bacterial invasion, as inhibitors wortmannin and LY294002 reduce cell entry by 81% and 50%, respectively. PI-3 kinase and Akt are activated at the site of infection, and high concentrations of these kinases are recruited to the periphery of the bacterial microcolonies. [PI(3,4,5)P3], a product of activated PI-3 kinase, is also clustered at these sites, exposed on the outer leaflet of the plasma membrane. Several lines of evidence strongly suggest that [PI(3,4,5)P3] modulates bacterial motility-associated behavior. In its presence, bacteria aggregate more quickly and the resultant microcolonies are much larger. Motile bacteria incorporate [PI(3,4,5)P3]-coated beads into their microcolonies, and the beads would subsequently move with the

bacterial aggregates. Finally, [PI(3,4,5)P3], but not [PI(4,5)P2], upregulates *pilT*, the pilus retraction locus. Thus, pilus retraction promotes GC infectivity by activating signal cascades via the PI-3 kinase pathway. Activated PI-3 kinase stimulates the infected cell to produce a lipid second messenger, [PI(3,4,5)P3], that, in turn, directly modulates bacterial motility behavior.

Introduction

Type IV pili are produced by a wide variety of bacteria including *Myxococcus xanthus*, *Synechocysitis spp*, *Vibrio cholerae*, Enteropathogenic and Enterohemorrhagic *E. coli, Pseudomonas aeruginosa, Neisseria gonorrhoeae* (GC) *and N. meningitidis* (see review, Strom and Lory, 1993). These structures play important roles in motility-associated behavior such as fruiting body and biofilm formation, phototaxis, phage sensitivity and virulence (Bacallao, 1989; Bhaya et al., 2000; Bhaya et al., 2001a; Bhaya et al., 2001b; Bradley, 1972a; Bradley, 1974; Brossay et al., 1994; Comolli et al., 1999; Maier et al., 2002; Merz et al., 2000a; Nassif et al., 1993; Swanson, 1973; Wu et al., 1997; Yoshida et al., 1999).

The GC type IV pilus binds human complement regulatory protein CD46 on the surface of nucleated human cells (Kallstrom et al., 1997). Pili preparations and piliated bacteria trigger tyrosine phosphorylation of the CD46 cytosolic tail 2 isoform (Lee et al., 2002). They induce the release of free Ca^{2+} from intracellular stores, resulting in a redistribution of Lamp1 compartments and the promotion of bacterial intracellular survival (Ayala et al., 2001; Kallstrom et al., 1997; Lin et al., 1997). These activities of the GC

type IV pilus strongly suggest that the structure modulates host cell signaling cascades that influence infectivity.

Type IV pili are retractile structures (Bradley, 1972a; Bradley, 1972b; Bradley, 1974; Merz et al., 2000; Skerker and Berg, 2001; Sun et al., 2000). Retraction of a single GC pilus often generates forces greater than 80 pN (Maier et al., 2002; Merz et al., 2000).Retraction requires PilT, a member of a large family of putative NTPases from type II and type IV secretion systems (Herdendorf et al., 2002; Wolfgang et al., 1998a; Wolfgang et al., 1998b). GC *pilT* null mutants are piliated but cannot retract pili (Merz et al., 2000b). They are nonmotile and cannot be transformed with DNA (Wolfgang et al., 1998a). They aggregate into microcolonies, though these structures have an aberrant appearance. They adhere normally to epithelial cells, but are unable to trigger the formation of cortical plaques- (Merz et al., 1999) structures in the host cell cortex beneath adherent microcolonies that contain high concentrations of cytoskeletal components, receptors and other signaling proteins. Finally, *pilT* mutants invade cells at a lower frequency than the wt parent strain. Thus, the retractile process of the GC type IV pilus plays a central role in Neisserial physiology, genetics and virulence.

How pilus retraction might influence infectivity is unknown. Mechanical forces less than 80 pN exerted on the plasma membrane of mammalian cells trigger Ca^{2+} fluxes and induce signaling cascades, promoting, among other events, cytoskeleton reorganization during cell-cell and cell-substrate adhesion and cellular adhesion plaque formation (Berridge et al., 2003; Raucher and Sheetz, 1999; Raucher and Sheetz, 2000; Raucher et al., 2000; Shao et al., 1998; Sheetz et al., 1998; Wang et al., 1993). Pilus retraction during bacterial attachment may, therefore, generate sufficient force on the membrane of the epithelial cell to trigger cellular signals that influence the course of infection.

One signal cascade that is activated through membrane tension is the phosphoinositide-3 (PI-3) kinase pathway (Miao et al., 2002; Raucher et al., 2000). PI-3 kinases orchestrate many cellular events, including cell growth, motility, differentiation, survival and intracellular trafficking (Cantley, 2002; Foster et al., 2003). Upon receptor activation, PI-3 kinases are recruited to the membrane through binding of the src-homology (SH) domain of its regulatory subunit to specific phosphorylated tyrosine residues in the receptor or associated proteins, placing the kinase in close proximity to its phosphoinositide substrates. Upon activation, PI-3 kinases phosphorylate

phosphoinositides at the 3-position of the inositol ring. Effector proteins are then recruited to the activation site through binding of their pleckstrin-homology (PH) domain to the phosphorylated phosphoinositides, and upon activation they initiate various local responses, including actin polymerization, assembly of signaling complexes and priming of kinase cascades. One effector protein activated by PI-3 kinase is Akt, or protein kinase B (PKB). Normally cytoplasmic, Akt is recruited to the membrane by the PI-3 kinase product [PI(3,4,5)P3] (Brazil et al., 2002). The coupling of PI-3 kinases to numerous receptors, the variety of lipid second messengers generated by these kinases, and the wide range of functions of the downstream effectors contribute to the central nature of the PI-3 kinase cascade in regulating cellular functions.

PI-3 kinases have been implicated in bacterial virulence. Efficient entry into host cells by *Listeria monocytogenes*, *Helicobacter pylori* and *Chlamydia pneumoniae* require PI-3 kinase activation (Coombes et al., 2002; Ireton and Cossart, 1997; Kwok et al., 2002). Internalization of Opa-expressing, nonpiliated GC through the CEACAM-3, but not the CEACAM-1, pathway is blocked by PI-3 kinase inhibitors (Booth et al., 2003). Thus, many

pathogens utilize the PI-3 kinase pathway for cell entry, although they are likely to induce this cascade through a variety of means.

In this study, we examined the role played by pilus retraction during GC infection of human epithelial cells in culture. In particular, we tested the hypothesis that pilus retraction activates the PI-3 kinase pathway. We show that infection of epithelial cells by wt GC activates PI-3 kinase while a *pilT* null mutant fails to do so. A *pilT* mutant with an IPTG (isopropyl β-D-thioglactopyranoside)-inducible promoter activates the PI-3 kinase pathway to much greater levels when the inducer is present. The PI-3 kinase plays an important role in GC virulence, as inhibitors wortmannin and LY294002 reduce bacterial invasion by 81% and 50%, respectively. High levels of PI-3 kinase, Akt and [PI(3,4,5)P3] are clustered at the site of infection. Furthermore, [PI(3,4,5)P3] is exposed on the outer leaflet of the membrane. [PI(3,4,5)P3] modulates bacterial behavior *in vitro*, accelerating bacterial aggregation/microcolony formation and upregulating the expression of the pilus retraction locus *pilT*. Our findings demonstrate that pilus retraction during GC infection activates a central host cell signaling cascade, leading to the generation of a lipid second messenger that directly communicates with the pathogen to modulate its motility behavior.

Experimental Procedures

Reagents

Wortmannin, LY294002, soluble [PI(3,4,5)P3] and [PI(4,5)P2] were purchased from Calbiochem. Antibodies to the PI-3 kinase p110 subunit and phospho-Akt were purchased from Cell Signaling Technologies. Ezrin antibody was purchased from BD Transduction Labs. Anti-[PI(3,4,5)P3] antibody was purchased from Echelon Biochemical. The PI-3 kinase detection ELISA kit was purchased from Echelon Biochemical. Alexa-488 and Alexa-572 labeled secondary antibodies were purchased from Molecular Probes.

Cell lines, bacterial strains and infections

A431 cells were maintained in DME with 10% heat-inactivated FBS at 37°C (Life Technologies) and 5% CO_2. GC wt strain MS11 (Segal et al., 1986) and an isogenic GC *pilT* mutant was used for infection experiments. The MS11 *pilT* null mutant was constructed by transforming chromosomal DNA from strain GT103(N400 P+, Opa-, *pilT*::mTnErm) (Wolfgang et al., 1998a) into MS11 and selecting transformants for erythromycin resistance. PilE expression was confirmed by Western blotting and piliation determined by

immunofluorescence microscopy using a rabbit polyclonal antibody raised against total GC proteins. Loss of *pilT* function was determined by loss of competence for DNA transformation. One *pilT* candidate was selected and chromsomal DNA isolated and used to backcross (re-transform) the same stock of MS11, with tests repeated. Transformation was used to transfer the isopropyl β-D-thioglactopyranoside (IPTG)-inducible *pilT* gene in strain MW4 (Wolfgang et al., 1998c) into MS11 to yield the MS11 inducible *pilT* mutant used in this study. Piliation and Opa phenotypes were monitored by colony morphology. For infection experiments, bacteria were resuspended and diluted into unsupplemented DME and then added to epithelial cells at a ratio of 100 colony forming units per cell or as specified.

Adhesion Assay

A431 cells were grown to 80% confluency in 12 well plates 24h prior to infection. Adhesion was measured as described previously (Shaw and Falkow, 1988; Waldbeser et al., 1994). Cells were washed three times with serum-free DMEM prior to infection and incubated for 1h in serum-free medium containing LY294002 (50 μM) , wortmannin (10 nM), or medium alone. Bacteria were then added to the cultures at a multiplicity of infection (MOI) of 50, and the cultures were incubated for 3 hr in the presence of

inhibitor. Supernatants were collected and saved for plating. Infected cultures were washed five times in PBS and the washes also saved and plated (see below). Washed cells were then lysed in liquid GCB medium with saponin (0.5%, wt/vol). Serial dilutions of the lysates were plated on supplemented GCB agar, the plates were grown for 48h at 37°C, 5% CO_2, and colony forming units were counted. Adhesion index was measured as the number of cell-associated bacteria divided by the total number of bacteria in the well at the end of the infection.

Gentamicin Protection Assay

Gentamicin protection assays were performed as described previously (Waldbeser et al., 1994). A431 cells were grown to 80% confluency in 12 well plates 24h prior to infection. Cells were washed three times with serum-free DMEM prior to infection and incubated in serum-free medium with LY294002 or wortmannin as described above. Cells were infected for 6 hr with MS11 at an MOI of 50. Cells were then incubated with Gentamicin (100μg/ml) for 1h at 37°C to kill extracellular and attached bacteria. After washing five times in PBS to remove nonadherent bacteria, the cells were lysed. The lysates were collected in GCB plus saponin (0.5%, wt/vol) and serially diluted and spread onto supplemented GCB plates. The plates were

incubated for 48h at 37°C, 5% CO_2, and colony forming units were determined. Invasion index was measured as the number of Gentamicin resistant bacteria divided by the total number of adhered and invaded bacteria in the well at the end of the infection.

Akt kinase Assay

Akt activity was assayed using an Akt kinase assay kit (Cell Signaling Technology). Akt activity was measured by using a phospho-GSK-3α/β antibody to detect kinase activity of immunoprecipitated Akt after incubation with a GSK fusion peptide. Phosho-Akt antibodies were used to confirm levels of Akt activity. Polyclonal antibodies to Akt were used to confirm total levels of Akt in lysates.

Immunofluorescence microscopy

A431 cells were grown on coverslips to 30-50% confluence and infected for 3h with MS11 at an MOI of 100. Coverslips were washed three times in PBS, fixed for 20min at room temperature in 4% paraformaldehyde, and blocked for 1h in isotonic PBS containing normal goat serum (3%, vol/ vol) and saponin (0.02%, vol/vol). To determine the presence of [PI(3,4,5)P3] on the outer leaflet of the membrane, saponin was omitted from all blocking

and incubation steps. Primary antibody was diluted as specified above in blocking buffer, added to samples, and incubated overnight at 4°C in a moist chamber. The coverslips were rinsed in PBS and incubated with an Alexa conjugated secondary antibody diluted 1:500 in blocking buffer for 1h at 25°C. Samples to visualize outer membrane proteins and phospholipids were all incubated at 4°C for all steps. The cells were also incubated with the DNA stain DAPI at 1:1000 for 10 minutes at 25°C to visualize the bacteria and nuclei. Samples were rinsed extensively in PBS before mounting in Fluoromount-G (Fisher Scientific). Negative control samples were processed identically except for the omission of primary antibodies. Optical sections in the z-axis plane were obtained with a Deltavision Restoration Microscope (Applied Precision Instruments, Inc.) fitted with a Nikon 60x oil immersion objective, and the images were processed at a Silicon Graphics workstation with accompanying API software. The images were subsequently exported to Adobe Photoshop and Adobe Illustrator for manuscript preparation.

PI-3 kinase ELISA

ELISA to determine levels of [PI(3,4,5)P3] in A431 cell supernatants was performed using a PI-3 kinase assay kit (Echelon BioSciences) per manufacturer's instructions except the supernatants from infected and

uninfected A431 cells were incubated with the [PI(3,4,5)P3] detection reagent incubated at 4°C for 18h. The reaction mixtures were then transferred to the detection plate and all other steps performed as indicated. [PI(3,4,5)P3] levels were detected using a MicroMax plate reader using absorbance at 450 nm. Levels of [PI(3,4,5)P3] were quantified by comparing values from wells containing supernatants from cells to the values in a standard curve run concomitantly with test wells.

Bacterial aggregation (microcolony formation) assay

Wt MS11 bacteria was diluted to approximately 5 x 10^8 CFU/ml in supplemented GCB medium in a total volume of 1ml. 10µg of soluble [PI(3,4,5)P3] (Calbiochem) was added at a concentration of 1mg/ml and the were samples vortexed for 1min. 100µl of the culture was added to a coverdish (Costar) and bacterial aggregation was monitored in real time at room temperature by Differential Interference Contrast microscopy using the Deltavision Restoration Microscope (API). Time lapsed movies were produced in Quicktime format using a Silicon Graphics workstation with accompanying API software.

[PI(3,4,5)P3] bead aggregation assays

1.0 μm yellow-green fluorescent polystyrene beads (Fluospheres, Molecular probes) were covalently coupled with soluble [PI(3,4,5)P3] (Calbiochem) using water-soluble caboiimide (EDAC) as recommended by the supplier. The beads were washed thoroughly in PBS and adsorbed onto a clean coverslip dish in the presence of PBS. BSA (bovine serum albumin) was used to coat mock beads for controls. GC cultures were added as stated for aggregation assays. Differential Interference Contrast and fluorescence microscopy was used to visualize aggregating bacteria and beads, respectively. Time lapsed movies were produced in Quicktime format using a SiliconGraphics workstation with accompanying API software.

RNA extraction and Real-time PCR assays

Bacterial RNA was extracted from MS11 using the RNAeasy kit (Qiagen) per manufacturer's instructions. Briefly, wt MS11was diluted in supplemented GCB medium and incubated with 20μg of [PI(3,4,5)P3], [PI(4,5)P2] or a vehicle control for 2h before collection. Cells were lysed in lysozyme (0.4 μg/μl wt/vol) containing Tris-EDTA buffer. DNA contamination was removed by treatment with DNA-free (Ambion) and samples were quantified by spectrophotometric analysis (Beckman DU 600).

Complementary DNA was generated using the iScript cDNA Synthesis Kit (Bio-Rad) as recommended by the manufacturer. Oligonucleotides complementary to *pilT* were designed using the primer express software (PE Applied biosystems) to obtain amplicons of the same size. Oligonucleotides to *pilT* (pilTin.630, 5' GTCGACCGTATCGTGGACGTATT 3'; pilTout.730, 5' TTCAGCAGGTTTTGGGAGATGAC 3') were purchased from Invitrogen.

Real-time PCR using SYBR GREEN PCR Master Mix (Applied Biosystems) was carried out on the ABI Prism 7000 Sequence Detector System (Applied Biosystems). Amplification plots were analyzed with the ABI Prism SDS Software package (Version 1.0) and the data were processed using TaqMan Turbocrunch (96) v3. Relative quantification of gene expression was performed by the comparative Ct (threshold cycle) method according to the manufacturer's instructions and as published (Schmittgen et al., 2000). The parameter Ct is defined as the cycle number at which fluorescence passes the fixed threshold. Two independent RTPCR experiments were performed. Dilutions of the reverse transcription product from each experiment were loaded in triplicate for each oligonucleotide couple in all experiments.

Results

PI-3 kinase and Akt are activated upon GC infection

We tested the hypothesis that pilus retraction during infection activates PI-3 kinase as this pathway is known to be activated by membrane tension. Akt activity was used as the indicator of PI-3 kinase activation, as Akt is a direct downstream target of PI-3 kinase. A431 cells (a human epidermoid carcinoma line used previously for *Neisseria* virulence studies) were infected for various lengths of time with wt GC strain MS11 or MS11 *pilT*, a piliated null mutant that adheres normally to cells, forms microcolonies, but fails to retract pili. Akt was immunoprecipitated from whole cell lysates and immune complexes were tested for their ability to phosphorylate GSK-3, a known Akt substrate. Akt was activated within 30 minutes after infection with wt GC, as judged by the presence of phospho-GSK, and Akt activity was detectable for 3 hours (Figure 1a). In contrast, Akt activity was barely detectable in cells infected with the *pilT* mutant (Figure 1b). Equal amounts of Akt were in all the samples, as illustrated by control immunoblots of total cell lysates using an anti-Akt antibody (Figure 1a and b, lower panels).

The dependence of Akt activation on pilus retraction was also examined using MS11 *placZ-pilT*, a piliated strain in which the *pilT* open reading frame has been placed under the control of the IPTG (isopropyl β-D-thioglactopyranoside)-inducible *lacZ* promoter (Figure 1c). As observed previously, phospho-GSK was detected within 30 minutes of infection by wt MS11. Levels of phospho-GSK were low in cells infected with MS11 *placZ-pilT* in the absence of IPTG. The addition of IPTG to these cells restored phospho-GSK levels to that in cells infected with wt bacteria. Akt activation in infected cells was confirmed by immunoblotting with an antibody that recognizes phospho-Akt Ser473. The appearance of phospho-Akt coincided with the activity of Akt upon GSK-3. Additional control immunoblots showed equal levels of Akt in all samples. The presence of 10 nM wortmannin blocked Akt activation (Figure 1c), verifying the dependence of Akt activation on PI-3 kinase. Taken together, these results demonstrate that infection of epithelial cells with piliated GC quickly activates PI-3 kinase. Activation is sustained over a 3hour period and the degree of activation is strongly influenced by pilus retraction.

Invasion of piliated GC is dependent on PI-3 kinase activation

We next determined whether the PI-3 kinase pathway influenced bacterial invasion. Cells were infected with wt MS11 for 6 hours with PI-3 kinase inhibitors wortmannin or LY294002 or medium alone, and the invasion index was determined by the Gentamicin protection assay (see Methods). Wortmannin reduced GC invasion by 81.6% ($p=0.005$), compared to untreated infected cells (Figure 2a). Wortmannin did not affect total bacterial counts or bacterial adhesion to cells (data not shown). Thus, the effect of wortmannin on cell entry was not due to reduced bacterial viability or adherence. Similarly, LY294002 reduced invasion by 50.0% ($p=0.001$), compared to untreated infected cell controls (Figure 2b). It should be noted that a small fraction of the *pilT* inoculum was capable of invading cells. LY294002 did not affect invasion by this fraction (Figure 2c). These results strongly suggest that GC capable of retracting their pili use the PI-3 kinase signaling pathway as an important means to gain entry into host cells.

PI-3 kinase and Akt form ring-like structures at the base of microcolonies

Piliated *Neisseria* trigger the formation of cortical plaques in infected cells directly beneath sites of bacterial attachment. These structures, which are visible for 3-4 hours after infection, are enriched in membrane and other signaling proteins and cytoskeletal components (Eugene et al., 2002; Hoffmann et al., 2001; Merz et al., 1999). We next determined the location of PI-3 kinase and Akt in infected cells. Cells were infected for 3 hours with wt MS11 and processed for immunofluorescence microscopy using antibodies recognizing the PI-3 kinase p110 catalytic subunit, Akt phosphorylated on serine 473, or ezrin, a cytoskeleton component present in Neisseria-induced cortical plaques. High levels of PI-3 kinase (Figure3a-c) and phospho-Akt (Figure 3d-f) were found in ring-like structures at the periphery of the adherent microcolonies. It should be noted that the strain of wt piliated MS11 used in our study adheres to the apical membrane of A431 cells for at least 4 hours before entering the cell. The annular structures formed by these two kinases are therefore very unlikely to be associated with intracellular bacteria.

Three dimensional reconstructions of images from 0.1 μm vertical (Z) sections of infected stained cells revealed that the annular pattern of phospho-Akt occurred mainly at the periphery of the attached microcolony. In contrast, ezrin was observed beneath the entire microcolony as well as in the microvilli between the bacteria within the microcolony (Figure 3g-i; see also Figure 3e). The *pilT* mutant, which is piliated and adheres normally to A431 cells, did not trigger the clustering of PI-3 kinase p110 or phospho-Akt in infected cells (Figure 4a). Taken together, these microscopy results demonstrate that wt MS11 causes the clustering of PI-3 kinase p110 catalytic subunit and phospho-Akt at sites of infection. These signaling proteins have a different distribution than other cortical plaque proteins, being confined mainly to the periphery of the microcolonies.

Infected cells have high levels of [PI(3,4,5)P3] that are accessible to adherent bacteria

Lipid second messengers are a product of activated PI-3 kinase. [PI(3,4,5)P3], in particular, serves as a signaling intermediary between PI-3 kinase and Akt. The clustering of activated PI-3 kinase and Akt at sites of bacterial attachment suggests that these sites may also have high levels of [PI(3,4,5)P3]. We therefore examined the location of [PI(3,4,5)P3] in

infected cells by immunofluorescence microscopy, using a [PI(3,4,5)P3] specific antibody. [PI(3,4,5)P3] accumulated at high levels in these cells, forming a ring-like structure at the periphery of microcolonies that colocalized with activated Akt. This result was observed again in a subsequent experiment in which the membrane-permeabilization step was omitted and all fixation steps were performed at 4°C to minimize entry of antibodies into the cell (Figure 4b). These results strongly suggest that [PI(3,4,5)P3] clusters in the outer leaflet at the base of the adherent microcolonies.

Although phosphoinositides are preferentially located at the cytoplasmic leaflet of the host plasma membrane, they and other lipid moieties can be translocated across the membrane bilayer and/or released into the medium (Daleke, 2003). Furthermore, extracellular [PI(3,4,5)P3] can influence signaling events. Physiological levels of exogenous [PI(3,4,5)P3], but not [PI(3,4)P(2)], PI[(4,5)P(2)] or PI[(3)P], induce Ca^{2+} fluxes in T cells (Hsu et al., 2000). That [PI(3,4,5)P3] is present at the outer face of the plasma membrane of infected cells, in close proximity to the attached microcolony, would imply that the colonizing bacteria may have access to this pool of lipids.

We next determined whether infected cells released [PI(3,4,5)P3] into the medium, using an ELISA-based assay to determine total [PI(3,4,5)P3] concentrations. A substantial level (26.4 +/- 1.7 SD) pmol of [PI(3,4,5)P3] was found in the supernate of both infected and uninfected cells in numerous assays. However, no significant differences in [PI(3,4,5)P3] levels were detected between infected and uninfected cell supernates.

Taken together, these results demonstrate that infected cells have high levels of [PI(3,4,5)P3] on the outer leaflet of the plasma membrane in close proximity to the adherent bacteria. Supernatants of infected cultures did not have higher levels of [PI(3,4,5)P3] than those of uninfected cultures. At this time it is unclear whether this result is a reflection of the insensitivity of the assay or whether infected cells do not release more [PI(3,4,5)P3] into the medium than uninfected cells.

[PI(3,4,5)P3] produced by PI-3 kinase modulates bacterial behavior

Lipids as signaling effectors for bacteria have gained attention recently. The *Pseudomonas aeruginosa* phosphatidylethanolamine (PE) serves as a chemoattractant for the bacteria (Kearns et al., 2001). Certain PE species

enhance the twitching velocity of *Pseudomonas* type IV pili and the gliding rates of *Myxococcus*, and promote the aggregation of Enteropathogenic and Enterohemorrhagic *E. coli* (Barnett Foster et al., 1999; Kearns and Shimkets, 2001a; Kearns and Shimkets, 2001b). Mammalian PE could bind directly to EHEC/EPEC bacteria, suggesting that host cell PE may act as a receptor during infection (Khursigara et al., 2001).

The accessibility of [PI(3,4,5)P3] to adherent GC led us to determine whether this phospholipid affected Neisserial behavior, in particular, microcolony formation and twitching motility. An aggregation assay was first performed. Wt MS11 were incubated for 1h with 10 μg of [PI(3,4,5)P3] or vehicle alone, and microcolony formation was assessed by live cell imaging and Differential Interference Contrast microscopy. Bacteria incubated with [PI(3,4,5)P3] or vehicle were motile. However, bacteria exposed to [PI(3,4,5)P3] aggregated into microcolonies at earlier time points than those in the presence of vehicle (Figure 5). Moreover, microcolonies formed in the presence of the lipid were much larger than those formed in the presence of the vehicle. These results suggest that [PI(3,4,5)P3] stimulates bacteria to aggregate into microcolonies.

To determine whether [PI(3,4,5)P3] associates with the aggregating bacteria, the assay was repeated using wt MS11 incubated with [PI(3,4,5)P3] convalently attached to fluorescent beads to. As before, the assay was performed over 60min. The samples were visualized by Differential Interference Contrast microscopy to reveal bacterial aggregates, and using the FITC channel to visualize the fluorescent beads (Figure 6A). As bacteria moved along the surface, they aggregated, actively acquiring lipid-coated beads into the developing microcolonies. In the FITC images (Figure 6A, upper panel), some of the [PI(3,4,5)P3]-beads have a diffuse fluorescence. This is due to diffraction of fluorescence emission by bacteria. The FITC image viewed by Differential Interference Contrast imaging reveal the presence of microcolonies at these sites (Figure 6A, lower panel). As observed in the previous assay, bacteria exposed to mock-coated beads aggregate to a lesser extent than those exposed to [PI(3,4,5)P3]-coated beads, and their microcolonies are much smaller. Bacteria rarely incorporate mock-coated beads into their microcolonies, even though they come into physical contact with the beads (Figure 6B).

Once a lipid-coated bead was "engulfed" by a microcolony, it remained with the microcolony and moved with it. Time-lapse video microscopy (see

supplementary video file) revealed the microcolony-incorporated beads in constant motion resembling twitching behavior. In contrast, mock-coated beads remained attached to the slide even if they had been touched by a passing microcolony.Taken together, these imaging studies demonstrate that host cell [PI(3,4,5)P3] alters the behavior of GC *in vitro* by enhancing microcolony formation.

[PI(3,4,5)P3] upregulates *pilT*

Pilus retraction drives GC motility. The effect of [PI(3,4,5)P3] on GC motility and microcolony formation would suggest that this phospholipid may affect pilus retraction. We therefore determined whether [PI(3,4,5)P3] affected *pilT* expression. Wt MS11 were incubated for 2h with soluble [PI(3,4,5)P3], [PI(4,5)P2], or vehicle alone, and the levels of *pilT* message were determined by real-time PCR using the appropriate primers. Expression of *pilT* increased 1.8 fold in cultures incubated with [PI(4,5)P3] (SD +/-0.2). The bacterial response to [PI(3,4,5)P3] is specific, as neither vehicle alone or [PI(4,5)P2], which differs from [PI(3,4,5)P3] by the absence of a phosphate group in the inositol ring, altered *pilT* expression. These results lend support to the above observations regarding the influence of [PI(3,4,5)P3] on microcolony formation behavior. They indicate that this

eukaryotic phospholipid has a direct effect on a fundamental aspect of GC twitching behavior, namely *pilT* expression.

Discussion

Retraction of the *Neisseria gonorrhoeae* type IV pilus is required for a number of functions, including motility on solid surfaces and DNA uptake/transformation (Wolfgang et al., 1998a). Recent reports indicate that the pilus modulates host cell functions during infection (Kallstrom et al., 1998; Lee et al., 2002; Merz and So, 2000). We tested the hypothesis that pilus retraction during infection influences bacterial interactions with the host cell. We present evidence that piliated GC activates the PI3-kinase/Akt signaling pathway in cultured A431 human epithelial cells. Pilus retraction greatly enhances PI-3 kinase/Akt activation (Figure 1). The PI-3 kinase pathway plays a major role in pilus-mediated invasion, as wortmannin and LY294002 substantially reduce the process (81.6% and 50.0%, respectively; Figure 2).

Activation of the PI-3 kinase/Akt pathway is sustained for at least 3 hours. This time span is exactly coincident with the length of time that microcolonies are observed on the infected cell. This signaling cascade is activated specifically at sites of bacterial attachment, since activated PI-3 kinase and Akt are concentrated at the periphery of adherent microcolonies

(Figure 3). [PI(3,4,5)P3], a product of PI-3 kinase that serves as a signaling intermediate with Akt, is also found at high levels at these sites (Figure 4).

Several lines of evidence suggest that GC responds to [PI(3,4,5)P3]. The phospholipid clusters are exposed on the outer leaflet of the plasma membrane, suggesting that it is accessible to the bacteria (Figure 4). [PI(3,4,5)P3] stimulates GC behavior *in vitro*. Bacteria form microcolonies more quickly in its presence, and the resultant microcolonies are noticeably larger (Figure 5). Motile, aggregating bacteria incorporate lipid-coated beads into their structures, and the beads move with the bacterial aggregate (Figure 6; see video file). Finally, [PI(3,4,5)P3] upregulates transcription of the *pilT* gene. Thus, activation of PI-3 kinase/Akt stimulates the infected cell to produce high levels of a lipid second messenger at a site that is accessible to the adhered bacteria. This lipid, at least *in vitro*, promotes bacterial aggegration/microcolony formation. Lending support to this model of bacteria-host cell communication is the recent discovery that the eukaryotic hormones epinephrine and norepinephrine induce quorum sensing among EHEC that regulate expression of virulence factors (Sperandio et al., 2003). These observations support the growing notion that chemicals such as lipids,

both mammalian and bacterial, can serve as effectors to modulate bacterial behavior.

The manipulation of host cell signaling pathways by pathogenic bacteria is a rapidly emerging theme in biology. Cross-talk between bacteria and the host cell promote bacterial cell entry and intracellular survival. In particular, PI-3 kinase is used by a number of bacterial pathogens to trigger cytoskeleton remodeling and/or modulate phagocytic events that promote invasion (Coombes et al., 2002; Ireton and Cossart, 1997; Kwok et al., 2002). How activation of PI-3 kinase/Akt pathway promotes GC cell entry remains to be investigated. PI-3 kinase phosphorylates the D-3 position of the inositol ring of the plasma membrane lipid [PI(4,5)P2], converting it to [PI(3,4,5)P3]. The latter recruits signaling proteins with plekstrin homology (PH) domains to the site. [PI(3,4,5)P3] recruits Akt, a cytosolic PH-domain serine/threonine kinase, to the lipid membrane where it is activated. Akt is a central signaling station for processes ranging from cell proliferation and differentiation to cell survival and regulation of endocytic processes. Other PH-domain containing proteins such as guanosine diphosphate (GDP)-GTP exchange factors for Rac and ARF6 play key roles in actin cytoskeleton remodeling. Other PH-domain proteins include Bruton's tyrosine kinase

(Btk) and Tec family tyrosine kinases. Members of the latter family regulate changes in cytosolic free calcium levels and gene expression (Cantley, 2002). In this context, it should be noted that purified GC porin and pili as well as live bacteria trigger a series of Ca^{2+} fluxes in epithelial cells upon infection, and these events, in turn, promote bacterial intracellular survival (Ayala et al., 2001; Bauer et al., 1999; Muller et al., 1999; Naumann et al., 1999; van Putten et al., 1998). It will be interesting to determine how these events intersect with the pilT-dependent activation of the PI-3 kinase pathway.

How does [PI(3,4,5)P3] influence the GC infection process? One possibility is that the lipid promotes localized adhesion by functioning as a receptor for the bacteria. This hypothesis is supported by data from other systems suggesting that lipids serve as receptors for *Helicobacter pylori, Chlamydia trachomatis, Campylobacter upsaliensis* (Barnett Foster et al., 1999; Busse et al., 1997; Huesca et al., 1996; Lingwood et al., 1992; Sylvester et al., 1996). Of note is the fact that both PE and phosphoinositides are preferentially localized to the cytoplasmic leaflet of the host plasma membrane but can be translocated across the lipid bilayer by a number of enzymes (Daleke, 2003).

Another attractive possibility is that [PI(3,4,5)P3] serves as chemoattractant, its accumulation at sites of infection creating a gradient that is recognized by the bacteria. A number of studies support this second hypothesis. Certain lipid species are chemoattractants for bacteria that exhibit twitching motility. *Myxococcus xanthus* migrates towards gradients of certain types of PE, and other PE moieties enhance the twitching velocity of the *P. aeruginosa* type IV pilus (Kearns and Shimkets, 1998). PE also induces aggregation of Enteropathogenic and Enterohemorrhagic *E. coli* (Barnett Foster et al., 1999). In light of these observations, it is tempting to speculate that [PI(3,4,5)P3] may alter the retractile process of the GC type IV pilus, perhaps by increasing its rate of retraction, the force of retraction, or both. This is supported by our data that *pilT* is upregulated when GC is incubated with [PI(3,4,5)P3]. One direct consequence of increased *pilT* expression would be to increase the number of retractile motors at the bacterial surface, which in turn would increase the overall rate of retraction. Increased retraction rates may explain the increased rate of microcolony formation as well as the size of microcolonies formed in the presence of the lipid. Increased retractile forces may also activate additional PI-3 kinases which, in turn, would enhance accumulation of [PI(3,4,5)P3], thereby forming a

lipid gradient that would serve to attract other bacteria to the site of infection.

A corollary to the above hypothesis is that GC may need to "pull" together, to exert sufficient membrane force on host cells to activate signal cascades. We have observed that a *pilT* mutant forms microcolonies and adheres to epithelial cells normally, yet cannot trigger the formation of cortical plaques and is reduced in invasiveness (Merz et al., 1999). These results support the speculation that bacteria in microcolonies are more efficient in triggering signals in the target cell. Pilus retraction from a large microcolony is likely to generate a greater force on the plasma membrane than from a single diplococcus. This supports the notion that microcolony formation may represent a type of coordinated social effort by GC to gain effective entry into host cells.

Coordinated social behavior is reminiscent of the behavior displayed by *M. xanthus* during fruiting body formation, in which gliding bacteria deposit trails of slime, and cells peferentially glide in the trails left by those in front (Spormann et al., 1999). Insects such as ants also exhibit analogous trail-following behavior, which is mediated by antennae-borne chemosensory systems (Skidmore et al.,1988). Interestingly, a derivative of dioleoyl PE

serves as an ant foraging pheromone as well as a chemoeffector for *M. xanthus* (Kearns and Shimkets, 1998).

In summary, our findings indicate that *Neisseria gonorrhoeae* activates the PI-3 kinase/Akt signal cascade upon infection of epithelial cells in culture and that this process is dependent on retraction of the bacterial type IV pilus. PI-3 kinase activation facilitates entry of bacteria into the target cell. It also generates [PI(3,4,5)P3], a host cell lipid that crosses species to communicate with the bacteria to alter their behavior. The specific mechanism by which a host lipid messenger engages signaling in bacteria has yet to be determined, and further studies on this system will likely present new insights into the role of interspecies signaling crosstalks in bacterial pathogenesis.

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References

Ayala, B. P., Vasquez, B., Clary, S., Tainer, J. A., Rodland, K., and So, M. (2001). The pilus-induced Ca2+ flux triggers lysosome exocytosis and increases the amount of Lamp1 accessible to Neisseria IgA1 protease. Cell Microbiol *3*, 265-275.

Bacallao, R., Stelzer, E.H. (1989). Preservation of biological specimens for observationin a confocal fluorescence microscope and operational principles of confocal fluorescence microscopy. Methods Cell Biol *31*, 437-452.

Barnett Foster, D., Philpott, D., Abul-Milh, M., Huesca, M., Sherman, P. M., and Lingwood, C. A. (1999). Phosphatidylethanolamine recognition promotes enteropathogenic E. coli and enterohemorrhagic E. coli host cell attachment. Microb Pathog *27*, 289-301.

Bauer, F. J., Rudel, T., Stein, M., and Meyer, T. F. (1999). Mutagenesis of the Neisseria gonorrhoeae porin reduces invasion in epithelial cells and enhances phagocyte responsiveness. Mol Microbiol *31*, 903-913.

Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003). Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol *4*, 517-529.

Bhaya, D., Bianco, N. R., Bryant, D., and Grossman, A. (2000). Type IV pilus biogenesis and motility in the cyanobacterium Synechocystis sp. PCC6803. Mol Microbiol *37*, 941-951.

Bhaya, D., Takahashi, A., and Grossman, A. R. (2001a). Light regulation of type IV pilus-dependent motility by chemosensor-like elements in Synechocystis PCC6803. Proc Natl Acad Sci U S A *98*, 7540-7545.

Bhaya, D., Takahashi, A., Shahi, P., and Grossman, A. R. (2001b). Novel motility mutants of synechocystis strain PCC 6803 generated by in vitro transposon mutagenesis. J Bacteriol *183*, 6140-6143.

Booth, J. W., Telio, D., Liao, E. H., McCaw, S. E., Matsuo, T., Grinstein, S., and Gray-Owen, S. D. (2003). Phosphatidylinositol 3-kinases in carcinoembryonic antigen-related cellular adhesion molecule-mediated internalization of Neisseria gonorrhoeae. J Biol Chem *278*, 14037-14045.

Bradley, D. E. (1972a). Evidence for the retraction of Pseudomonas aeruginosa RNA phage pili. Biochem Biophys Res Commun *47*, 142-149.

Bradley, D. E. (1972b). Shortening of Pseudomonas aeruginosa pili after RNA-phage adsorption. J Gen Microbiol *72*, 303-319.

Bradley, D. E. (1974). The adsorption of Pseudomonas aeruginosa pilus-dependent bacteriophages to a host mutant with nonretractile pili. Virology *58*, 149-163.

Brazil, D. P., Park, J., and Hemmings, B. A. (2002). PKB binding proteins. Getting in on the Akt. Cell *111*, 293-303.

Brossay, L., Paradis, G., Fox, R., Koomey, M., and Hebert, J. (1994). Identification, localization, and distribution of the PilT protein in Neisseria gonorrhoeae. Infect Immun *62*, 2302-2308.

Busse, J., Hartmann, E., and Lingwood, C. A. (1997). Receptor affinity purification of a lipid-binding adhesin from Haemophilus influenzae. J Infect Dis *175*, 77-83.

Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. Science *296*, 1655-1657.

Comolli, J. C., Waite, L. L., Mostov, K. E., and Engel, J. N. (1999). Pili binding to asialo-GM1 on epithelial cells can mediate cytotoxicity or bacterial internalization by Pseudomonas aeruginosa. Infect Immun *67*, 3207-3214.

Coombes, B. K., Johnson, D. L., and Mahony, J. B. (2002). Strategic targeting of essential host-pathogen interactions in chlamydial disease. Curr Drug Targets Infect Disord *2*, 201-216.

Daleke, D. L. (2003). Regulation of transbilayer plasma membrane phospholipid asymmetry. J Lipid Res *44*, 233-242.

Eugene, E., Hoffmann, I., Pujol, C., Couraud, P. O., Bourdoulous, S., and Nassif, X. (2002). Microvilli-like structures are associated with the internalization of virulent capsulated Neisseria meningitidis into vascular endothelial cells. J Cell Sci *115*, 1231-1241.

Foster, F. M., Traer, C. J., Abraham, S. M., and Fry, M. J. (2003). The phosphoinositide (PI) 3-kinase family. J Cell Sci *116*, 3037-3040.

Herdendorf, T. J., McCaslin, D. R., and Forest, K. T. (2002). Aquifex aeolicus PilT, homologue of a surface motility protein, is a thermostable oligomeric NTPase. J Bacteriol *184*, 6465-6471.

Hoffmann, I., Eugene, E., Nassif, X., Couraud, P. O., and Bourdoulous, S. (2001). Activation of ErbB2 receptor tyrosine kinase supports invasion of endothelial cells by Neisseria meningitidis. J Cell Biol *155*, 133-143.

Hsu, A. L., Ching, T. T., Sen, G., Wang, D. S., Bondada, S., Authi, K. S., and Chen, C. S. (2000). Novel function of phosphoinositide 3-kinase in T cell Ca2+ signaling. A phosphatidylinositol 3,4,5-trisphosphate-mediated Ca2+ entry mechanism. J Biol Chem *275*, 16242-16250.

Huesca, M., Borgia, S., Hoffman, P., and Lingwood, C. A. (1996). Acidic pH changes receptor binding specificity of Helicobacter pylori: a binary adhesion model in which surface heat shock (stress) proteins mediate sulfatide recognition in gastric colonization. Infect Immun *64*, 2643-2648.

Ireton, K., and Cossart, P. (1997). Host-pathogen interactions during entry and actin-based movement of Listeria monocytogenes. Annu Rev Genet *31*, 113-138.

Kallstrom, H., Islam, M. S., Berggren, P. O., and Jonsson, A. B. (1998). Cell signaling by the type IV pili of pathogenic Neisseria. J Biol Chem *273*, 21777-21782.

Kallstrom, H., Liszewski, M. K., Atkinson, J. P., and Jonsson, A. B. (1997). Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic Neisseria. Mol Microbiol *25*, 639-647.

Kearns, D. B., Robinson, J., and Shimkets, L. J. (2001). Pseudomonas aeruginosa exhibits directed twitching motility up phosphatidylethanolamine gradients. J Bacteriol *183*, 763-767.

Kearns, D. B., and Shimkets, L. J. (1998). Chemotaxis in a gliding bacterium. Proc Natl Acad Sci U S A *95*, 11957-11962.

Kearns, D. B., and Shimkets, L. J. (2001a). Directed movement and surface-borne motility of Myxococcus and Pseudomonas. Methods Enzymol *336*, 94-102.

Kearns, D. B., and Shimkets, L. J. (2001b). Lipid chemotaxis and signal transduction in Myxococcus xanthus. Trends Microbiol *9*, 126-129.

Khursigara, C., Abul-Milh, M., Lau, B., Giron, J. A., Lingwood, C. A., and Foster, D. E. (2001). Enteropathogenic Escherichia coli virulence factor bundle-forming pilus has a binding specificity for phosphatidylethanolamine. Infect Immun *69*, 6573-6579.

Kwok, T., Backert, S., Schwarz, H., Berger, J., and Meyer, T. F. (2002). Specific entry of Helicobacter pylori into cultured gastric epithelial cells via a zipper-like mechanism. Infect Immun *70*, 2108-2120.

Lee, S. W., Bonnah, R. A., Higashi, D. L., Atkinson, J. P., Milgram, S. L., and So, M. (2002). CD46 is phosphorylated at tyrosine 354 upon infection of epithelial cells by Neisseria gonorrhoeae. J Cell Biol *156*, 951-957.

Lin, L., Ayala, P., Larson, J., Mulks, M., Fukuda, M., Carlsson, S. R., Enns, C., and So, M. (1997). The Neisseria type 2 IgA1 protease cleaves LAMP1 and promotes survival of bacteria within epithelial cells. Mol Microbiol *24*, 1083-1094.

Lingwood, C. A., Huesca, M., and Kuksis, A. (1992). The glycerolipid receptor for Helicobacter pylori (and exoenzyme S) is phosphatidylethanolamine. Infect Immun *60*, 2470-2474.

Maier, B., Potter, L., So, M., Long, C. D., Seifert, H. S., and Sheetz, M. P. (2002). Single pilus motor forces exceed 100 pN. Proc Natl Acad Sci U S A *99*, 16012-16017.

Merz, A. J., Enns, C. A., and So, M. (1999). Type IV pili of pathogenic neisseriae elicit cortical plaque formation in epithelial cells. Mol Microbiol *32*, 1316-1332.

Merz, A. J., and So, M. (2000). Interactions of pathogenic neisseriae with epithelial cell membranes. Annu Rev Cell Dev Biol *16*, 423-457.

Merz, A. J., So, M., and Sheetz, M. P. (2000a). Pilus retraction powers bacterial twitching motility. Nature *407*, 98-102.

Merz, A. J., So, M., and Sheetz, M. P. (2000b). Pilus retraction powers bacterial twitching motility. Nature *407*, 98-102.

Miao, H., Yuan, S., Wang, Y., Tsygankov, A., and Chien, S. (2002). Role of Cbl in shear-activation of PI 3-kinase and JNK in endothelial cells. Biochem Biophys Res Commun *292*, 892-899.

Muller, A., Gunther, D., Dux, F., Naumann, M., Meyer, T. F., and Rudel, T. (1999). Neisserial porin (PorB) causes rapid calcium influx in target

cells and induces apoptosis by the activation of cysteine proteases. Embo J *18*, 339-352.

Nassif, X., Lowy, J., Stenberg, P., O'Gaora, P., Ganji, A., and So, M. (1993). Antigenic variation of pilin regulates adhesion of Neisseria meningitidis to human epithelial cells. Mol Microbiol *8*, 719-725.

Naumann, M., Rudel, T., and Meyer, T. F. (1999). Host cell interactions and signalling with Neisseria gonorrhoeae. Curr Opin Microbiol *2*, 62-70.

Raucher, D., and Sheetz, M. P. (1999). Membrane expansion increases endocytosis rate during mitosis. J Cell Biol *144*, 497-506.

Raucher, D., and Sheetz, M. P. (2000). Cell spreading and lamellipodial extension rate is regulated by membrane tension. J Cell Biol *148*, 127-136.

Raucher, D., Stauffer, T., Chen, W., Shen, K., Guo, S., York, J. D., Sheetz, M. P., and Meyer, T. (2000). Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion [In Process Citation]. Cell *100*, 221-228.

Schmittgen, T. D., Zakrajsek, B. A., Mills, A. G., Gorn, V., Singer, M. J., and Reed, M. W. (2000). Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. Anal Biochem *285*, 194-204.

Segal, E., Hagblom, P., Seifert, H. S., and So, M. (1986). Antigenic variation of gonococcal pilus involves assembly of separated silent gene segments. Proc Natl Acad Sci U S A *83*, 2177-2181.

Shao, J. Y., Ting-Beall, H. P., and Hochmuth, R. M. (1998). Static and dynamic lengths of neutrophil microvilli. Proc Natl Acad Sci U S A *95*, 6797-6802.

Shaw, J. H., and Falkow, S. (1988). Model for invasion of human tissue culture cells by *Neisseria gonnorrhoeae*. *56*, 1625-1632.

Sheetz, M. P., Felsenfeld, D. P., and Galbraith, C. G. (1998). Cell migration: regulation of force on extracellular-matrix-integrin complexes. Trends Cell Biol *8*, 51-54.

Skerker, J. M., and Berg, H. C. (2001). Direct observation of extension and retraction of type IV pili. Proc Natl Acad Sci U S A *98*, 6901-6904.

Sperandio, V., Torres, A. G., Jarvis, B., Nataro, J. P., and Kaper, J. B. (2003). Bacteria-host communication: the language of hormones. Proc Natl Acad Sci U S A *100*, 8951-8956.

Strom, M. S., and Lory, S. (1993). Structure-function and biogenesis of the type IV pili. Annu Rev Microbiol *47*, 565-596.

Sun, H., Zusman, D. R., and Shi, W. (2000). Type IV pilus of Myxococcus xanthus is a motility apparatus controlled by the frz chemosensory system. Curr Biol *10*, 1143-1146.

Swanson, J. (1973). Studies on gonococcus infection. IV. Pili: their role in attachment of gonococci to tissue culture cells. J Exp Med *137*, 571-589.

Sylvester, F. A., Philpott, D., Gold, B., Lastovica, A., and Forstner, J. F. (1996). Adherence to lipids and intestinal mucin by a recently recognized human pathogen, Campylobacter upsaliensis. Infect Immun *64*, 4060-4066.

van Putten, J. P., Duensing, T. D., and Carlson, J. (1998). Gonococcal invasion of epithelial cells driven by P.IA, a bacterial ion channel with GTP binding properties. J Exp Med *188*, 941-952.

Waldbeser, L. S., Ajioka, R. S., Merz, A. J., Puaoi, D., Lin, L., Thomas, M., and So, M. (1994). The opaH locus of Neisseria gonorrhoeae MS11A is involved in epithelial cell invasion. Mol Microbiol *13*, 919-928.

Wang, N., Butler, J. P., and Ingber, D. E. (1993). Mechanotransduction across the cell surface and through the cytoskeleton [see comments]. Science *260*, 1124-1127.

Wolfgang, M., Lauer, P., Park, H. S., Brossay, L., Hebert, J., and Koomey, M. (1998a). PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated Neisseria gonorrhoeae. Mol Microbiol *29*, 321-330.

Wolfgang, M., Park, H. S., Hayes, S. F., van Putten, J. P., and Koomey, M. (1998b). Suppression of an absolute defect in type IV pilus biogenesis by loss-of-function mutations in pilT, a twitching motility gene in Neisseria gonorrhoeae. Proc Natl Acad Sci U S A *95*, 14973-14978.

Wolfgang, M., Park, H. S., Hayes, S. F., van Putten, J. P. M., and Koomey, M. (1998c). Suppression of an absolute defect in type IV pilus biogenesis by loss- of-function mutations in pilT, a twitching motility gene in Neisseria gonorrhoeae. Proc Natl Acad Sci U S A *95*, 14973-14978.

Wu, S. S., Wu, J., and Kaiser, D. (1997). The Myxococcus xanthus pilT locus is required for social gliding motility although pili are still produced. Mol Microbiol *23*, 109-121.

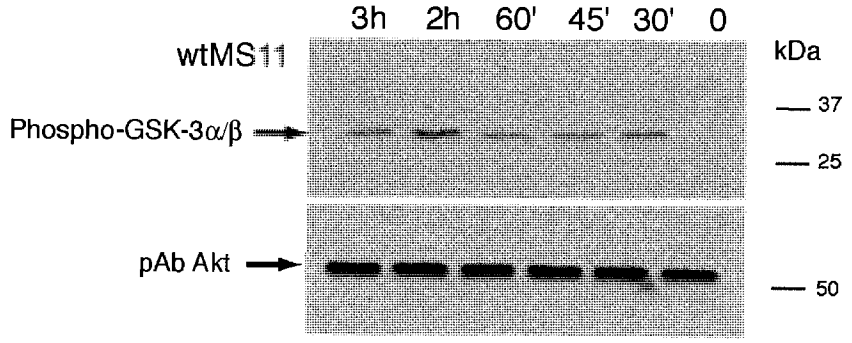
Yoshida, T., Kim, S. R., and Komano, T. (1999). Twelve pil genes are required for biogenesis of the R64 thin pilus. J Bacteriol *181*, 2038-2043.

Figure 1. Akt activation in GC-infected cells.

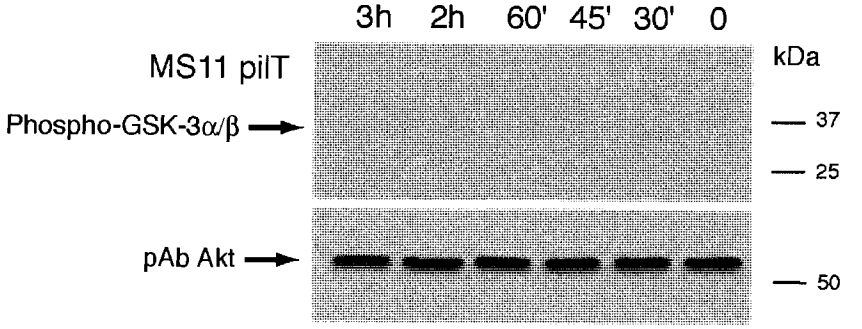
A431 (human epidermoid carcinoma) cells were infected with wt GC MS11 at an MOI of 50 for various lengths of time. Akt was immunoprecipitated from infected cells using an Akt kinase assay kit (Cell Signaling). Akt activity was determined by its ability to phosphorylate GSK-3α/β, and phosphorylated GSK-3α/β was measured using an antibody to phospho-GSK-3α/β. Total levels of Akt in the precipitates (lower panel) were determined by probing lysates with polyclonal antibodies to Akt. (B) Akt activity in A431 cells infected with GC MS11*pilT*. (C) Akt activity in uninfected cells (lane 1), cells infected with wt MS11 (lane 2), wt MS11 pretreated with 10 nM wortmannin (lane 3), MS11*pilT* (lane 4), MS11$pilT_i$ (IPTG-inducible *pilT*) without IPTG (lane 5), MS11$pilT_i$ in the presence of 2 μm IPTG. Total levels of Akt in the precipitates (middle panel) were determined by probing lysates with polyclonal antibodies to Akt. Immunoprecipitated Akt was probed with a phospho-Akt Ser 473 as an additional control to confirm levels of Akt activity (lower panel).

FIGURE 1

A.


3h 2h 60' 45' 30' 0
wtMS11
Phospho-GSK-3α/β
pAb Akt
kDa
37
25
50

B.


3h 2h 60' 45' 30' 0
MS11 pilT
Phospho-GSK-3α/β
pAb Akt
kDa
37
25
50
IP: Akt
IB: Phospho-GSK 3α/β
IB: pAkt

C.

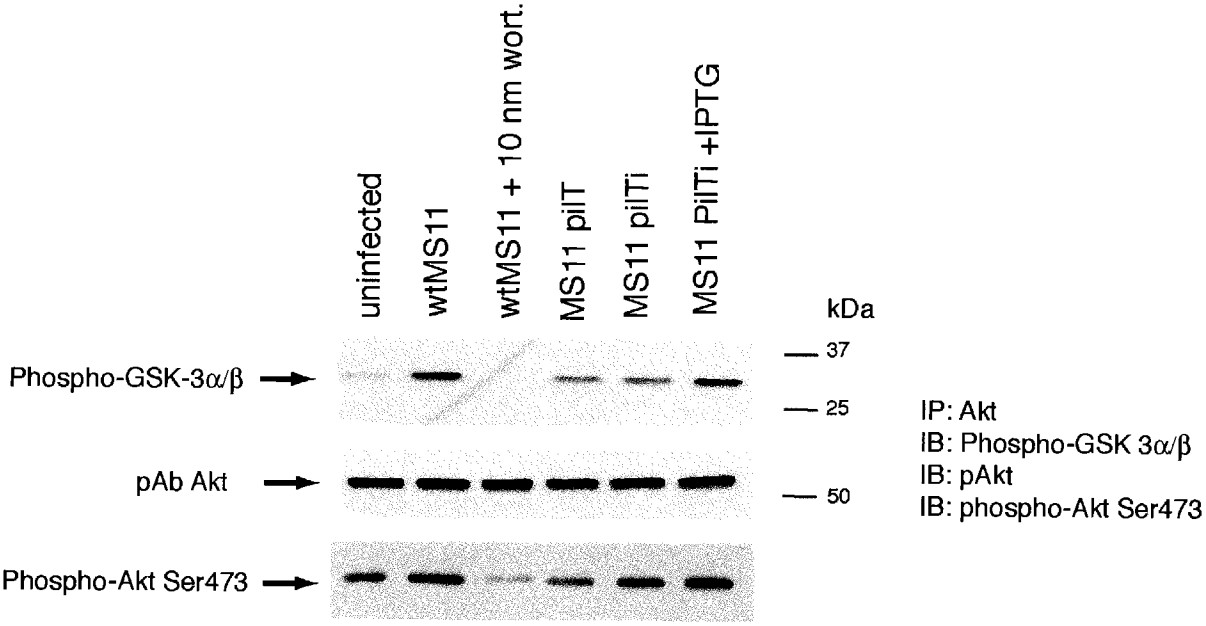
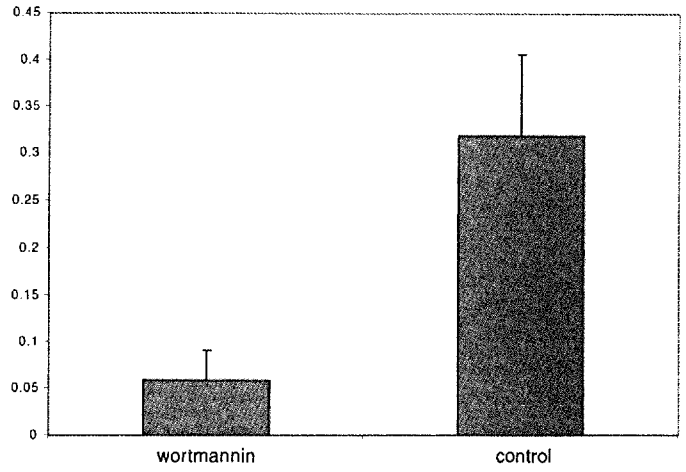

uninfected
wtMS11
wtMS11 + 10 nm wort.
MS11 pilT
MS11 pilTi
MS11 PilTi +IPTG
kDa
37
25
50
Phospho-GSK-3α/β
pAb Akt
Phospho-Akt Ser473
IP: Akt
IB: Phospho-GSK 3α/β
IB: pAkt
IB: phospho-Akt Ser473

Figure 2. Inhibition of PI-3 kinase affects bacterial invasion of wt GC but not a *pilT* mutant.

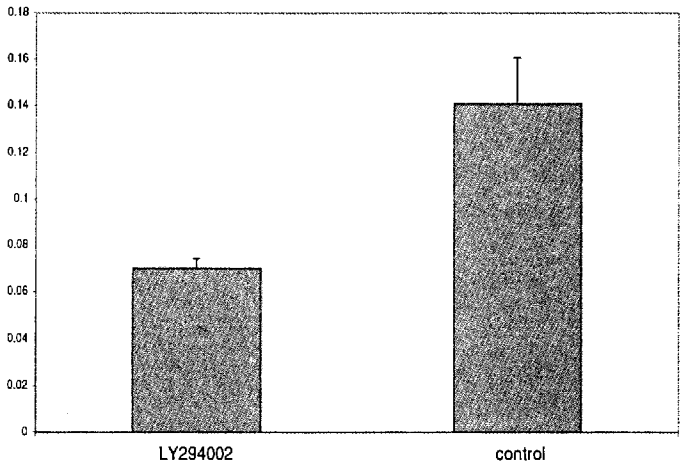
A431 cells were infected with MS11 for 6 h and intracellular (Gentamicin-protected) bacteria determined. All infections were done in triplicate. Bars indicate mean + SD of triplicate determinations from a representative experiment. (A): Invasion index of wt MS11 in the presence (left) or absence (right) of 10 nM wortmannin. (B): Invasion index of Wt MS11 in the presence (left) or absence (right) of 50 μM LY294002. (C) Invasion index of MS11*pilT* mutant in the presence (left) or absence (right) of 50 μM LY294002. DMSO served as a vehicle control for untreated lanes.

FIGURE 2

A.


0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
wortmannin
control

B.


0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
LY294002
control

C.

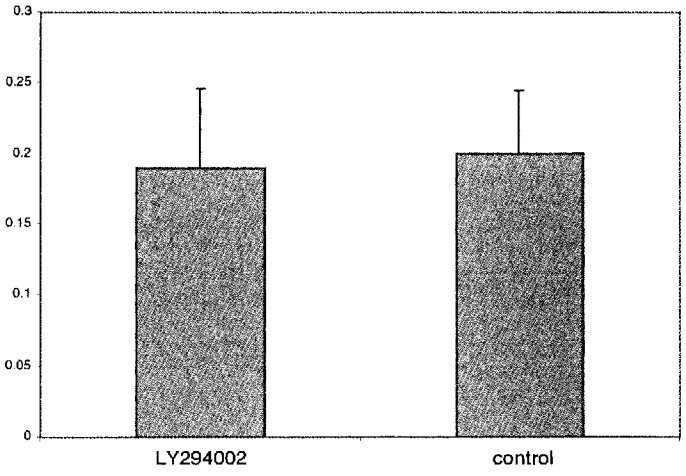

0.3
0.25
0.2
0.15
0.1
0.05
0
LY294002
control

Figure 3. PI3K p110 and phospho-Akt staining of A431 cells infected with wt GC MS11.

A431 endocervical epithelial cells were infected with wt MS11 for 3h and then fixed and processed for immunofluorescence microscopy. DAPI was used to visualize bacteria. Staining is as follows: (A) PI-3 kinase p110; (B) ezrin; (C) overlay of panels A and B. (D)phospho-Akt; (E) ezrin; (F) overlay of panels D and E.

Three dimensional reconstructions of phospho-Akt and ezrin from panels D-E (G-I). Images were deconvolved and the Volume Builder algorithm was performed using Deltavision Softworx software. Colocalization of phospho-Akt (red) and ezrin(green) can be seen at the base of the microcolony in yellow.

FIGURE 3

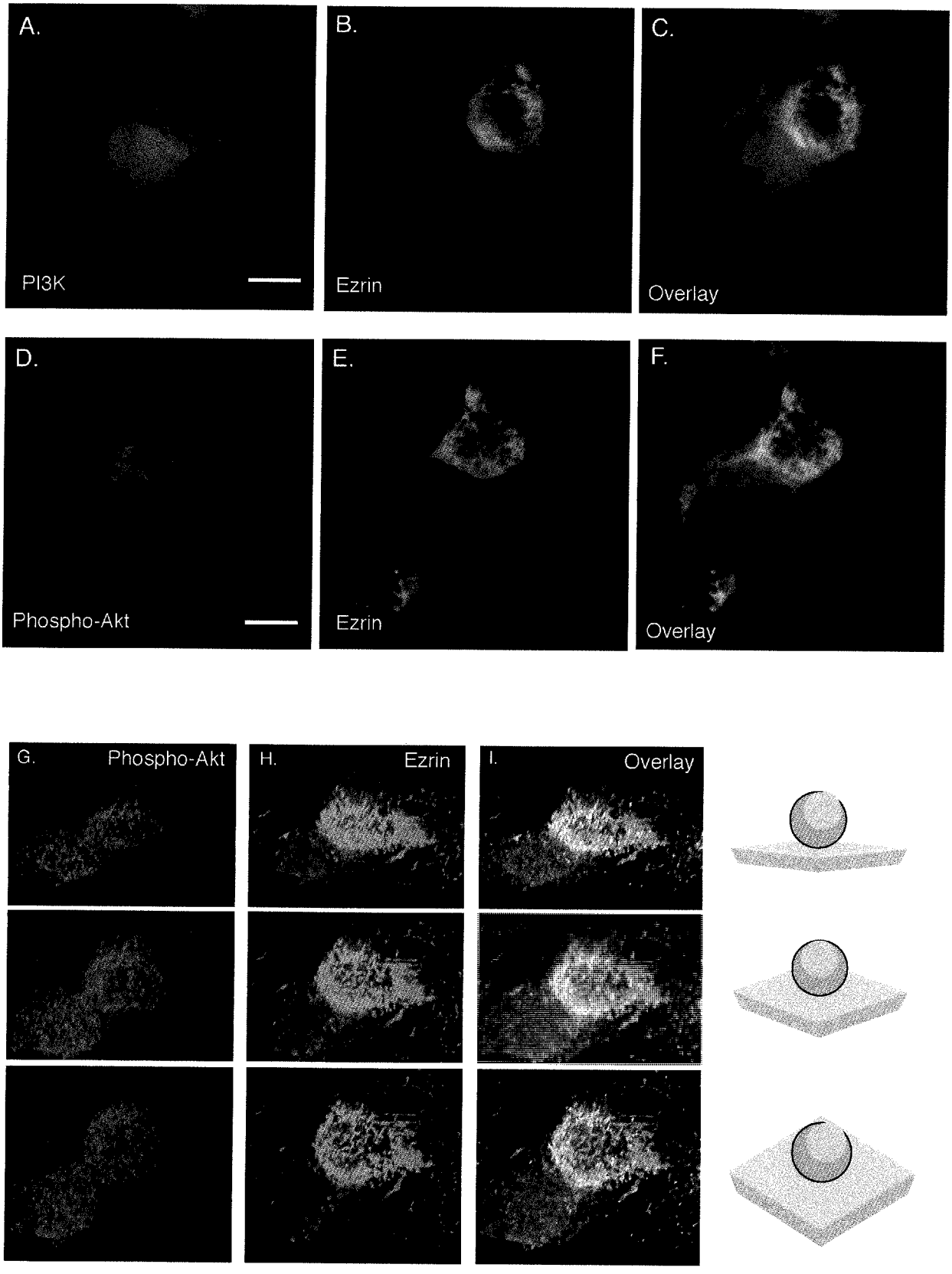
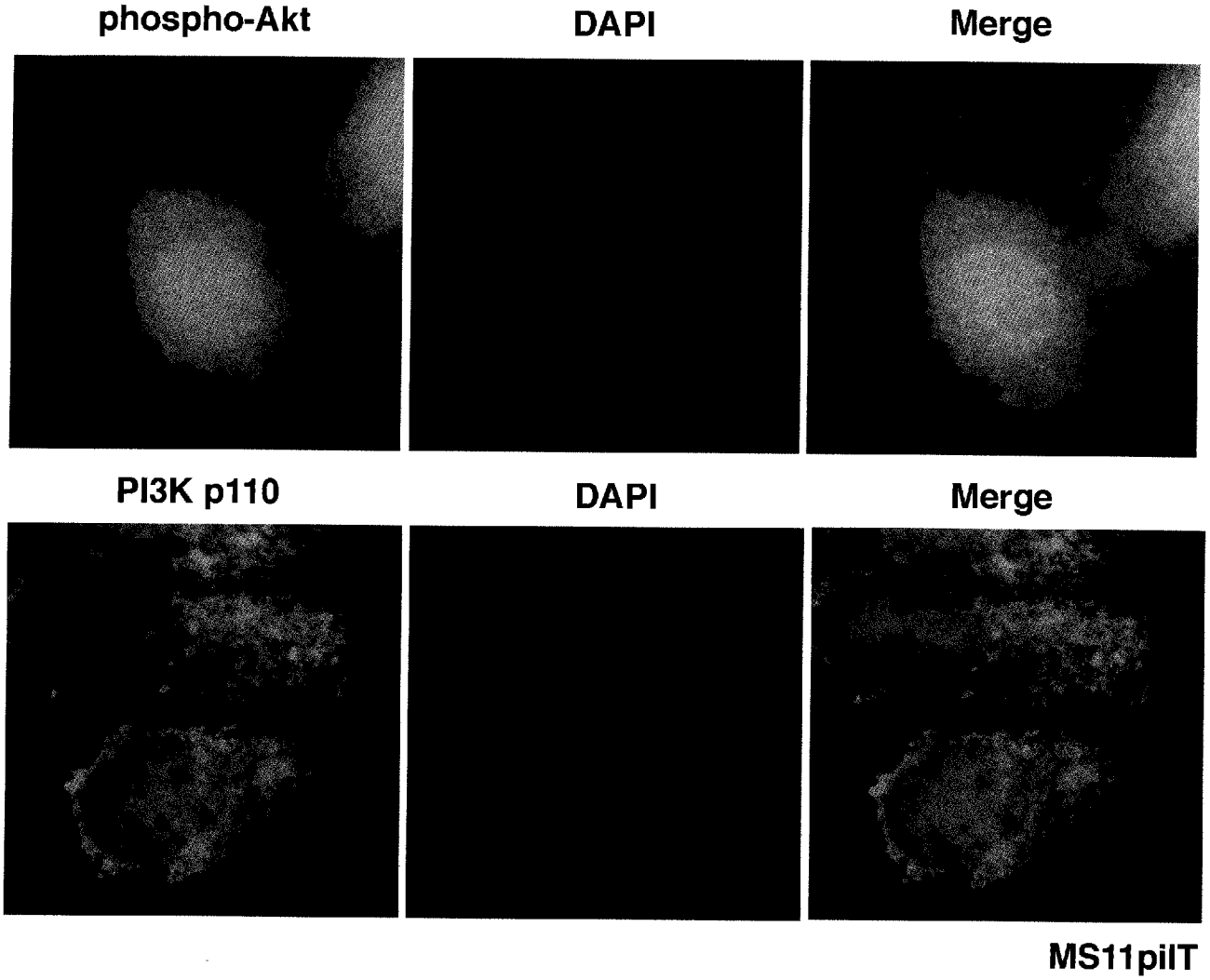

A.
PI3K
B.
Ezrin
C.
Overlay
D.
Phospho-Akt
E.
Ezrin
F.
Overlay
G.
Phospho-Akt
H.
Ezrin
I.
Overlay

Figure 4. Clustering of PI-3 kinase, phospho-Akt and [PI(3,4,5)P3] in GC-infected cells. A431 cells were infected with wt MS11 or MS11*pilT* for 3h, then fixed and processed for immunofluorescence microscopy using the appropriate antibodies. Saponin was included in all steps for panel A. DNA was stained with DAPI to visualize bacteria and nuclei. (A). PI-3 kinase p110 and phospho-Akt staining in cells infected with MS11*pilT*.
(B). Accumulation of [PI(3,4,5)P3] at the cell membrane outer leaflet of cells infected with wt MS11. To avoid permeabilization of the cell membrane and entry of antibodies into the cell, saponin was excluded from the fixation/processing reagents and all incubations were performed at 4°C. Middle panel serves as control to show decreased phospho-Akt signal, indicating minimal entry of antibody into cells.

FIGURE 4

A.


phospho-Akt
DAPI
Merge
PI3K p110
DAPI
Merge
MS11pilT

B.

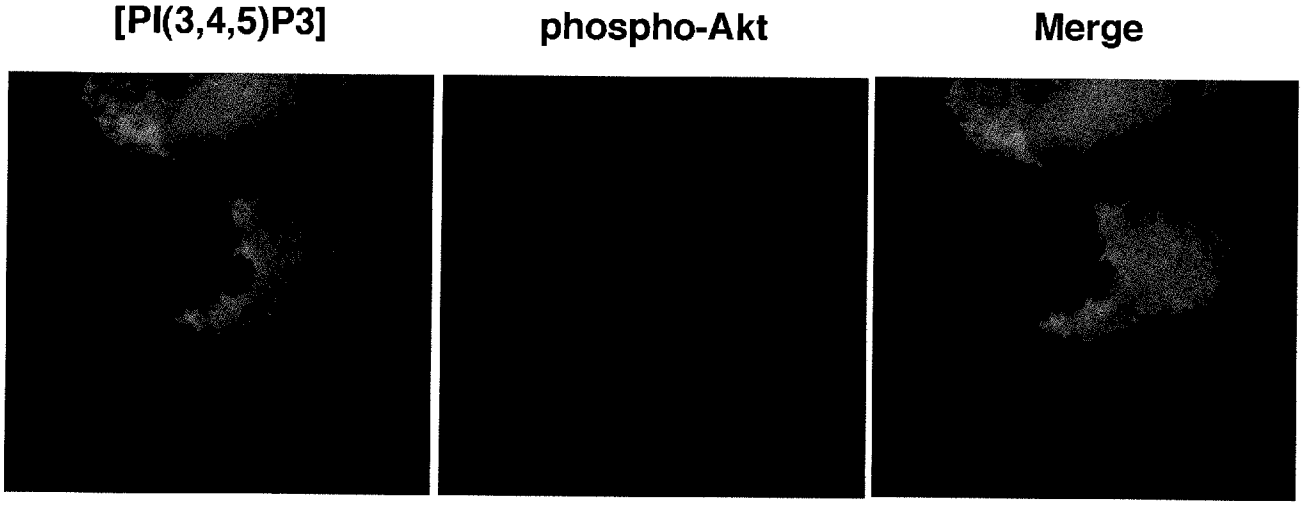
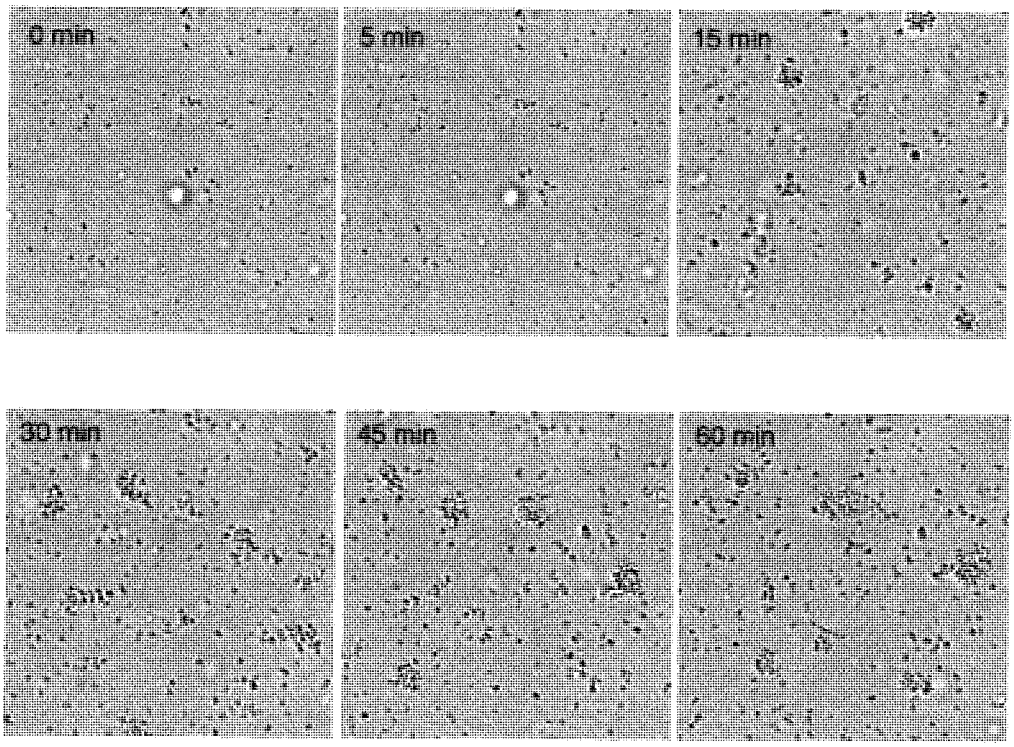

[PI(3,4,5)P3]
phospho-Akt
Merge
wtMS11

Figure 5. Microcolony formation in the presence of [PI(3,4,5)P3].

Wt MS11 were incubated for various lengths of time with [PI(3,4,5)P3] (Panel A) or vehicle (Panel B) and microcolony formation was visualized by Differential Interference Contrast time-lapse microscopy.

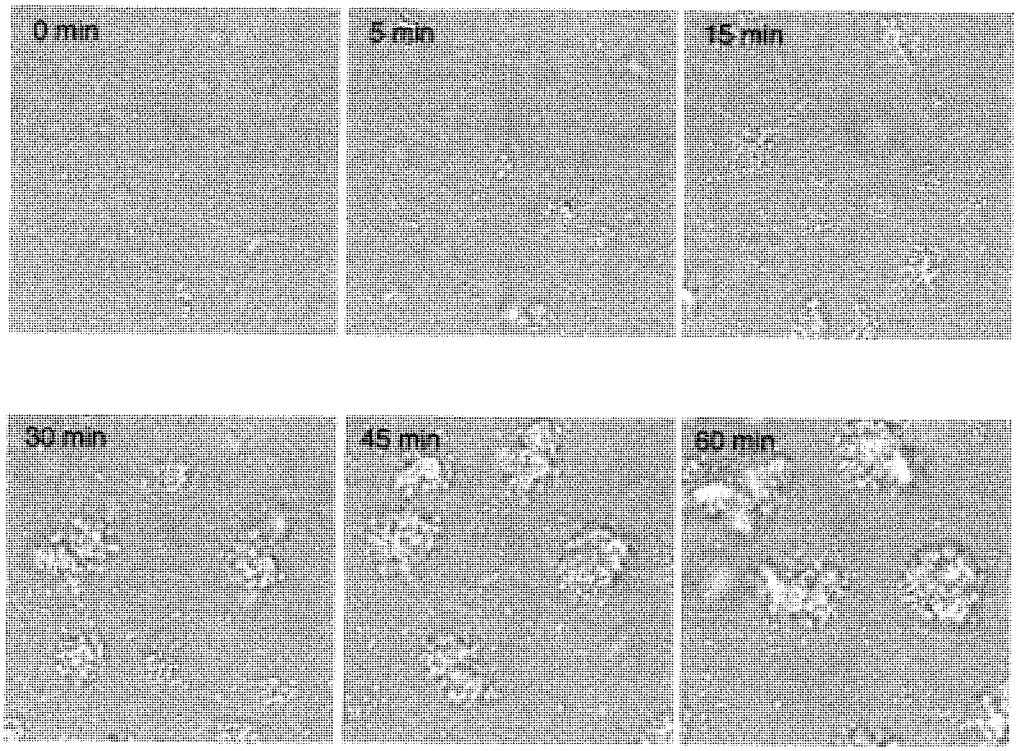
FIGURE 5

A.



wtMS11 + DMSO

B.

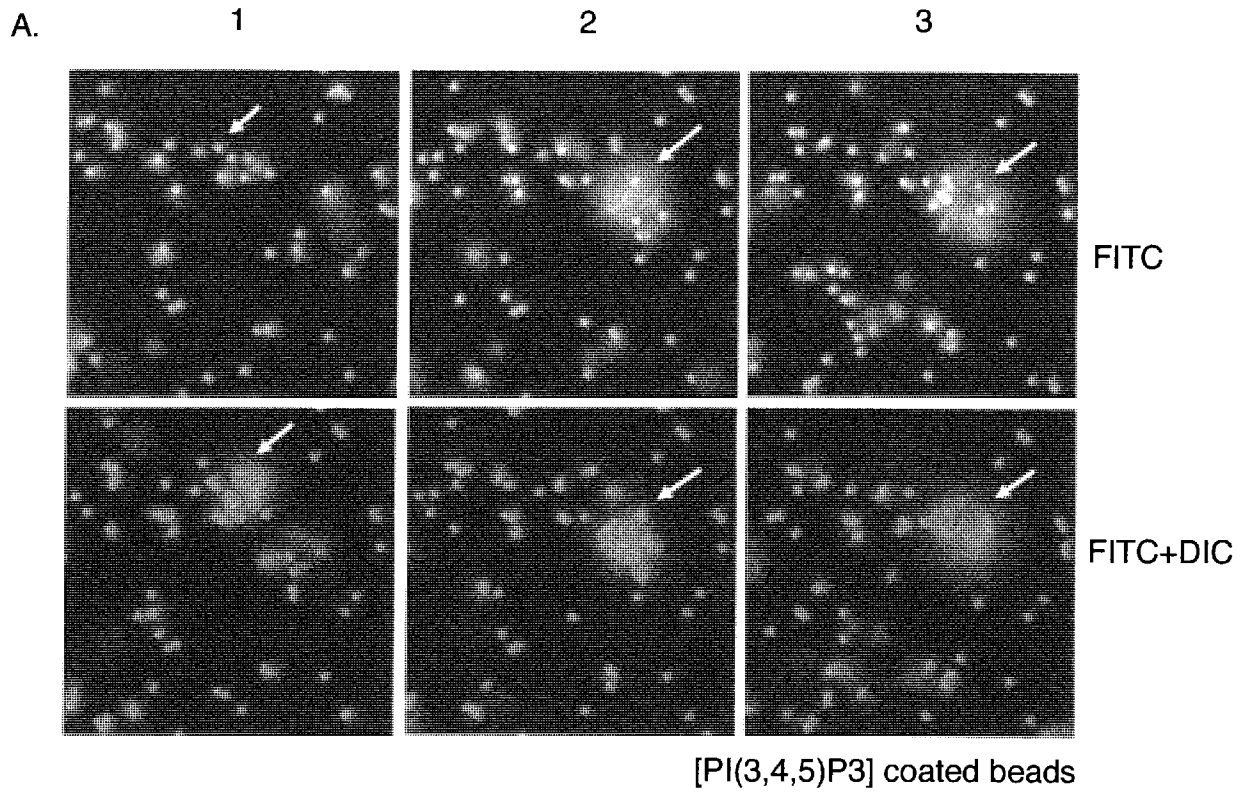


wtMS11 + [PI(3,4,5)P3]

Figure 6. Time-lapse microscopy of wt MS11 in the presence of [PI(3,4,5)P3]- or mock-coated fluorescent beads.

1.0 μm yellow-green fluorescent polystyrene beads (Fluospheres, Molecular Probes) were covalently coupled with soluble [PI(3,4,5)P3] or BSA and added to cultures of wt MS11 for various lengths of time. Differential Interference Contrast and flurorescence microscopy was used to visualize aggregating bacteria and beads, respectively. Time lapsed movies were produced in Quicktime format using a Silicon Graphics workstation with accompanying API software (see video). (A) wt MS11 + [PI(3,4,5)P3]-coated beads; (B) wt MS11 + mock-coated beads. Upper panels show images using the FITC channel to indicate bead position. Lower panels contain images visualized by FITC overlayed by Differential Interence Constrast imaging (DIC) to illustrate bacterial aggregation. "Flared" signals, representing acquisition of fluorescent beads into microcolonies, were observed at the earliest time points in cultures incubated with [PI(3,4,5)P3]-beads. These signals were not seen in cultures incubated with mock-coated beads.

FIGURE 6


A.
1
2
3
FITC
FITC+DIC
[PI(3,4,5)P3] coated beads
B.
1
2
3
FITC
FITC +DIC
Mock coated beads

Chapter 4: Manuscript III

Involvement of the CD46 cytoplasmic tail isoforms Cyt1 and Cyt2 in Type IV pilus-dependent *Neisseria gonorrhoeae* attachment to host cells.

Shaun W. Lee*, Nathan Weyand*, Paul Yoshihara & Magdalene So

Department of Molecular Microbiology & Immunology, L220

Oregon Health and Science University

Portland, Oregon, 97201-3098

In Preparation

*These authors contributed equally to this work.

Summary

CD46 (membrane cofactor protein; MCP) is a type I transmembrane glycoprotein that inhibits complement activation on host cells. CD46 serves as a receptor for numerous viral and bacterial pathogens. CD46 has been shown to be a cellular pilus receptor for pathogenic *Neisseria*. Lines of evidence indicate that the CD46 cytoplasmic tails play an important role in Neisserial pathogenesis. CD46 mutants with truncated cytoplasmic tails fail to support bacterial adhesion. Infection by *Neisseria gonorrhoeae* (GC) rapidly phosphorylates the cytoplasmic 2 tail isoform of CD46. Here, we report the construction of monoclonal antibodies generated using synthesized peptides specific to the two major cytoplasmic tail isoforms of CD46. Immunofluorescence microscopy revealed differential clustering of CD46 cytoplasmic tails identified using the cytoplasmic tail-specific antibodies. A CD46 Cyt1 specific antibody revealed periodic clustering of CD46 Cyt1 isoforms in GC-induced plaques. In contrast, CD46 Cyt2 staining revealed a prominent zone of negative staining at the site of GC-induced plaques, indicating that the CD46 Cyt2 tail either undergoes tail-specific proteolysis and/or engages signaling complexes that mask the epitope to the cytoplasmic-specific region of the CD46 Cyt2 tail.

Furthermore, different cell lines displayed different staining patterns of the individual CD46 tail domains, in appearance and intensity. These data indicate that the two cytoplasmic CD46 tails likely play distinct and cell-type specific roles in *Neisseria* infection.

Introduction

Membrane cofactor protein (MCP), or CD46, is a widely expressed type I transmembrane regulatory glycoprotein that serves a complement regulatory function in nucleated human cells. CD46 binds the complement activation products C3b and C4b that is deposited on host cells, serving as a cofactor to initiate their proteolytic cleaveage by the serine protease factor I, and ultimately allowing host cells to avoid complement attack (for review, see Liszewski et al., 1991; Liszewski et al., 1994; Lublin and Atkinson, 1989).

CD46 structure

The ectodomain of CD46 consists of four modules known as short consensus repeats, or complement control protein repeats (CCPs), followed by a segment of alternatively spliced serine-threonine-proline rich domains (STP-A, -B, -C) that are involved in cytoprotection (Liszewski and Atkinson, 1996; Liszewski et al., 1998). Common isoforms of CD46 contain the BC or C domains of the STP, although rarer forms exist (Post et al., 1991; Russell et al., 1992). SCR2, 3, and 4 are important for complement regulatory functions (Adams et al., 1991; Iwata et al., 1995). Following the STP domain is a short region of unknown function, a transmembrane anchor, a juxtamembrane cytoplasmic segment (JxM), and a short (16-23 amino acid)

cytoplasmic tail domain. The two major cytoplasmic isoforms Cyt1 and Cyt2 arise by alternative splicing (Post et al., 1991).

CD46 and cell signaling

The cytoplasmic domains of CD46 contain various signaling motifs likely to be involved in intracellular signaling events (Liszewski et al., 1994). Cyt1 consists of 16 unique amino acids and contains possible motifs for phorylation by Casein kinase-2 (CK-2) and protein kinase C (PKC). The Cyt2 tail consists of 23 amino acids and contains signals for phosphorylation by Src-kinases and CK-2. In the RAW264.7 mouse macrophage cell line, the CD46 tail interacts with multiple kinases, and this interaction correlates with tyrosine phosphorylation of the CD46 cytoplasmic domains (Wong et al., 1997). In Jurkat cells, the CD46 Cyt2 isoform is tyrosine phosphorylated by the Src kinase Lck following antibody ligation (Wang et al., 2000). The CD46 Cyt1 tail isoform has been shown to interact directly with Dlg4, a PDZ domain-containing scaffolding protein (PDZ proteins act to tether membrane proteins directly or indirectly to the actin cytoskeleton), in transfection experiments using MDCK cells (Ludford-Menting et al., 2002). Furthermore, this interaction is important for asymmetric targeting of CD46 in polarized cells. CD46 has also been shown to interact directly with

integrins and indirectly with tetraspanins (Lozahic et al., 2000). Thus, CD46 may engage a variety of signaling complexes through its association with integrins and the tetraspanin family of proteins.

CD46 as a pathogen receptor

Much attention has focused on CD46 as a cell surface receptor for human pathogens, including group A strains of measles virus (Dorig et al., 1993), *Streptococcus pyogenes* (Okada et al., 1995), and human herpesvirus 6 (Santoro et al., 1999). Recently, CD46 has been identified as a cellular receptor for group B adenoviruses (Gaggar et al., 2003). The interaction between the Ad35 fiber knob protein of group B adenovirus and CD46 was found to be essential for mediating adenovirus infection *in vitro*.

CD46 has been identified as a pilus receptor for pathogenic *Neisseria* (Kallstrom et al., 1997). Efficient adherence of *N. gonorrhoeae* (GC) requires SCR-3 and the STP domain (Kallstrom et al., 2001). Recent *in vivo* studies using transgenic mice found that mice expressing human CD46 were more susceptible to meningococcal disease by *Neisseria meningitidis* (Johansson et al., 2003). Mortality was more rapid in mice infected with piliated strains delivered intranasally than non-piliated strains, suggesting

that human CD46 mediates pilus-dependent interactions at the epithelial mucosa important for entry.

Reports have suggested that the cytoplasmic domains are important for Nesserial infection, though the exact mechanism is not understood. Human epithelial cells expressing CD46 with truncated tails failed to support bacterial adhesion (Kallstrom et al., 2001). GC adhesion to epithelial cells was found to be inversely proportional to the level of expression of CD46 (Tobiason and Seifert, 2001), indicating that pilus-mediated adhesion is not likely mediated through passive binding to CD46 alone. Infection of A431 epithelial cells by piliated GC triggered tyrosine phosphorylation of the Cyt2 tail isoform via activation of the src-family kinase c-yes (Lee et al., 2002). Inhibition of Src-family kinases was found to inhibit adhesion of GC host cells by 50%. These observations strongly suggest that pilus-mediated cellular adhesion may occur through signaling cascade(s) generated through the CD46 cytoplasmic tail, and that these signaling cascades likely activate host kinases important for infection.

Pathogenic *Neisseria* express type IV pili, a retractile structure that mediates bacterial motility (Brossay et al., 1994; Merz et al., 2000), DNA uptake (Wolfgang et al., 1998) and adhesion to host cells (Nassif et al., 1993;

Swanson, 1973). Piliated, live bacteria induce the formation of adhesion-promoting cortical plaques directly beneath the site of bacterial attachment. These plaques contain high concentrations of cortical actin and ezrin, membrane glycoproteins ICAM1, EGFR and CD44, c-yes and other tyrosine phosphorylated proteins (Merz et al., 1999; Merz and So, 1997; Lee et al., 2002; Hoffman et al. 2001). Purified pili trigger the release of Ca^{2+} from intracellular stores (Kallstrom et al., 1998) and a subsequent redistribution of lysosomes and lysosome-associated membrane proteins to the plasma membrane of target cells (Ayala et al., 1998).

Together, these reports support the notion that GC type IV pili may promote attachment through initiation of signaling transduction pathways in the host cell. This may occur in two ways, without exclusivity: (1). Via a signaling cascade initiated by direct binding to CD46, (2). Through the generation of membrane tension or shear forces by retractile pili that cause signaling changes in the host cell.

To better understand the roles of the individual cytoplasmic domains of CD46 in the signaling events triggered by *Neisseria* infection, we generated monoclonal antibodies against synthetic peptides derived from the cytoplasmic tails of the Cyt1 and Cyt2 isoforms and used these Mabs to

follow the fate of the tail isoforms in infection with *Neisseria gonorrhoeae* (GC). Various epithelial cell lines were infected with wt GC and examined for the presence of the Cyt1 or Cyt2 isoforms of CD46 in cortical plaques. Indirect immunofluorescence microscopy revealed differential clustering of CD46 cytoplasmic tails. Consistent with the varying distribution of the CD46 isoforms among various cell types, different cell lines exhibited different staining patterns of the Cyt1 and Cyt2 isoforms. A CD46 Cyt1 specific antibody revealed clustering of Cyt1 isoforms of CD46 underneath GC-induced plaques in some of the tested cell lines. In contrast, CD46 Cyt2 staining was noticeably absent from GC-induced plaques in most of our cell lines; however, staining with a monoclonal antibody that specifically recognizes the ectodomain of CD46 found CD46 to be clustered within GC cortical plaques, indicating that either the CD46 Cyt2 tail undergoes tail-specific proteolysis and/or engages signaling complexes that mask the epitope to cytoplasmic specific tails. The data presented here suggest that the cytoplasmic tail isoforms of CD46 likely play distinct roles in *Neisseria* infection and that these differences are influenced by the differential expression of these isoforms in disparate cell lines.

Results and discussion

CD46 antibody construction

Monoclonal antibodies specific to the Cyt1 and Cyt2 isoforms of the CD46 cytoplasmic tail were generated using synthetic peptides purchased from Global peptides services (Fort Collins, CO). The CD46 Cyt1 and Cyt2 tail sequences are listed in Table 1. The peptides were conjugated to KLH for direct use as immunogens for monoclonal antibody production. All of the CD46 cytoplasmic tail antibodies were generated by Paul Yoshihara at the Monoclonal Antibody Core Facility, Vaccine and Gene Therapy Institute (VGTI; Beaverton, Oregon). Antibody specificity was determined by ELISA.

Western blot analysis

We performed western blot analysis to test the specificity of the CD46 tail antibodies in lysates of A431 epithelial cells. Cells were collected in lysis buffer and centrifuged for 20 minutes at 13,000g to pellet nuclear material and debris. Supernatants were diluted in SDS running buffer and approximately 5μg of total proteins were loaded onto 10 % SDS polyacrylamide gels and probed using the CD46 Cyt1 or the Cyt2 antibody

at a dilution of 1:3000. Initial mouse bleeds, hybridoma cell supernatants as well as the purified antibodies were tested for reactivity. Figure 1 shows immunoblot stainings for the purified antibody clones, demonstrating specificity.

There are many CD46 antibodies available commercially. However, currently there are no widely available antibodies that can discriminate between the cytoplasmic tails. Furthermore, all commercially available antibodies are generated against the ectodomain and usually migrate as a broad doublet of 55-70 kDa on western blot, depending on cell type. Though the doublets are thought to arise from expression of the BC and C forms of the STP splice variant, it is difficult to identify cytoplasmic isoform differences in CD46 in these bands. Our western blot data show a clear staining pattern identified by using tail-specific antibodies that heretofore have not been reported in literature.

Immunofluorescence microscopy of CD46 tail variants in GC infection

We used the monoclonal CD46 cytoplasmic tail antibodies to examine the role played by each tail isoform in GC infection of epithelial cells. Piliated *Neisseria* induces the formation of cortical plaques- structures beneath the sites of bacterial attachment that are enriched in membrane proteins,

signaling proteins, and cytoskeletal components (Hoffmann et al., 2001; Merz et al., 1999; Merz and So, 1997).

One component that was previously identified to be clustered in cortical plaques was c-yes, a src family kinase particularly enriched in epithelial cells (Lee et al., 2002). It was also demonstrated that c-yes is most likely responsible for the tyrosine phosphorylation of CD46 *in vivo*, and we sought to determine initially if CD46 was also clustered in the cortical plaques. When we previously used a commercial antibody clone (J4-48) generated against the ectodomain of CD46 to look for the presence of CD46 in cortical plaques, we saw sporadic staining of CD46 clustered underneath the sites of bacterial attachment (Lee, S., Merz, A., So, M. unpublished data) in A431 epithelial cells. One possible explanation for this data was that there was a preferential clustering of one specific tail isoform that could not be discriminated using the ectodomain-specific CD46 antibody.

We used the CD46 tail isoform antibodies described in this paper to probe GC-induced cortical plaques to gain a better understanding of the role of the individual tails in GC infection.

The CD46 Cyt1 tail isoform is found in GC-induced cortical plaques

The location of the CD46 Cyt1 isoform was determined by immunofluorescence microscopy. Epithelial cells were infected for 3h with wt GC MS11 and incubated with the CD46 Cyt1 specific antibody clone 2F-1. Slides were also stained with a polyclonal antibody to phospho-ezrin as a positive control to visualize cortical plaques and with DAPI to visualize bacteria. CD46 Cyt1 was clustered beneath GC microcolonies (Figure 2). This clustering occured on many but not all microcolonies. We could not conclude whether this staining was due to microcolony size, or any other noticeable differences in morphology of the microcolonies.

Furthermore, the clustering of CD46 Cyt1 was variable, depending on the cell type examined (Table 2). CD46 Cyt1 did not cluster appreciably in Hec-1B (adenocarcimona uterine endometrium) cells, or End1 (endocervical epithelial carcinoma) cells. In contrast, HjCE (immortalized conjunctival epithelial) and A431 (endocervical carcinoma) cells clustered CD46 Cyt1, athough HjCE cells were found to have more robust Cyt1 staining than A431 cells in most cases. All cell types studied supported GC infection and promoted microcolony formation. As different cell types are known to express different amounts of each tail isoform, this is one possible explanation for the staining differences in our data.

The CD46 Cyt2 tail isoform is noticeably absent underneath GC induced cortical plaques

We repeated the experiments in our tested cell lines to look for the presence of the Cyt2 tail isoform of CD46 in cortical plaques (Figure 3). CD46 Cyt2 was noticeably absent from the cortical plaques in some of our tested cell lines. Upon closer examination, we discovered that staining infected cells with CD46 Cyt2 produced a zone of negative staining, present directly underneath the adhered microcolony. This "exclusion" phenotype was observed in Hec1B, End1, and HjCE cells (Table 2). Conjunctival cells showed the most pronounced negative zone underneath sites of GC infection.

To examine this further, we repeated the experiments with the same conditions using a commercially available antibody clone that recognizes the ectodomain of CD46, and found appreciable clustering. As the commercial antibody was monoclonal, we could not co-stain with the Cyt2 specific antibody. However, when the slides were costained with a polyclonal antibody to phospho-ezrin, cortical plaques were visible and phospho-ezrin accumulation was evident in the regions that were negatively stained using the CD46 Cyt2 antibody.

Descriptions of staining using the CD46 cytoplasmic specific antibodies or the commercially available ectodomain CD46 antibody are summarized in Table 2.

Discussion

In this report we describe the first use of newly generated monoclonal antibodies directed against the two major cytoplasmic tail isoforms of CD46. Our data indicate that CD46 cytoplasmic tail isoforms are most likely expressed in different amounts depending on the cell line. This is consistent with previous studies showing differential expression of CD46 ectodomains in various cell types. Furthermore, cortical plaques induced by GC showed differential staining of CD46 cytoplasmic tails, suggesting that the two cytoplasmic tails likely play distinct roles during infection.

It has been reported that adhesion of pathogenic *Neisseria* is dependent on expression of the cytoplasmic domain of CD46 (Kallstrom et al., 2001). However, the mechanism by which each individual tail isoform promotes infection is unclear. The importance of Neisserial signaling through CD46 was demonstrated previously (Lee et al., 2002). Because Cyt2 is tyrosine phosphorylated by c-yes, the localization of c-yes to sites of infection may recruit other signaling proteins to generate downstream signals important for host cell invasion. Notably, c-yes contains a src-homology (SH) domain, which can recruit many proteins through binding interactions. Many candidate proteins such as PI-3 kinase contain SH domains. It will be

interesting to determine whether other proteins that are recruited either by kinases or by CD46 activation will play a role in the overall infection process.

One possible explanation for the complete absence of staining of the CD46 Cyt2 tail in our cortical plaques is that following infection, CD46 binds signaling proteins that mask the epitope domain of our Cyt2 monoclonal antibody. This is supported by our data which show that CD46 remains clustered in cortical plaques using identical conditions when stained with an ectodomain antibody. Numerous signaling proteins have been found associated with the Cyt2 tail, including src family kinases Lck, c-yes, and various macrophage kinases (Wang et al., 2000; Wong et al., 1997; Lee et al., 2002).

Another possibility is that the CD46 Cyt2 tail undergoes a proteolytic event that releases the cytoplasmic tail from the membrane. The exact function of this is unclear; however, it is known that bacteria secrete proteases that can act on host cell proteins (Farley et al., 1986; Hauck and Meyer, 1997; Klauser et al., 1993; Lin et al., 1997; Vandeputte-Rutten and Gros, 2002). Thus it remains possible that a bacterial protease could modify local concentrations of CD46 at the surface to change its membrane distribution.

Additionally, host proteases such as secretases might also be recruited to sites of infection to activate cleavage (Steiner et al., 2000). Future experiments will clarify the role of CD46 as a potential proteolytic substrate in the overall process of *Neisseria* infection.

CD46 staining in cortical plaques using Cyt1 antibodies was visible, but periodic, and the intensity of the signal was variable depending on the cell type. The appearance of periodic staining might represent a pool of CD46 Cyt1 that have not complexed to other proteins, and/or have complexed to proteins that do not mask the epitope recognized by our CD46 Cyt1 antibody. One possibility is that CD46 Cyt1 recruits or binds a PDZ-containing protein such as Dlg4 in our cell lines that bridge CD46 to other PDZ binding proteins such as ezrin. This is an attractive hypothesis, as ezrin has been identified previously as a component of cortical plaques (Merz et al., 1999). Furthermore, all of the images in our immunofluorescence data were costained with a polyclonal antibody that recognizes the phosphorylated form of ezrin as a positive control. It will be interesting to determine if CD46, ezrin and other PDZ-proteins form complexes in response to infection, and whether the nature and timeline of this interaction promotes infectivity.

In summary, we report here the construction and use of monoclonal antibodies generated using synthesized peptides specific to the cytoplasmic tail isoforms of CD46. Staining with CD46 Cyt1 specific antibody revealed periodic clustering of Cyt1 isoforms of CD46 underneath GC-induced plaques, while CD46 Cyt2 staining was noticeably absent from GC-induced plaques. Furthermore, different cell lines displayed different staining patterns of the indivual CD46 tail domains, in apprearance and intensity. These data indicate that the two cytoplasmic CD46 tails likely play distinct roles in *Neisseria* infection. Cell type-specific expression of the cytoplasmic isoforms of CD46 may also influence the role of CD46 in overall *Neisseria* pathogenesis.

Materials and Methods

Cell lines, bacterial strains and infections

All cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) at 37°C (Life Technologies) and 5% CO_2 unless otherwise recommended by ATCC. Antibodies to phospho-ezrin were purchased from Cell signaling technologies. CD46 ectodomain antibody J4-48 was obtained from Immunotech.

N. gonorrhoeae strain MS11 (Segal et al., 1986; Wolfgang et al., 1998) was used for all infection experiments. Piliation and Opa phenotypes were monitored by colony morphology.

Construction of CD46 Cyt 1 and Cyt2 monoclonal antibodies

Peptides encoding the CD46 cytoplasmic tail 1 and tail 2 isoforms were synthesized by Global Peptide Services (Fort Collins, CO) for use in raising antibodies specific for each tail subunit.

The sequences of the Cyt1 and Cyt2 peptides generated are listed in Table 1. The peptides were purified to 95% purity by HPLC and each peptide was

conjugated to KLH. These peptides were subsequently used directly for immunization without further modifications.

The peptide immunogen was inoculated into four BALB/c mice, followed by booster inoculations at determined intervals. At approximately four weeks, serum samples were collected from each mouse and tested by ELISA. Positive results were followed with continued boosters until titers were deemed acceptable for hybridoma fusions. The mouse with the highest titer antibody response was selected for splenectomy, polyethylene glycol fusion and hybridoma selection. All hybridomas were screened by ELISA, and cell lines demonstrating specific IgG antibody production against the immunogen was cryopreserved. These parent cell lines were selected for cloning and antibody production. All antibodies were purified using protein A chromatography (1M NH_4SO_4 binder buffer, pH 9.6, 0.1 M glycine elution buffer, pH 2.8), and finally diluted for use in PBS (0.1M, pH 7.2).

Immunofluorescence microscopy

A431 cells were grown on coverslips to 30-50% confluence and infected for 3h with wt MS11 (Wolfgang et al., 1998) at an MOI of 100. Coverslips were washed 3X in PBS, fixed for 20 min at room temperature in 4%

paraformaldehyde and blocked for 1h in isotonic PBS containing 3%(v/v) normal goat serum (Gibco BRL) and 0.03% saponin (Sigma). Primary antibodies were diluted 1:100 for all of the CD46 cytoplasmic antibodies in blocking buffer, added to samples and incubated overnight at 4°C in a moist chamber. The coverslips were rinsed in PBS and incubated with an Alexa-488 or Alexa-572 conjugated secondary antibody (Molecular Probes) diluted 1:1000 in blocking buffer for 1h at 25°C. The cells were also incubated with DAPI for 10 min at 25°C to visualize the bacteria and nuclei. Samples were rinsed extensively in PBS before mounting in Fluromount-G (Fisher Scientific). 0.2 μm optical sections in the Z-axis plane were obtained with a Nikon 60X oil immersion objective and the images were processed using a Deltavision Restoration Microscope (Applied Precision Instruments, Inc., Issaquah, WA) and Silicon graphics workstation with accompanying API software. The images were subsequently exported to Adobe Photoshop and Adobe Illustrator for manuscript preparation.

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References

Adams, E. M., Brown, M. C., Nunge, M., Krych, M., and Atkinson, J. P. (1991). Contribution of the repeating domains of membrane cofactor protein (CD46) of the complement system to ligand binding and cofactor activity. J Immunol *147*, 3005-3011.

Ayala, P., Lin, L., Hopper, S., Fukuda, M., and So, M. (1998). Infection of epithelial cells by pathogenic neisseriae reduces the levels of multiple lysosomal constituents. Infect Immun *66*, 5001-5007.

Dorig, R. E., Marcil, A., Chopra, A., and Richardson, C. D. (1993). The human CD46 molecule is a receptor for measles virus (Edmonston strain). Cell *75*, 295-305.

Farley, M., Stephens, D., Mulks, M., Cooper, M., Bricker, J., Mirra, S., and Wright, A. (1986). Pathogenesis of IgA1 Protease-Producing and - Nonproducing *Haemophilus influenzae* in Human Nasopharyngeal Organ Cultures. J Infect Dis *154*, 752-759.

Fortini, M. E. (2002). Gamma-secretase-mediated proteolysis in cell-surface-receptor signalling. Nat Rev Mol Cell Biol *3*, 673-684.

Gaggar, A., Shayakhmetov, D. M., and Lieber, A. (2003). CD46 is a cellular receptor for group B adenoviruses. Nat Med *9*, 1408-1412.

Hauck, C. R., and Meyer, T. F. (1997). The lysosomal/phagosomal membrane protein h-lamp-1 is a target of the IgA1 protease of Neisseria gonorrhoeae. FEBS Lett *405*, 86-90.

Hoffmann, I., Eugene, E., Nassif, X., Couraud, P. O., and Bourdoulous, S. (2001). Activation of ErbB2 receptor tyrosine kinase supports invasion of endothelial cells by Neisseria meningitidis. J Cell Biol *155*, 133-143.

Iwata, K., Seya, T., Yanagi, Y., Pesando, J. M., Johnson, P. M., Okabe, M., Ueda, S., Ariga, H., and Nagasawa, S. (1995). Diversity of sites for measles virus binding and for inactivation of complement C3b and C4b on membrane cofactor protein CD46. J Biol Chem *270*, 15148-15152.

Johansson, L., Rytkonen, A., Bergman, P., Albiger, B., Kallstrom, H., Hokfelt, T., Agerberth, B., Cattaneo, R., and Jonsson, A. B. (2003). CD46 in meningococcal disease. Science *301*, 373-375.

Kallstrom, H., Blackmer Gill, D., Albiger, B., Liszewski, M. K., Atkinson, J. P., and Jonsson, A. B. (2001). Attachment of Neisseria gonorrhoeae to the cellular pilus receptor CD46: identification of domains important for bacterial adherence. Cell Microbiol *3*, 133-143.

Kallstrom, H., Islam, M. S., Berggren, P. O., and Jonsson, A. B. (1998). Cell signaling by the type IV pili of pathogenic Neisseria. J Biol Chem *273*, 21777-21782.

Kallstrom, H., Liszewski, M. K., Atkinson, J. P., and Jonsson, A. B. (1997). Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic Neisseria. Mol Microbiol *25*, 639-647.

Klauser, T., Pohlner, J., and Meyer, T. F. (1993). The secretion pathway of IgA protease-type proteins in gram-negative bacteria. Bioessays *15*, 799-805.

Lee, S. W., Bonnah, R. A., Higashi, D. L., Atkinson, J. P., Milgram, S. L., and So, M. (2002). CD46 is phosphorylated at tyrosine 354 upon infection of epithelial cells by Neisseria gonorrhoeae. J Cell Biol *156*, 951-957.

Lin, L., Ayala, P., Larson, J., Mulks, M., Fukuda, M., Carlsson, S. R., Enns, C., and So, M. (1997). The Neisseria type 2 IgA1 protease cleaves LAMP1 and promotes survival of bacteria within epithelial cells. Mol Microbiol *24*, 1083-1094.

Liszewski, M. K., and Atkinson, J. P. (1996). Membrane cofactor protein (MCP; CD46). Isoforms differ in protection against the classical pathway of complement. J Immunol *156*, 4415-4421.

Liszewski, M. K., Leung, M. K., and Atkinson, J. P. (1998). Membrane cofactor protein: importance of N- and O-glycosylation for complement regulatory function. J Immunol *161*, 3711-3718.

Liszewski, M. K., Post, T. W., and Atkinson, J. P. (1991). Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. Annu Rev Immunol *9*, 431-455.

Liszewski, M. K., Tedja, I., and Atkinson, J. P. (1994). Membrane cofactor protein (CD46) of complement. Processing differences related to alternatively spliced cytoplasmic domains. J Biol Chem *269*, 10776-10779.

Lozahic, S., Christiansen, D., Manie, S., Gerlier, D., Billard, M., Boucheix, C., and Rubinstein, E. (2000). CD46 (membrane cofactor protein) associates with multiple beta1 integrins and tetraspans. Eur J Immunol *30*, 900-907.

Lublin, D. M., and Atkinson, J. P. (1989). Decay-accelerating factor: biochemistry, molecular biology, and function. Annu Rev Immunol *7*, 35-58.

Ludford-Menting, M. J., Thomas, S. J., Crimeen, B., Harris, L. J., Loveland, B. E., Bills, M., Ellis, S., and Russell, S. M. (2002). A functional

interaction between CD46 and DLG4: a role for DLG4 in epithelial polarization. J Biol Chem *277*, 4477-4484.

Merz, A. J., Enns, C. A., and So, M. (1999). Type IV pili of pathogenic neisseriae elicit cortical plaque formation in epithelial cells. Mol Microbiol *32*, 1316-1332.

Merz, A. J., and So, M. (1997). Attachment of piliated, Opa- and Opc- gonococci and meningococci to epithelial cells elicits cortical actin rearrangements and clustering of tyrosine-phosphorylated proteins. Infect Immun *65*, 4341-4349.

Nassif, X., Lowy, J., Stenberg, P., O'Gaora, P., Ganji, A., and So, M. (1993). Antigenic variation of pilin regulates adhesion of Neisseria meningitidis to human epithelial cells. Mol Microbiol *8*, 719-725.

Post, T. W., Liszewski, M. K., Adams, E. M., Tedja, I., Miller, E. A., and Atkinson, J. P. (1991). Membrane cofactor protein of the complement system: alternative splicing of serine/threonine/proline-rich exons and cytoplasmic tails produces multiple isoforms that correlate with protein phenotype. J Exp Med *174*, 93-102.

Russell, S. M., Loveland, B. E., Johnstone, R. W., Thorley, B. R., and McKenzie, I. F. (1992). Functional characterisation of alternatively spliced CD46 cytoplasmic tails. Transplant Proc *24*, 2329-2330.

Santoro, F., Kennedy, P. E., Locatelli, G., Malnati, M. S., Berger, E. A., and Lusso, P. (1999). CD46 is a cellular receptor for human herpesvirus 6. Cell *99*, 817-827.

Segal, E., Hagblom, P., Seifert, H. S., and So, M. (1986). Antigenic variation of gonococcal pilus involves assembly of separated silent gene segments. Proc Natl Acad Sci U S A *83*, 2177-2181.

Steiner, H., Kostka, M., Romig, H., Basset, G., Pesold, B., Hardy, J., Capell, A., Meyn, L., Grim, M. L., Baumeister, R., *et al.* (2000). Glycine 384 is required for presenilin-1 function and is conserved in bacterial polytopic aspartyl proteases. Nat Cell Biol *2*, 848-851.

Swanson, J. (1973). Studies on gonococcus infection. IV. Pili: their role in attachment of gonococci to tissue culture cells. J Exp Med *137*, 571-589.

Vandeputte-Rutten, L., and Gros, P. (2002). Novel proteases: common themes and surprising features. Curr Opin Struct Biol *12*, 704-708.

Wang, G., Liszewski, M. K., Chan, A. C., and Atkinson, J. P. (2000). Membrane cofactor protein (MCP; CD46): isoform-specific tyrosine phosphorylation. J Immunol *164*, 1839-1846.

Wolfgang, M., Lauer, P., Park, H. S., Brossay, L., Hebert, J., and Koomey, M. (1998). PilT mutations lead to simultaneous defects in competence

for natural transformation and twitching motility in piliated Neisseria gonorrhoeae. Mol Microbiol *29*, 321-330.

Wong, T. C., Yant, S., Harder, B. J., Korte-Sarfaty, J., and Hirano, A. (1997). The cytoplasmic domains of complement regulatory protein CD46 interact with multiple kinases in macrophages. J Leukoc Biol *62*, 892-900.

Table 1. Sequences of CD46 Cyt1 and Cyt2 peptides used to generate monoclonal antibodies.

TABLE 1

CD46 Cytoplasmic Tail Sequences Used to Generate Monoclonal Antibodies
CD46 Cyt1 NH_2-(CG)PYRYLQRRKKKGTYLTDETHREVKFTSL-COOH CD46 Cyt2 NH_2-(CG)PYRYLQRRKKKGKADGGAEYATYQTKSTT-COOH

Figure 1. CD46 monoclonal cytoplasmic antibodies generate distinct bands between 50-75 kDa. A431 cell lysates were loaded onto SDS polyacrylamide gels and probed with either CD46 Cyt 2 specific antibody clones (13G10, 5H8, 8D4) or Cyt1 antibody clone (2F-1).

FIGURE 1

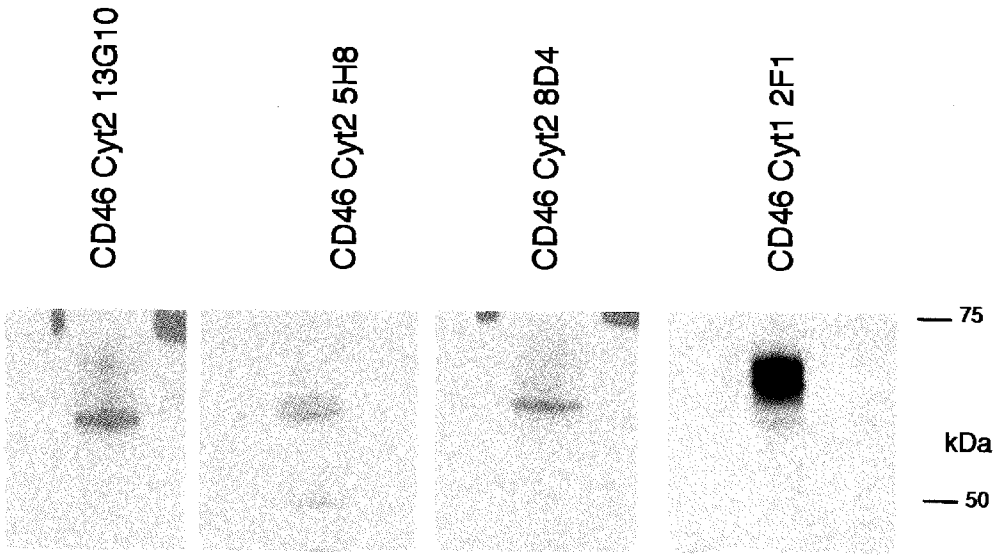

CD46 Cyt2 13G10
CD46 Cyt2 5H8
CD46 Cyt2 8D4
CD46 Cyt1 2F1
75
kDa
50

Figure 2. Cortical plaque staining of GC microcolonies in infected HjCE cells with CD46 Cyt1-specific antibody 2F-1. Cells were also stained with the CD46 monoclonal antibody J448 that recognizes the ectodomain of CD46 isoforms. HjCE and Hec1B cells were infected with wt MS11 for 3h, fixed, and processed for immunofluorescence deconvolution microscopy. Phospho-Erm staining is depicted in red. Images were obtained using a Deltavision restoration microscope (API) with a 60x oil objective.

FIGURE 2

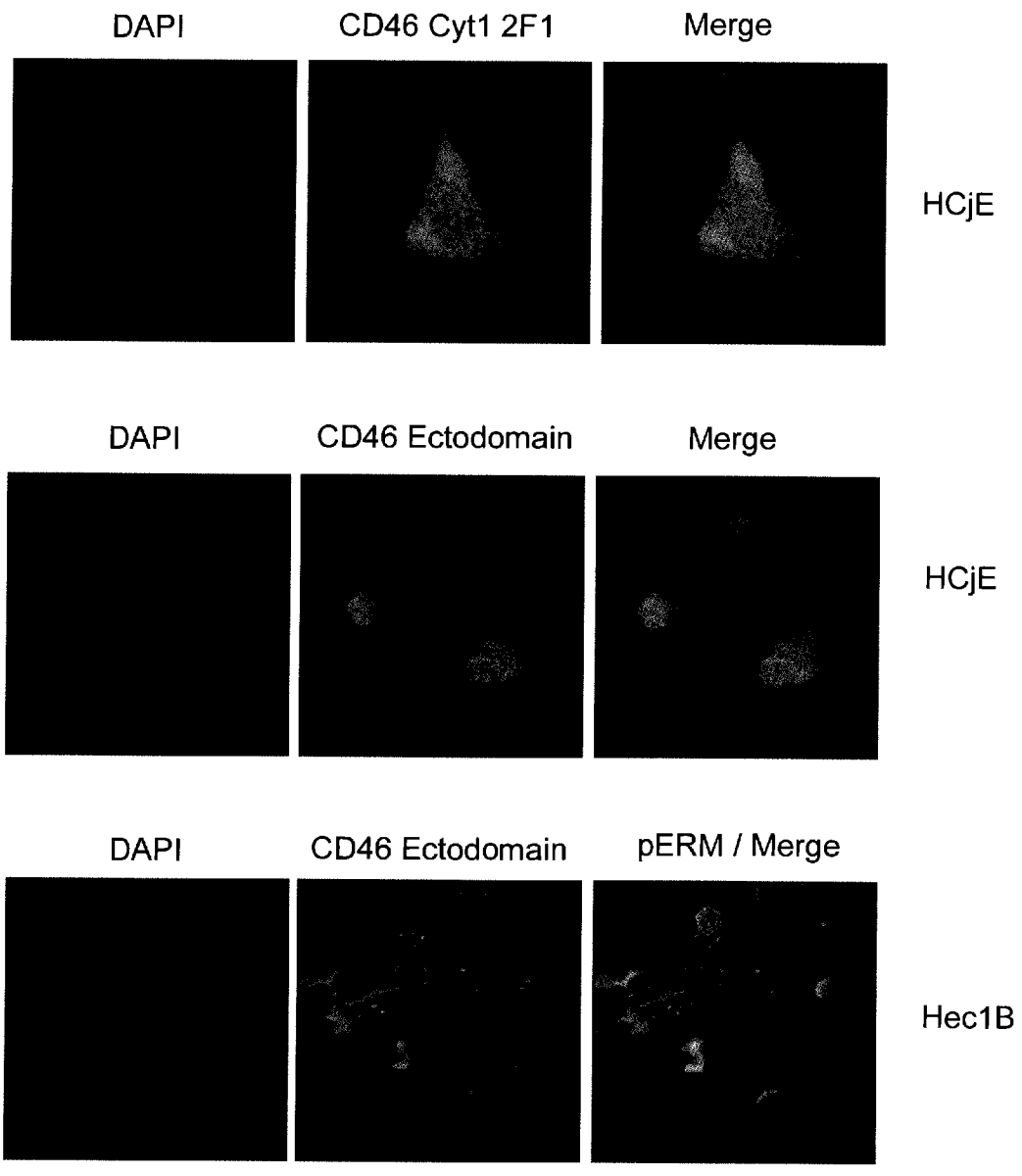

DAPI
CD46 Cyt1 2F1
Merge
HCjE
DAPI
CD46 Ectodomain
Merge
HCjE
DAPI
CD46 Ectodomain
pERM / Merge
Hec1B

Figure 3. Cortical plaque staining of GC microcolonies in infected cell lines with CD46 Cyt2-specific antibody clones 8D4, 13G10, and 5H8. HjCE and Hec1B cells were infected with wt MS11 for 3h, fixed, and processed for immunofluorescence deconvolution microscopy. Phospho-Erm staining is depicted in red. Images were obtained using a Deltavision restoration microscope (API) with a 60x oil objective.

FIGURE 3

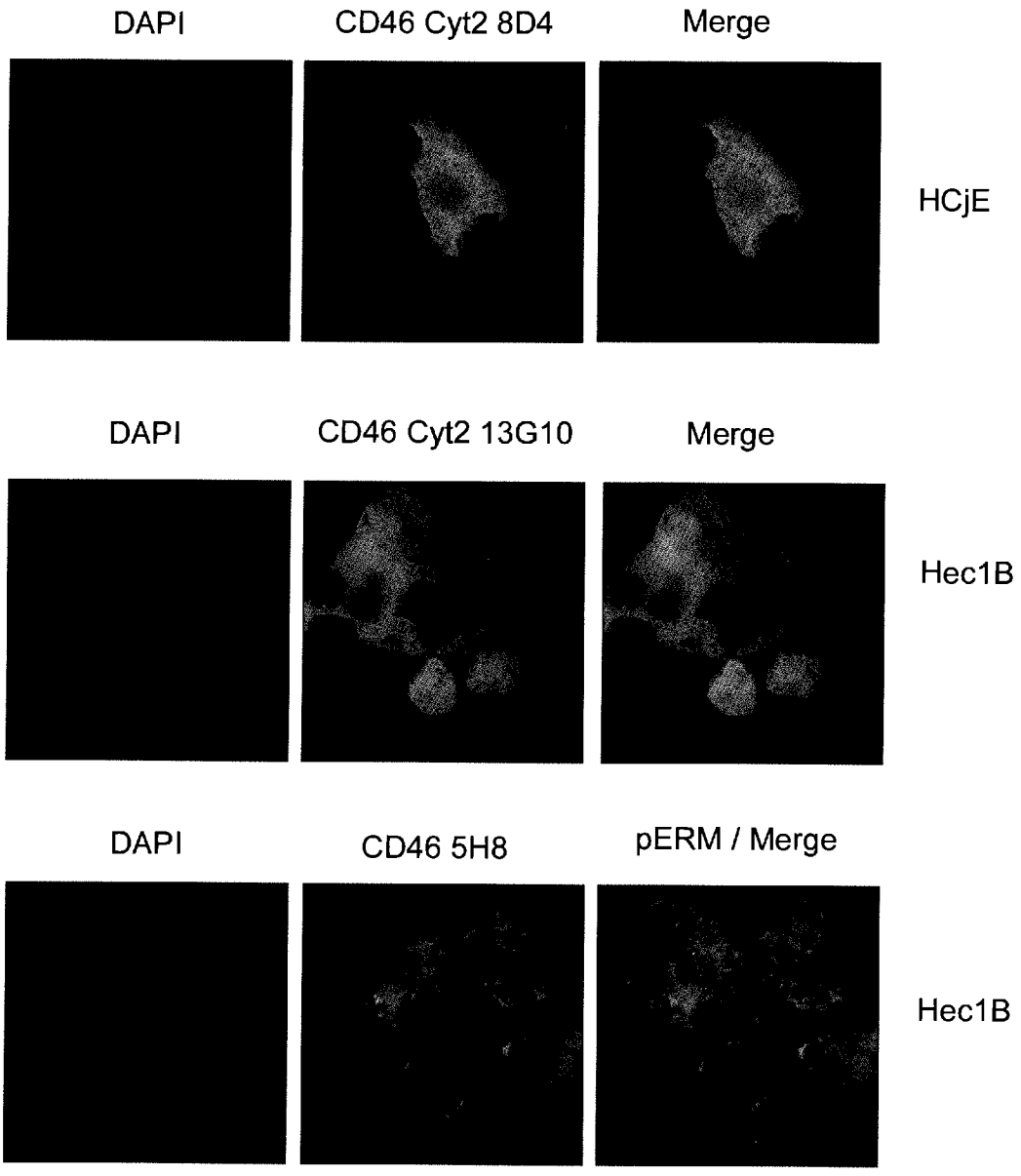

DAPI
CD46 Cyt2 8D4
Merge
HCjE
DAPI
CD46 Cyt2 13G10
Merge
Hec1B
DAPI
CD46 5H8
pERM / Merge
Hec1B

Table 2. Summary of reactivities of the CD46 monoclonal antibodies to various cell lines by immunofluorescence microscopy. (+) indicates strong clustering in GC-induced cortical plaques. (-) indicates weak clustering phenotype. (E) indicates exclusion in cortical plaques, showing prominent negative staining for CD46 underneath GC-induced cortical plaques.

TABLE 2

Cell Type	CD46 Antibody	Cortical Plaque formation
A431	Cyt1- 2F1	+, Variable
	Cyt2-13G10	+, Variable
	Cyt2- 8D4	-
	Cyt2- 5H8	-
End1	Cyt2- 8D4	E
Hec1B	Ectodomain-J4.48	+
	Cyt1-2F1	-
	Cyt2-13G10	E
	Cyt2- 5H8	E
	Cyt2-8D4	E
HCjE	Ectodomain-J4.48	+, Very robust
	Cyt1-2F1	+
	Cyt2-13G10	E
	Cyt2- 5H8	E
	Cyt2-8D4	E

Chapter 5: Conclusions

5.1. *Neisseria gonorrhoeae* type IV pili trigger signaling cascades

The work presented in this thesis demonstrate that *Neisseria gonorrhoeae* type IV pili induce host cell signaling that play important roles in overall infectivity. As discussed in manuscript I, infection of human epithelial cells by piliated *N. gonorrhoeae* (GC) leads to rapid tyrosine phosphorylation of the CD46 cyt 2 isoform, at tyrosine 354. It was further demonstrated that the src-family kinase c-yes is involved in this reaction. Inhibitors of src-family kinase decreased bacterial adhesion by 50%, demonstrating that c-yes activation is an important event in GC infection. These findings support a model in which pilus interaction with CD46 initiates signaling cascades important for *Neisseria* infectivity.

In manuscript II, GC infection of human epithelial cells was shown to activate the PI-3 kinase/Akt (PKB) pathway. Moreover, pilus retraction greatly enhanced this activity. [PI(3,4,5)P3], a host lipid product of activated PI-3 kinase, was found located directly underneath sites of bacterial attachment. Addition of [PI(3,4,5)P3] to GC cultures had a direct effect on

bacterial motility-associated behavior, and increased transcription of the GC *pilT* gene. The data presented in this manuscript demonstrate that retractile force generated by GC type IV pili can exert sufficient membrane tension to trigger a specific signaling cascade in host cells. Importantly, it presents evidence that the eukaryotic chemical [PI(3,4,5)P3] generated through activation of this signaling pathway can cross species to communicate directly with the bacteria to alter their behavior.

In this chapter, I discuss some recent findings by other groups that support and further the work presented here, and propose some major emerging themes in bacteria-host interations important to my thesis work.

5.2 GC Type IV pili "touch" host cells: early events in signaling

As demonstrated in Manuscript I, CD46 phosphorylation occurs within 5 minutes after GC infection, and is deactivated 30 min post infection. This is consistent with findings reported by Wang et al., in which CD46 Cyt2 tail tyrosine phosphorylation in Jurkat cells occurs minutes after antibody ligation and is completely absent by 20 min, likely through the action of tyrosine phosphatases (Wang et al., 2000). Recently, a report by Ting et al. used genetically encoded FRET (Fluorescence resonance energy transfer)-based reporters to observe the motion of real-time tyrosine phosphorylation of a cell in response to outside ligand (Ting et al., 2001). The genetic reporters consisted of a fusion protein containing: a cyan fluorescent protein (CFP) at one end, a phosphotyrosine binding domain (SH2), a linker sequence, a consensus substrate for the relevant kinase, and a yellow fluorescent protein (YFP) at the opposite end. Phosphorylation of the reporter construct by a tested kinase results in a conformational "bending"of the reporter protein, bringing the two arms containing the CFP and YFP together, culminating in an observable FRET response. When B82 mouse fibroblasts were transfected with the reporter construct containing a consensus substrate for the tyrosine kinase EGFR (epidermal growth factor

receptor) and stimulated with exogenous EGF, maximal kinase activity was measured to occur 5 min after ligand addition, and decayed to near baseline levels by 30 minutes after addition.

Taken together, these findings suggest that *Neisseria gonorrhoeae* trigger an early phosphorylation event with similar spatiotemporal profiles as those mediated by normal cellular signaling events.

5.2.1 Calcium: The first wave

Pilus addition to epithelial cells has been shown to induce a Ca^{2+} response (Kallstrom et al., 1998). This response depended on interactions with CD46, as blocking pilus interactions with CD46 were shown to inhibit calcium signaling. It was further demonstrated that depletion of intracellular Ca^{2+} stores reduced GC adhesion to host epithelial cells. Data obtained in our lab suggest *Neisseria* can induce more than one Ca^{2+} flux. Pili and the GC outer membrane protein porins induced different and independent Ca^{2+} responses in epithelial cells (Ayala et al., 2002). An early Ca^{2+} flux was observed 1-2 minutes after the addition of purified porins to the host cell, while purified pili induced a calcium release 10 min post addition. Pili-induced calcium activity had a relatively slow rate of decay (basal levels restored – 10 min)

while the calcium spike in response to porins was rapid, had a narrow peak, and was immediately inactivated.

These data suggest that earliest signaling events (within minutes) in *Neisseria gonorrhoeae* infection may involve an extracellular calcium influx from the action of porins. Porins are trimeric ion channels that can insert into host membranes without assistance from colonizing bacteria to induce a calcium influx (Massari et al., 2003; Muller et al., 1999).

It is possible that this early Ca^{2+} event is required to prime kinases for activation of other proteins. We plan to test this hypothesis, by using selective calcium inhibitors to block a Ca^{2+} signal during infection, and determining whether the activation of host kinases such as c-yes would require an initial Ca^{2+} response. We can also determine whether calcium-dependent kinases, such as protein kinase C (PKC), serve as the intermediary between a calcium response and the activation of kinases such as c-yes that are not strictly dependent on calcium activity. Finally, we can determine the importance of the initial Ca^{2+} response in the phosphorylation of CD46 by GC. We speculate that suppression of c-yes or other src-kinase activation through inhibition of Ca^{2+} may result in lower levels of phosphorylation of CD46 by GC upon infection.

5.2.2 Calcium: the second wave

The second sustained calcium response involves intracellular signaling and the moblilization of internal calcium stores. The second Ca^{2+} response is likely mediated through CD46 signaling, as data by Kallstrom et al. indicate that this is most likely the Ca^{2+} response inhibited by blocking pilus interaction with CD46 (Kallstrom et al., 1998).

It is not yet clear how this second calcium response is influenced by any antecedent signals, nor how this second calcium spike influences downstream signaling events in *Neisseria* infection. It may be possible that an "early event" kinase such as c-yes, once activated, can modulate downstream kinases, such as phospholipase C (PLC) , that generate IP-3 (Inositol 3-phosphate) that in turn activate an intracellular-stored calcium response. It is also possible that the initial first wave of calcium can have a direct role in activating the second wave of calcium through calcium-activated calcium channels. The specific paths by which GC utilize these calcium responses to promote pathogenicity will give valuable insight and fill important gaps in signaling responses elicited by pathogenic *Neisseria*.

5.2.3 CD46 cytoplasmic tails: different roles, different partners

In manuscript III, isoform-specific differences in CD46 clustering to cortical plaques were examined using monoclonal antibodies generated against the two cytoplasmic tail domains. Cytoplasmic tail 1 and tail 2 isoforms of CD46 were found to exhibit different levels of clustering in GC-induced cortical plaques. Furthermore, the appearance of each CD46 cytoplasmic variant in GC-induced cortical plaques differed depending on the cell type infected. The noticeable absence of CD46 Cyt2 staining evident in some of the tested cell lines can be explained by the following possibilities. The CD46 Cyt2 isoform undergoes tail-specific proteolysis during infection, and this cleavage event is localized to the site of GC attachment, resulting in the absent Cyt2 phenotype by antibody staining. Alternatively, the Cyt2 epitope recognized by our antibodies may be masked by the interaction with kinase complexes recruited to sites of infection. Co-immunoprecipitation and previous data certainly support this possiblity.

In contrast, the Cyt1 tail, was shown to plaque in several cell lines in response to GC infection. It is possible that CD46 Cyt1 also undergoes protein-protein interactions, but that the epitope recognized by our antibodies remains exposed. Despite putative kinase sites, CD46 Cyt1 has

only been shown to interact *directly* with one protein, Dlg4 (Ludford-Menting et al., 2002). The Dlg family of proteins contain three PDZ (Post-synaptic density 95/ *Discs Large*/ZO-1) domains- protein-protein interaction domains- that serve to interact with membrane bound receptors and the cytoskeleton to regulate the polarization of receptors and coordinate signaling networks. The sequence of the last four amino acids of CD46 Cyt 1, FTSL, follows the sequence motif required for interacting with PDZ proteins, X-S/T-X-I/L/V. The binding domain motif, FTSL is located at the extreme end of the Cyt1 tail, and thus it remains possible that this interaction does not affect recognition by the Cyt1-specific antibody. We have performed *in vitro* binding assays and confirmed this using IKEPP, an intestinal and kidney enriched PDZ containing protein (Thelin, W. , Lee, S., Milgram, S., and So, M. unpublished data- see Figure 1). Given this information, it is likely that a PDZ-containing protein that is yet unidentified interacts with the Cyt1 tail of CD46 in our cell lines.

One attractive candidate for a PDZ-domain containing protein is EBP50 (Ezrin binding protein-50). EBP50 contains three PDZ domains and has been shown to interact with ezrin, a known component of cortical plaques (Reczek et al., 1997; Reczek and Bretscher, 1998). This would allow the

following chain of structural connections. Pili would bind to CD46, and CD46 cyt1 would be localized in cortical plaques and bind to EBP50. EBP50 would bind to ezrin, which would link the complex to the actin cytoskeleton and therefore stabilize the growing microvilli around the microcolony.

This hypothesis is supported by findings that show CD46 associates with the linker molecule moesin, a linker protein related to ezrin and part of the ERM (ezrin-radixin-moesin) family of proteins that serve to tether membrane proteins to the actin cytoskeleton (Schneider-Schaulies et al., 1995). An interesting question is whether the interaction between CD46, a PDZ-linker protein such as EBP50, and a cytoskeletal linker protein such as ezrin engage in complex formation specifically in response to GC infection, and how other signaling processes modulate this event.

That CD46 may play tail-specific roles in *Neisseria* infection has also been proposed recently by experiments demonstrating that transgenic mice expressing human CD46 were found susceptible to meningococcal infection (Johansson et al., 2003). They discovered that piliated *Neisseria meningitidis* was found to cause more severe disease than nonpiliated strains in intranasal

challenge of CD46 transgenic mice, supporting the notion that CD46-pilus interactions serve a critical first step in crossing the blood-brain barrier and establishing meningococcal disease. They propose that since CD46 Cyt2 is preferentially expressed in brain tissue, it is possible that signaling by the Cyt2 tail, or the enhanced inflammatory response that is specific to CD46 Cyt2 activation (Marie et al., 2002) may play a significant role in meningococcal infection.

These findings suggest that the cytoplasmic variants of CD46 contribute important but distinctive roles in mediating *Neisseria* infection. The generation of monoclonal antibodies described in manuscript III that discriminates between the two CD46 cytoplasmic isoforms will thus serve as important tools for evaluating the specific mechanisms these tails contribute to the infection process of *Neisseria.*

5.3 GC Type IV pili "pull" on host cells: middle events in signaling

As described in manuscript II, piliated GC activate the PI-3 kinase/Akt (PKB) signaling pathway. This activity was reduced in nonretractile *pilT* mutants, and abolished in the presence of 10 nM wortmannin. The activation of Akt was restored in an inducible *pilT* mutant upon inducing expression of

the *pilT* gene, supporting the hypothesis that PI-3 kinase activation by piliated GC involves signaling through membrane tension generated by the process of pilus retraction.

Akt activity was detected 30 min after infection, and this activity was sustained over 3 hours. At this time, robust cortical plaques are evident in host cells directly underneath the sites of infection. Activated PI-3 kinase and Akt also accumulate at the site of infection. [PI(3,4,5)P3], a product of activated PI-3 kinase, also clusters at these sites, consistent with the notion that activation of PI-3 kinase generates a local concentration of [PI(3,4,5)P3], at sites of adherent GC microcolonies. It is intriguing that PI-3 kinase, Akt and [PI(3,4,5)P3], all exhibit similar ring-like patterns around the adherent microcolony. These ring-like staining patterns interestingly resemble plasma membrane "phagocytic cups"- regions of [PI(3,4,5)P3] localization that were identified using a PH (pleckstrin homology) domain containing GFP fusion proteins to probe the location of [PI(3,4,5)P3] at the plasma membrane (Devreotes and Janetopoulos, 2003).

5.3.1 Why is PI3/Akt activity in GC infection sustained over hours?

[PI(3,4,5)P3] directly influenced the aggregation of GC in culture (manuscript II). [PI(3,4,5)P3] incubation was also demonstrated to increase

transcription of the *pilT* gene. Thus, we can hypothesize that one purpose of prolonged PI-3 kinase /Akt activation is to sustain a constant level of [PI(3,4,5)P3] at the sites of bacterial adhesion.

This would have two important consequences. One possibility is that increased local concentrations of lipid would promote localized adhesion by functioning as a receptor for the bacteria. This hypothesis is supported by data from other systems suggesting that lipids serve as receptors for *Helicobacter pylori, Chlamydia trachomatis, Campylobacter upsaliensis* (Busse et al., 1997; Huesca et al., 1996; Lingwood et al., 1992; Sylvester et al., 1996). As bacteria continue to engage host cells, localized production of [PI(3,4,5)P3] at sites of adhesion would be an effective way to generate a bacterial receptor. As more bacteria bind, the retractile force generated would continue to be sustained, and thus more [PI(3,4,5)P3] would be generated through PI-3 kinase activation. This mode of prolonged activation of PI-3 kinase has been recently observed in the process of T cell activation, where antigen presenting cells (APC) maintain contact with T cells for several hours through peptide major histocompatibility (MHC) complexes on the cell surface (Costello et al., 2002). Costello et al. observed that the production of [PI(3,4,5)P3] was sustained for hours as T cells responded to antigen, and that elevated levels of [PI(3,4,5)P3] needed to be maintained

for T cell proliferation. Similar to the local clustering of [PI(3,4,5)P3] that was observed in our GC-induced cortical plaques, [PI(3,4,5)P3] accumulation was also seen directly at the T cell-APC contact zone. Another consequence of [PI(3,4,5)P3] buildup is that [PI(3,4,5)P3] could function as a bacterial chemoattractant, its accumulation at sites of infection creating a gradient that is recognized by the colonizing bacteria. A number of studies support this second hypothesis. Certain lipid species are chemoattractants for bacteria that exhibit twitching motility. *Myxococcus xanthus* migrates towards gradients of certain types of PE (phosphatidylethanolamine), and other PE moieties enhance the twitching velocity of the *Pseudomonas aeruginosa* type IV pilus (Kearns and Shimkets, 1998). PE also induces aggregation of Enteropathogenic and Enterohemorrhagic *E. coli* (Khursigara et al., 2001). Our data demonstrating the increased transcription of the *pilT* gene in GC upon [PI(3,4,5)P3] incubation lends strong support for this idea. One direct consequence of increased *pilT* expression would be to increase the number of retractile motors at the bacterial surface, which in turn would increase the overall rate of retraction. Increased retraction rates may lead to increases in overall motility or speed, and may explain the greater rate of microcolony formation as well as the size of microcolonies formed in the presence of the lipid.

Increased retractile forces may also activate additional PI-3 kinase which, in turn, would enhance accumulation of [PI(3,4,5)P3] and create a local chemotactic source that would serve to attract other bacteria to the site of a growing microcolony.

5.3.2 GC pull together: microcolonies allow cooperative behavior

In light of this hypothesis, it may be necessary for GC to join as microcolonies in order to cooperatively exert sufficient membrane force on host cells to activate signal cascades (See figure 2). In this model, a single diplococcus would be relatively ineffective in generating membrane tension. Pilus retraction by a single organism would serve to pull the bacteria toward the host cell, since the host cell would remain stationary given the ratio of mass betwen a typical host cell and a single diplococcus. However, as a microcolony grows, the sphere of microcolonies would then serve as an anchor (the tangent point where the microcolony contacts the host cell) providing a pivot from which pulling by bacteria at the edge of the microcolony would then generate sufficient tension. It is intriguing that given this cartoon model, pilus binding and retraction on average would generate tension at a radial circumference from the center of the microcolony. Indeed this is exactly where we observe the localization of

both active forms of PI-3 kinase and Akt, and [PI(3,4,5)P3]—the annular staining.

If [PI(3,4,5)P3] is indeed a bacterial chemoattractant, activation of additional PI-3 kinase in response to pilus retraction from a microcolony would generate higher levels of [PI(3,4,5)P3] locally, thus attracting more bacteria to those sites of infection, effectively increasing the size of the microcolony. This in turn, would generate more membrane tension, activate more PI-3 kinase, create additional [PI(3,4,5)P3], and effectively create a feedback loop. Such a scenario could explain the sustained activation of Akt at the sites of infection. In this context, it is interesting to note that the length of time of PI-3 kinase activation is exactly coincident with the span of time that microcolonies and robust cortical plaques are observed on the infected cell.

5.4 Merging early events in GC infection to middle events

In light of the findings discussed here, we can begin to construct a timecourse of the early steps in GC infection of host cells, and connect a series of signaling events initated by *Neisseria* important for infectivity.

Initial pilus-mediated contact triggers CD46 phosphorylation, which recruits c-yes kinase directly beneath attached bacteria. In a coimmunoprecipitation experiment I show that c-yes is complexed directly or indirectly with PI-3 kinase 30 minutes following infection (Figure 3 Lee, S., and So, M, unpublished data). Since both kinases contain SH (src-homology) interaction domains, it is possible that this association is direct.

C-yes kinase may then be responsible for directing PI-3 kinase to those same sites of infection, where bacteria continue to colonize. Activation of PI-3 kinase would then generate local concentrations of [PI(3,4,5)P3], and some of the [PI(3,4,5)P3] would be translocated to the extracellular face, to be directly recognized by the bacteria to affect aggregative behavior. The middle stage would then proceed (30 min to 3 hours post-infection), with sustained PI-3 kinase activation generating prolonged levels of [PI(3,4,5)P3]. Other proteins would be recruited to the plaques, and cytoskeletal proteins would be recruited and linked to membrane proteins, some of which may be recuited to these sites through interactions with the CD46 Cyt1 tails, allowing microvilli elongation to proceed.

Figure 4 illustrates the stepwise model of GC infection to host epithelial cells as described.

5.5 Bacterial-host communication

The manipulation of host cell signaling pathways by pathogenic bacteria is rapidly becoming an established tenet in biology. Cross-talk between bacteria and the host cell promote bacterial cell entry and intracellular survival in numerous pathogens.

One theme that emerges from the findings presented in this thesis is that bacteria are capable of engaging precise signaling cascades that are important for its infectivity. From this also follows the idea that bacteria can recognize and respond to chemicals specific to the host, as evidenced by the finding that [PI(3,4,5)P3], a host cell second lipid product, can be directly recognized by the bacteria to alter its behavior.

Lending support for this model of bacteria-host cell communication is a recent discovery that the eucaryotic hormones epinephrine and norepinephrine induce quorum sensing among EHEC that regulate expression of virulence factors (Sperandio et al., 2003). The specific mechanism by which a host lipid messenger engages signaling in bacteria has yet to be determined, and further studies on this system will likely present new insights into the role of interspecies signaling crosstalks in bacterial pathogenesis.

5.6 Bacterial homologues (PilD and PilF) in humans

The notion that interspecies communication between bacteria and humans exist is made even more intriguing by recent reports of functional bacterial homologues found in humans.

A gene responsible for the human disorder Bardet-Biedl syndrome (BBS) was recently cloned, and amazingly, found to contain a prokaryotic PilF domain (Ansley et al., 2003). Individuals afflicted with this disease had a deletion mutation in this gene, which disrupted proper ciliary body formation.The PilF homologue, BBS8, localized to centrosomes and basal bodies and interacted with another protein involved in ciliogenesis. This raises the possibility that PilF may contain a universal ATPase motor that is conserved in eukaryotic and prokaryotic systems. Furthermore, it is interesting to speculate that mechanisms that regulate the function of BBS8 in eukaryotic cells may also cross species to affect PilF function in prokaryotes.

Mutations in presenilin have been associated with familial Alzheimer's disease (Selkoe, 1999). Recently, it has been proposed that presenilin-1 may be an aspartyl protease similar to γ-secretase. Steiner et al. identified the

active site of the presenilin protease and unexpectedly found homology to a family of bacterial aspartyl proteases, known as type IV prepilin peptidases (TFPP) (Steiner et al., 2000). Type IV prepilin peptidases have been identified in many gram-negative and gram-positive bacteria species. Interestingly, a prototypic TFPP is PilD, from *P. aeruginosa*, involved in pilin processing (Strom and Lory, 1993).

These findings may serve as valuable clues in revealing potential new insights into the relationships between pathogenic bacteria and their eukaryotic host. They raise the important possibility that commonalities exist between prokaryotic and eukaryotic biological mechanisms that allow bacterial-host interactions to share similarities in language.

5.7 Conclusion

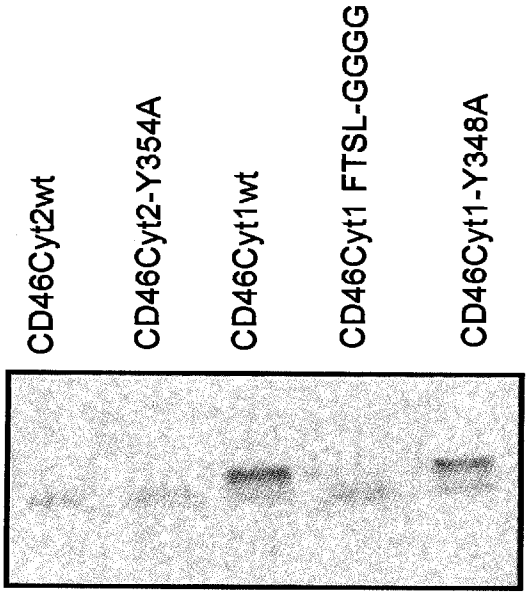
The results presented in this thesis allow important insights into the signaling pathways utilized by pathogenic *Neisseria*. Specifically, I describe how *Neisseria gonorrhoeae* use type IV pili to trigger specific host cell signaling cascades, through receptor-based activation, and through the generation of host cell membrane tension using retractile force, that are important for the infection process. *Neisseria gonorrhoeae* exploit host cell signaling to generate a eukaryotic chemical that crosses species to act directly on infecting bacteria. This may reveal an emerging theme in bacterial-host interactions: the ability of prokaryotes to recognize and respond to eukaryotic signals and chemicals.

Further studies to identify important cellular signaling pathways involved in infection may reveal novel targets for pharmacologic intervention of disease. These studies may therefore also shed light on how Type IV pili may function in other bacterial-host systems.

Figure 1. IKEPP Binds to FTSL motif on CD46 Cyt1 tail. CD46 GST-cytoplasmic tail proteins were loaded onto SDS polyacrylamide gels and incubated with ^{35}S labeled IKEPP (Intestinal and kidney enriched PDZ-Protein) in a protein overlay assay. Lanes indicate location of CD46-GST fusion proteins. Lane 1: CD46 Cyt2-wt. Lane 2: CD46 Cyt2-Y354A. Lane 3: CD46 Cyt1wt. Lane 4: CD46 Cyt1 FTSL-GGGG. Lane 5: CD46 Cyt1-Y348A.

(Lee, S., Thelin W., Milgram, S. and So, M. unpublished data)

FIGURE 1



(Lee, S. Thelin, W., Milgram, S. So, M., unpublished data)

Figure 2. Cartoon model demonstrating cooperative pulling by GC microcolonies. A single diplococcus is pushed against the host cell plasma membrane upon retraction, generating little tension. A GC microcolony with extended pili contains an anchor point from which retraction generates substantial amounts of membrane tension, radially extended from the sphere of the microcolony.

FIGURE 2

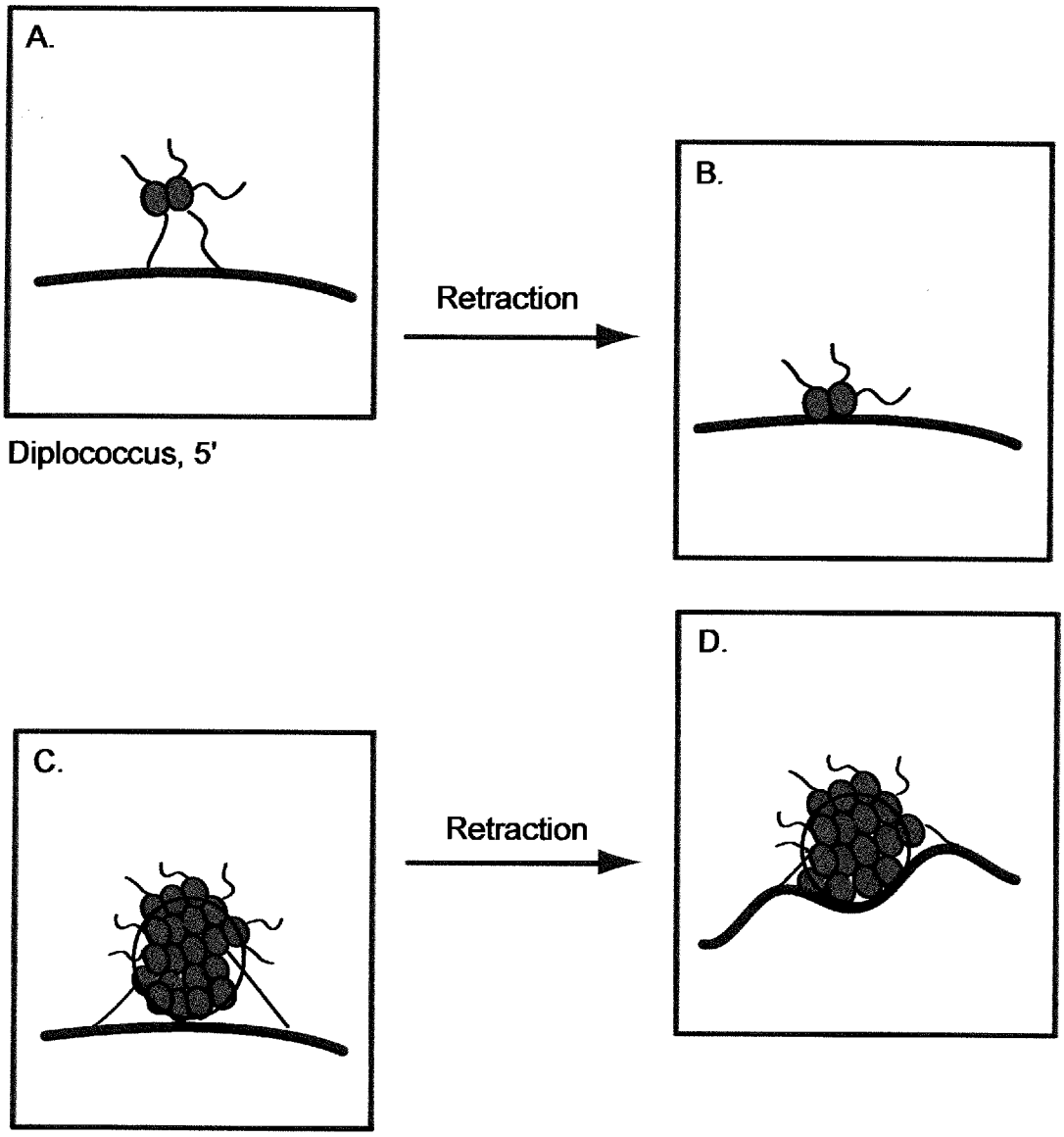
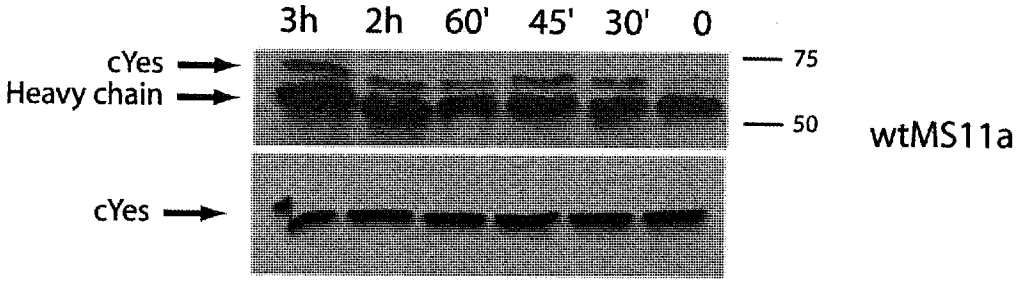


Figure 3. Association of c-Yes tyrosine kinase with PI-3 kinase upon infection. Co-immunoprecipitation of c-Yes with PI3-kinase in wt MS11 infected cells. PI3-kinase p85 was immunoprecipitated from A431 cells infected with MS11 for various lengths of time. The presence of c-Yes in the precipitates was determined by immunoblotting with monoclonal anti-c-Yes antibodies (upper panel). Inputs (total cell lysates from each time point) were immunoblotted with anti-c-Yes antibodies to confirm uniform levels of the kinase in the samples (lower panel).

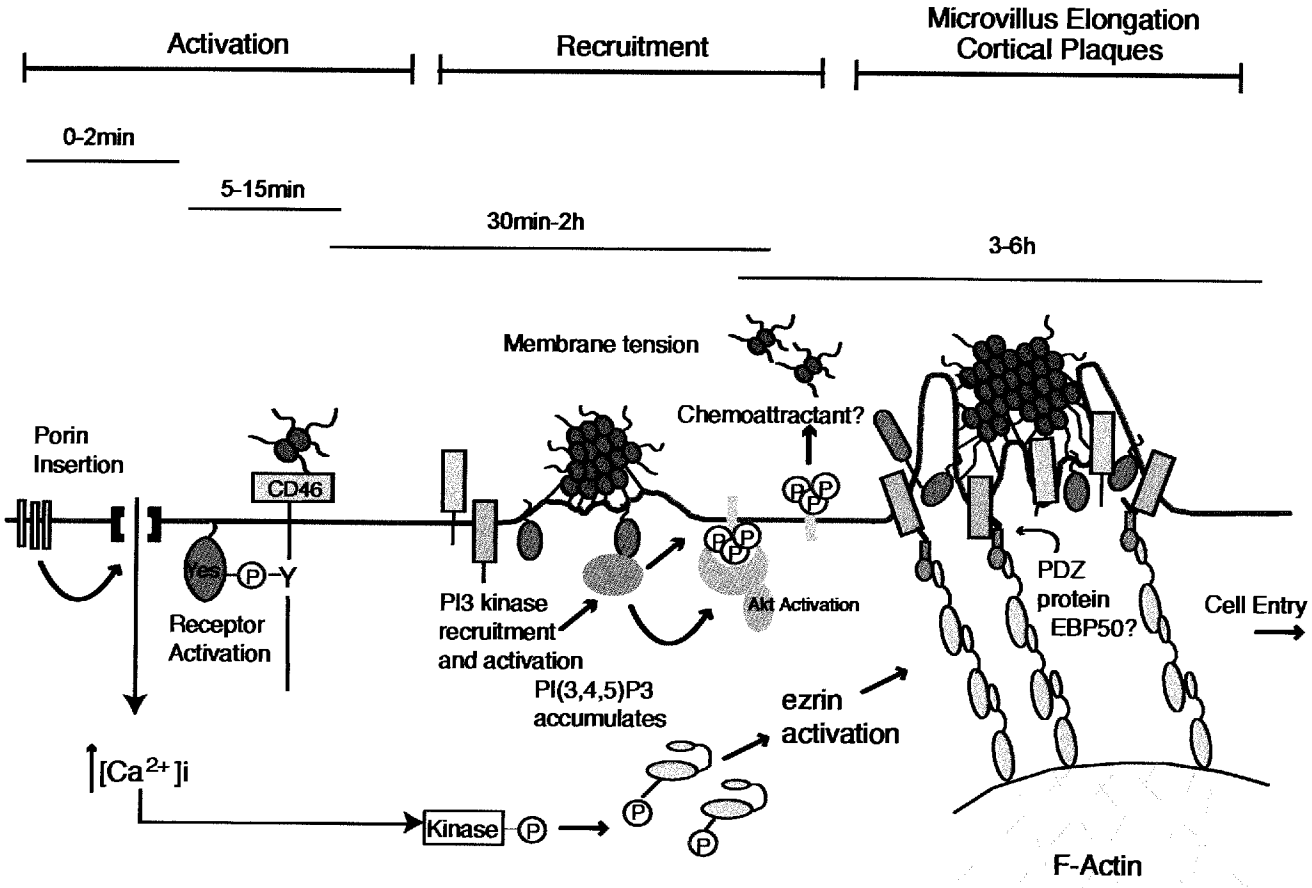
FIGURE 3



IP: PI 3 kinase p85
IB: cYes

Figure 4. Model of early host cell signaling cascades induced by GC infection of epithelial cells. Bars indicate relative timepoints when signaling events are likely to occur.

FIGURE 4


Activation
Recruitment
Microvillus Elongation
Cortical Plaques
0-2min
5-15min
30min-2h
3-6h
Membrane tension
Chemoattractant?
Porin
Insertion
CD46
Receptor
Activation
PI3 kinase
recruitment
and activation
Akt Activation
PDZ
protein
EBP50?
Cell Entry
PI(3,4,5)P3
accumulates
ezrin
activation
[Ca2+]i
Kinase
F-Actin

References

Ansley, S. J., Badano, J. L., Blacque, O. E., Hill, J., Hoskins, B. E., Leitch, C. C., Kim, J. C., Ross, A. J., Eichers, E. R., Teslovich, T. M., *et al.* (2003). Basal body dysfunction is a likely cause of pleiotropic Bardet-Biedl syndrome. Nature *425*, 628-633.

Busse, J., Hartmann, E., and Lingwood, C. A. (1997). Receptor affinity purification of a lipid-binding adhesin from Haemophilus influenzae. J Infect Dis *175*, 77-83.

Costello, P. S., Gallagher, M., and Cantrell, D. A. (2002). Sustained and dynamic inositol lipid metabolism inside and outside the immunological synapse. Nat Immunol *3*, 1082-1089.

Devreotes, P., and Janetopoulos, C. (2003). Eukaryotic chemotaxis: distinctions between directional sensing and polarization. J Biol Chem *278*, 20445-20448.

Huesca, M., Borgia, S., Hoffman, P., and Lingwood, C. A. (1996). Acidic pH changes receptor binding specificity of Helicobacter pylori: a binary adhesion model in which surface heat shock (stress) proteins mediate sulfatide recognition in gastric colonization. Infect Immun *64*, 2643-2648.

Johansson, L., Rytkonen, A., Bergman, P., Albiger, B., Kallstrom, H., Hokfelt, T., Agerberth, B., Cattaneo, R., and Jonsson, A. B. (2003). CD46 in meningococcal disease. Science *301*, 373-375.

Kallstrom, H., Islam, M. S., Berggren, P. O., and Jonsson, A. B. (1998). Cell signaling by the type IV pili of pathogenic Neisseria. J Biol Chem *273*, 21777-21782.

Kearns, D. B., and Shimkets, L. J. (1998). Chemotaxis in a gliding bacterium. Proc Natl Acad Sci U S A *95*, 11957-11962.

Khursigara, C., Abul-Milh, M., Lau, B., Giron, J. A., Lingwood, C. A., and Foster, D. E. (2001). Enteropathogenic Escherichia coli virulence factor bundle-forming pilus has a binding specificity for phosphatidylethanolamine. Infect Immun *69*, 6573-6579.

Lingwood, C. A., Huesca, M., and Kuksis, A. (1992). The glycerolipid receptor for Helicobacter pylori (and exoenzyme S) is phosphatidylethanolamine. Infect Immun *60*, 2470-2474.

Ludford-Menting, M. J., Thomas, S. J., Crimeen, B., Harris, L. J., Loveland, B. E., Bills, M., Ellis, S., and Russell, S. M. (2002). A functional interaction between CD46 and DLG4: a role for DLG4 in epithelial polarization. J Biol Chem *277*, 4477-4484.

Marie, J. C., Astier, A. L., Rivailler, P., Rabourdin-Combe, C., Wild, T. F., and Horvat, B. (2002). Linking innate and acquired immunity: divergent role of CD46 cytoplasmic domains in T cell induced inflammation. Nat Immunol *3*, 659-666.

Massari, P., Ram, S., Macleod, H., and Wetzler, L. M. (2003). The role of porins in neisserial pathogenesis and immunity. Trends Microbiol *11*, 87-93.

Muller, A., Gunther, D., Dux, F., Naumann, M., Meyer, T. F., and Rudel, T. (1999). Neisserial porin (PorB) causes rapid calcium influx in target cells and induces apoptosis by the activation of cysteine proteases. Embo J *18*, 339-352.

Reczek, D., Berryman, M., and Bretscher, A. (1997). Identification of EBP50: A PDZ-containing phosphoprotein that associates with members of the ezrin-radixin-moesin family. J Cell Biol *139*, 169-179.

Reczek, D., and Bretscher, A. (1998). The carboxyl-terminal region of EBP50 binds to a site in the amino- terminal domain of ezrin that is masked in the dormant molecule. J Biol Chem *273*, 18452-18458.

Schneider-Schaulies, J., Dunster, L. M., Schwartz-Albiez, R., Krohne, G., and ter Meulen, V. (1995). Physical association of moesin and CD46 as a receptor complex for measles virus. J Virol *69*, 2248-2256.

Selkoe, D. J. (1999). Translating cell biology into therapeutic advances in Alzheimer's disease. Nature *399*, A23-31.

Sperandio, V., Torres, A. G., Jarvis, B., Nataro, J. P., and Kaper, J. B. (2003). Bacteria-host communication: the language of hormones. Proc Natl Acad Sci U S A *100*, 8951-8956.

Steiner, H., Kostka, M., Romig, H., Basset, G., Pesold, B., Hardy, J., Capell, A., Meyn, L., Grim, M. L., Baumeister, R., *et al.* (2000). Glycine 384 is required for presenilin-1 function and is conserved in bacterial polytopic aspartyl proteases. Nat Cell Biol *2*, 848-851.

Strom, M. S., and Lory, S. (1993). Structure-function and biogenesis of the type IV pili. Annu Rev Microbiol *47*, 565-596.

Sylvester, F. A., Philpott, D., Gold, B., Lastovica, A., and Forstner, J. F. (1996). Adherence to lipids and intestinal mucin by a recently recognized human pathogen, Campylobacter upsaliensis. Infect Immun *64*, 4060-4066.

Ting, A. Y., Kain, K. H., Klemke, R. L., and Tsien, R. Y. (2001). Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells. Proc Natl Acad Sci U S A *98*, 15003-15008.

Wang, G., Liszewski, M. K., Chan, A. C., and Atkinson, J. P. (2000). Membrane cofactor protein (MCP; CD46): isoform-specific tyrosine phosphorylation. J Immunol *164*, 1839-1846.