ABSTRACT

Title of Document: THE ROLE OF ERBB RECEPTORS IN *NEISSERIA GONORRHOEAE* INVASION OF GENITAL EPITHELIAL CELLS

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Neisseria gonorrhoeae, the causative agent of the sexually transmitted infection gonorrhea, adheres to and invades genital epithelial cells. This study investigates host components that are used by the bacteria for their entry into epithelial cells. I found that the interaction of gonococci with the surface of HEC-1-B, a human endometrial carcinoma, and ME180, a human cervical epidermoid carcinoma, caused redistribution of both epidermal growth factor receptor (EGFR) and ErbB2, a related family member. Both EGFR and ErbB2 were translocated from the basolateral to the apical membrane in polarized HEC-1-B cells and concentrated under the microcolonies. Gonococcal infection increased EGFR and ErbB2 phosphorylation, indicating activation of the receptors. Kinase inhibitors of EGFR and ErbB2 inhibited and enhanced bacterial invasion, respectively, but had no effect

on gonococcal adherence or the recruitment of EGFR and ErbB2 to the microcolonies. Gonococcal inoculation upregulated the transcription levels and matrix metalloproteinases (MMP)-mediated surface shedding of ligands of EGFR. Inhibition of the surface shedding of EGFR ligands by an MMP inhibitor and by heparin wash reduced gonococcal invasion without altering their adherence. *N. gonorrhoeae* induced the activation of the MAP Kinase ERK, PI3K/AKT and PLCγ signaling pathways in an EGFR tyrosine kinase-dependent manner. Blocking Ca^{2+} flux, the downstream pathway of PLCγ, but not ERK and PI3K by inhibitors reduced gonococcal invasion. These data indicate that *N. gonorrhoeae* utilizes host signaling pathways to drive its invasion. The bacteria modulates host signaling by recruiting and activating EGFR and ErbB2. *N. gonorrhoeae* induces EGFR activation by increasing the expression and MMP-mediated shedding of EGFR ligands.

THE ROLE OF ERBB RECEPTORS IN *NEISSERIA GONORRHOEAE* INVASION OF GENITAL EPITHELIAL CELLS.

By

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Dedication

I dedicate this dissertation to my mother, Lois Swanson who always believed and to my grandmother Lillian Borowick.

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Abbreviations

ADAM	a disintegrin and metalloproteinase
AR	amphiregulin
BT	betacellulin
CaMK	calmodulin kinase
CEACAM	carcinoembryonic antigen
CR3	complement receptor 3
Cofilin	cofilin-1
DAG	diacylglycerol
DGI	Disseminated gonococcal infection
EGFR	epidermal growth factor receptor
ERK	extracellular signal related kinase
FBS	fetal bovine serum
GCK	gonococcal media base
GCPR	G-protein-coupled receptors
gonococci	*Neisseria gonorrhoeae*
HB-EGF	heparin binding epidermal growth factor-like growth factor
HEC-1-B	human epithelial endometrial adenocarcinoma cell line
Hep	heptose
HSPG	heparan sulfate proteoglycans
IC_{50}	50% inhibitory concentration
ID_{50}	50% infectious dose
IP_3	inositol triphosphate
KDO	2-keto-3-deoxy-mannooctulosonic acid
LIMK	LIM Kinase
LOS	lipooligosaccharide
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MKC	$MS11_{MKC}$
ME180	human cervical epidermal carcinoma cell line
MEK1/2	MAPK kinase
MLCK	myosin light chain kinase
MMP	matrix metalloproteinase
MOI	multiplicity of infection
NRG	neuregulin
Opa	opacity-associated proteins
OS	oligosaccharide
p44/p42	ERK1/2
pAKT	phosphorylated AKT
PDK	phosphoinositide dependent kinase
pERK	phosphorylated ERK
pex	immortalized primary endocervical cell

PFA	paraformaldehyde
PI3K	phosphoinositide 3-kinase
PID	pelvic inflammatory disease
Pil	pili
PIP_2	phosphoinositol 4,5 bisphosphate
PIP_3	phosphoinositol 3,4,5 bisphosphate
PKC	Protein Kinase C
PLCγ	phospholipase-gamma
PMN	polymorphonuclear leukocytes
PTB	phosphotyrosine binding
pY	phosphorylated tyrosine
Raf	MAPK kinase kinase
ROCK	Rho-associated kinase
RTK	receptor tyrosine kinase
SH2	Src homology 2
sHB-EGF	soluble HB-EGF
TACE	TNFα-converting enzyme
Tfp	type IV pili
TER	transepithelial resistance
TGF-α	transforming growth factor-alpha
ZO	zonula occludens

Chapter 1 Introduction

1.1 Background

Neisseria gonorrhoeae (gonococci), a Gram-negative diplococcus, is the causative agent of the sexually transmitted infection gonorrhea. Gonococci are obligate pathogens of humans that have no other natural host. *N. gonorrhoeae* causes significant disease in the U.S. and worldwide. Gonorrhea is the second most commonly reported infectious disease in the US at over 300,000 reported cases annually, although there are estimated to be approximately 700,000 cases in the US and 80,000,000 cases worldwide annually (106). Because of increasing incidence of antibiotic resistance, cephalosporins are the only remaining class of antibiotics that are available to treat gonorrhea (107). Additionally, the significance of this disease is exacerbated by the findings that gonococcal infection increases the risk of HIV transmission (42).

N. gonorrhoeae primarily infects epithelial cells of the genitourinary tract of both men and women, but also can infect epithelial cells of the conjunctiva, pharynx, and rectal mucosa. Primary infection differs between men and women. Nearly 90% of infected men are symptomatic. Symptoms usually appear within 2-5 days after infection, but can take up to 30 days to arise. These include dysurea and purulent discharge. During the initial infection of men, gonococci are thought to invade urethral epithelial cells. Proinflammatory cytokines are released, causing an influx of polymorphonuclear leukocytes (PMN) which engulf gonococci by phagocytosis.

Women are exposed to *N. gonorrhoeae* by their infected partners. Most infected women, however, are asymptomatic or have symptoms so mild that they are disregarded (101). Since asymptomatic women fail to seek treatment, this allows women to become chronically infected. Chronic infection allows the bacteria to spread to secondary sites and cause more serious disease. One of the outcomes of this is pelvic inflammatory disease (PID), which occurs in 10-20% of infected women (106). PID occurs when the infection moves into the upper reproductive tract. Infection of the Fallopian tubes, salpingitis, causes apoptosis of the ciliated cells, leading to the loss of function of the tubes. PID is also known to induce the over production of proinflammatory cytokines, which ultimately can lead to scarring of the reproductive organs, resulting in chronic pelvic pain, ectoptic pregnancy, and infertility. Disseminated gonococcal infection (DGI) occurs in 1-3% of infections and typically manifests as arthritis and dermatitis, but can also cause endocarditis, meningitis, and adult respiratory distress syndrome (1, 8, 16, 69, 98). Gonococcal infection during pregnancy has been shown to enhance the chances of acquiring DGI (1, 98). Men also are susceptible to DGI, although it occurs at a much lower incidence than in women (1).

Productive infection by *N. gonorrhoeae* of genital epithelial cells consists of four sequential steps: adherence, invasion, intracellular survival, and exocytosis. Since the anatomy of the male and female reproductive tracts is quite different, gonococci have had to develop methods to survive in both environments. Additionally the female reproductive tract remodels itself monthly. Therefore, in order to maintain infection in women, gonococci have had to develop strategies that

allow it to adhere to and possibly invade deeper into tissues as older cells are shed and newer epithelial cells differentiate in order to adapt to a constantly changing environment. One of the mechanisms that gonococci have developed to adapt to different environments is the ability to phase vary multiple genes including many of its surface molecules. A recent genomic search found that *N. gonorrhoeae* strain FA1090 had 72 genes that are putatively phase variable (130). Besides being able to adapt to changing environments, the ability to phase vary multiple genes has also been suggested to be important for avoiding immune detection (89).

1.2 Gonococcal Virulence Factors

Pili (Pil), opacity-associated proteins (Opa) and lipooligosaccharide (LOS) are three of the most studied gonococcal virulence factors, all of which are phase variable. *N. gonorrhoeae* express type IV pili (Tfp) that mediate the initial adherence to genital epithelial cells (68, 97). The gonococcal pilus is composed of multiple subunits of identical pilin monomers, PilE. There is both interstrain and intrastrain variability in pilus expression. Intrastrain variation is due to reciprocal homologous recombination of *pilE* with any of the multiple copies of *pilS*, the silent pilin loci. The *pilS* loci contain one or more partial copies of pilin genes that lack the N-terminal coding region and are tandemly arranged. *N. gonorrhoeae* strain MS11 has five *pilS* loci that contain up to 16 partial pilin gene copies (137).

Expression of the PilC protein is necessary for pilus fiber stabilization (154). The *pilC* gene is phase variable, which thereby controls pilus expression phase

variation. Besides being necessary for pilus stabilization, PilC is the major adhesin located at the tip of the pilus (112, 118). Loss of *pilC* expression through phase variation does not alter expression of the pilin monomers. When PilC is not expressed, PilE is processed by removal of approximately 39 amino acids N-terminal and secreted as a soluble pilin molecule called S pilin (54, 64). Gonococcal pili are highly immunogenic, so it has been postulated that secretion of S pilin might act as a decoy for the anti-pilus antibody response (25, 136).

After gonococci adhere to epithelial cells via their pili, they retract their pili allowing for a more intimate association between the bacterium and host cell surface (108). The twitching of all Tfp and pilus retraction is under the control of the ATPase protein PilT (154). The ability of the gonococci to retract their pili enhances the infection process, as gonococci that are piliated but do not express PilT invade 40% less well than gonococci that express PilT (79). Although CD46, a membrane protein, initially was identified as the receptor for gonococcal pili via PilC (77), recent studies have suggested that there is no relationship between CD46 and gonococcal adherence (71, 141). Thus there have been no definitive host receptor(s) identified for gonococcal pilus.

After initial attachment by pili, more intimate attachment is thought to be mediated via interaction of Opa and LOS with host cells. Opa comprise a family of closely related outer membrane proteins (7, 135). Most strains of *N. gonorrhoeae* have 10-11 *opa* genes, and through phase variation at any time can express none or several. (10, 15, 143). Opa proteins are lectins that increase both inter-gonococcal adherence by binding to the oligosaccharide portion of LOS on adjacent bacteria (14)

and gonococcal binding to host receptors.

Both Opa^+ and Opa^- gonococcal variants are thought to be virulent, since both can be isolated from sites of active gonococcal infection. Gonococci isolated from male urethrae and the cervices of women near the time of ovulation are mostly Opa^+, whereas gonococci isolated from the cervices of women near the time of menstruation, from Fallopian tubes and from blood during DGI are Opa^-.

There have been several host cell receptors identified for Opa proteins. Heparan sulfate proteoglycans (HSPG) are membrane proteins that are glycosylated primarily with heparan sulfate but also with chondroitan sulfate. CD44v3, syndecan-1 and syndecan-4 are all HSPGs that bind to at least one Opa variant on the surface of gonococci, which then can lead to the internalization of bacteria (44). All three HSPGs are localized at the basolateral side of epithelial cells. Since infection is initiated from the apical side, it is still unclear how these proteins may be utilized during the infection process. Opa proteins also have been shown to interact with members of the carcinoembryonic antigen (CEACAM) family on the surface of neutrophils and direct bacterial invasion (22). Neutrophil infiltration is a hallmark of gonococcal disease in men, but seldom occurs in women. About 70% of cervical epithelial cells have been shown to express CEACAM (93, 138), but it hasn't been found on Fallopian tube epithelial cells (138). While binding to CEACAM expressed on genital epithelial cells leads to internalization of gonococci by Opa:CEACAM mediated endocytosis, internalized gonococci via this mechanism are transported to lysosomes where they are efficiently killed (85). CEACAM mediated endocytosis by neutrophils does not lead to killing of the bacteria, which may be important for

disease progression in men. CEACAM-mediated endocytosis by female genital epithelial cells could lead to intracellular killing of gonococci and therefore may be protective.

N. gonorrhoeae express LOS, which is similar to the lipopolysaccharide (LPS) of enteric bacteria except that it lacks the O-antigen sugar repeats. Gonococcal LOS has a triantennary oligosaccharide (OS) structure that mimics human glycosphingolipids (52, 82, 83). Gonococcal LOS consists of a membrane anchored lipid A that is attached to two 2-keto-3-deoxy-mannooctulosonic acid (KDO) molecules. Two heptoses (Hep) extend from KDO1. The alpha-OS chain is attached to Hep1, and both the beta- and gamma-OS chains are attached to Hep2. Additionally the heptoses may be decorated with one or two phosphoethanolamines. The genes for the glycosyltransferases that encode for the stepwise addition of the sugars are known. The glycosyltransferases that produce the alpha-OS chain is encoded by the *lgtA-E* gene cluster and *lgtF* of which *lgtA, lgtC* and *lgtD* are phase variable (49). The shortest naturally occurring alpha-OS is a lactosyl moiety which occurs by the action of the invariant *lgtF* and *lgtB*. Extension of the beta-OS is under the control of the phase variable *lgtG* expression. The gamma-OS consists of a single *N*-acetylglucosamine and is controlled by the expression of rfaK which is coexpressed with *lgtF* and is invariant (65).

In a human challenge study designed to follow the variation of LOS in disease, men were intraurethrally challenged with various amounts of gonococcal strain MS11mk varA (MKA) (125). This strain produces a single LOS with a lactosyl alpha-chain. Only men who had been inoculated with the most organisms, 10^8, had

symptoms of disease. At the onset of leukorrhea it was noted that the LOS had varied to a higher MW species MS11varC (MKC). This change was found to be the alpha-chain lactose with one or more additions of *N*-acetyllactosamine called lacto-*N*-neotetraose or paraglobosyl LOS (63). The same study also found that 34/36 naturally acquired infections expressed paraglobosyl LOS or larger LOS species, the remaining two produced both paraglobosyl and lactosyl LOS. This and sequential human challenge studies demonstrated that paraglobosyl LOS is the virulent phenotype, and that ID_{50} of these virulent strains is only $2.5x10^3$ gonococci (124, 125).

Wang et al. showed that the lacto-*N*-neotetraose OS could inhibit the invasion of HEC-1-B cells by gonococci, suggesting that this LOS is important for invasion of epithelial cells (149). The importance of the OS portion of LOS in gonococcal invasion was further investigated in two similar in vitro studies using *N. gonorrhoeae* OS mutants of two different gonococcal strains and ME180 cervical epithelial cell line. In vitro studies with strain F62 using alpha-chain OS mutants that are invariant in the expression of a single LOS species in a Pil^+ Opa^- background showed that although there was no difference in the ability of the LOS mutants to adhere to human cervical epithelial cells only lacto-*N*-neotetraose LOS promoted efficient invasion into the epithelial cells (131). Contrary to this finding, another study using LOS mutants of strain MKC in a Pil^- Opa^+ background showed that there was no difference in adherence or invasion of gonococci with the truncated LOS alpha-chain mutants as compared to those with lacto-*N*-neotetraose LOS into ME180 cells. Only gonococci that express LOS lacking an alpha-chain OS were found to have both poor adherence and invasion. Clearly the background expression of two known adhesins,

pili and Opa, are reversed for the two studies. This could be one explanation for the disparity between the two studies. Consistent with a role of LOS in infection, clinical studies show that men expose women to *N. gonorrhoeae* that express pili, Opa and lacto-*N*-neotetraose LOS.

1.3 Gonococcal Infection Process

Gonococcal invasion of genital epithelial cells is an active process that requires participation of both the host cells and gonococci (Fig. 1). Killed bacteria are not endocytosed (13, 114). Unlike other bacterial intracellular pathogens in which endocytosis is rapid, *N. gonorrhoeae* infection of genital epithelial cells in vitro takes approximately 4-6 hours. Maximal adherence is seen at about 2 hours. New protein synthesis after initial adherence is required in both the gonococci and host cell in order to induce its endocytosis (53, 114, 127). After 2 hours, the host cell microvilli elongate, surround the gonococcal microcolonies, and attach to the gonococci along their full lengths. (33, 53). The gonococci appear to sink into the membrane without the benefit of lamellipodia or filopodia formation. Their invasion most closely resembles Type II phagocytosis like the complement receptor 3 (CR3)-mediated phagocytosis of C3bi opsonized particles (18).

CR3 dependent uptake of microorganisms happens in the absence of proinflammatory responses, a situation that exists in gonococcal infection of women. Although CR3 initially was believed to be expressed only on the surface of professional phagocytes, it has recently been found to be expressed on the surface of primary epithelial cells, including cervical and endometrial cells (38, 61). Gonococcal

LOS has been demonstrated to bind C3. The subsequent conversion of C3 to C3bi allows the engagement of CR3 and phagocytosis of the bacteria(37).

Gonococcal invasion of host cells is an actin-mediated process that is dependent upon rearrangement of the host cell cytoskeleton. The addition of the actin perturbing agents, cytochalasin B and cytochalasin D, inhibits gonococcal invasion of epithelial cells (114, 127). Pili-mediated initial adherence of gonococci to genital epithelial cells induces cortical plaque formation (87). The formation of cortical plaques requires host cytoskeletal rearrangement. Pili mediated adherence causes the recruitment of ezrin, a protein that links the actin cytoskeleton with the membrane and the assembly of F-actin beneath the gonococci (48, 86). EGFR (ErbB1), CD44v3 and ICAM-1 all were shown to be enriched in gonococcal induced cortical plaques and along elongated host microvilli (87).

Hoffmann *et al*. (59) did similar studies to examine the surface redistribution of ErbB receptors on the surface of endothelial cells in response to *N. meningitidis*. Unlike gonococcal adherence of epithelial cells, the study showed that in endothelial cells there is no recruitment of EGFR (ErbB1) to the site of adherent meningococci. EGFR and the ErbB family receptors, ErbB3 and ErbB4, all showed punctate staining over the entire surface of endothelial cells before and after the addition of meningococci. However the study showed that the addition of meningococci induces the recruitment of ErbB2 to the site of the meningococcal microcolonies.

In the past twenty years a lot of work has been published examining the mechanisms and signal transduction pathways that gonococci use in order to invade epithelial cells and cause disease. Pil^- Opa^- variants have been shown to be unable to

adhere, invade or cause primary disease. Therefore, much of the work has used gonococcal variants that where either Pil^+ Opa^- or Opa^+ Pil^- in an attempt to tease out the host signaling components that were specific to each adhesin. The results, although garnering a lot of new and overlapping information, also has led to contradictory information. This has led to the ever increasing view in the field that gonococci have multiple mechanisms for invasion and causing disease (109).

Adherence of gonococci to female genital epithelial cells is first initiated by pili binding to the host cell surface. The pili then retract bringing the gonococci in close intimate contact with the host cell. This pili retraction under the control of the PilT protein is able to induce host signaling events culminating in the invasion of the gonococci. Retraction of a single gonococcal pilus can exert forces up to 100 pN (81). Gonococci that are in a Pil^+ Opa^- background induces a Ca^{2+} flux in epithelial cells within 10 min of adherence, and this Ca^{2+} flux is enhanced by PilT expression (6, 76). Pilus retraction induces the activation of ERK conferring cytoprotection (60). Finally, Pil^+ gonococci induce phosphoinositide 3-kinase (PI3K) signaling pathways that are enhanced by PilT expression. PI3K activation enhances microcolony formation on the surface of the host cell (79). The importance of PI3K activation for invasion by Pil^+ Opa^- gonococci is not clear, as it has been reported both to be (79) and not be important for the invasion of epithelial cells. (36).

Opa proteins mediate intimate interactions between the epithelial cell surface and gonococci. In addition, they mediate inter-gonococcal interactions by binding to LOS, promoting microcolony formation. There are over 50 different molecules on the surface of HeLa cells that can bind Opa proteins, although most remain unknown (9).

Opa_{50} binds to the serum components, vitronectin and fibronectin, which can bind integrin receptors at the apical side (30). Opa_{50} also binds HSPGs on the basolateral side of epithelial cells leading to the internalization of gonococci. Similar to pili, Opa_{50} induces actin cytoskeleton rearrangement, microvilli extension, and the accumulation of phosphotyrosine proteins beneath the bacterial microcolony (44). Opa_{50} binding to HSPGs induces the activation of phosphatidylcholine dependent phospholipase C and acidic sphingomyelinase, generating the second messengers diacylglycerol (DAG) and ceramide and results in the invasion of gonococci (50). Vitronectin bridges the binding of Opa_{50} expressing gonococci to α_v containing integrin receptors and fibronectin bridges the binding to the $\alpha_5\beta_1$ integrin receptor. Integrin-mediated gonococcal invasion are Protein Kinase C (PKC) dependent.

Most Opa proteins that are expressed by gonococci can bind to any of three CEACAMs, -1, -5 or -6, found on the surface of genital epithelial cells to promote their invasion (94). CEACAMs are not ubiquitously expressed on all genital epithelial cells, 30% of normal cervical epithelial cells do not express CEACAM (138). Although Opa binding to any of these three CEACAMs on epithelial cells induces gonococcal endocytosis, the gonococci that entered host cells through CEACAM have been shown to be killed intracellularly (85). This suggests that expression of CEACAM is potentially a protective mechanism of cervical epithelial cells.

N. gonorrhoeae

Initial Adherence

Intimate Adherence

Invasion

Adhesins/Invasins

Pili

Opa, LOS

Pili, Opa, LOS, Por,

Genital epithelial cell

Receptors

CEACAM, HSPG, Integrins

CR3, AGPR

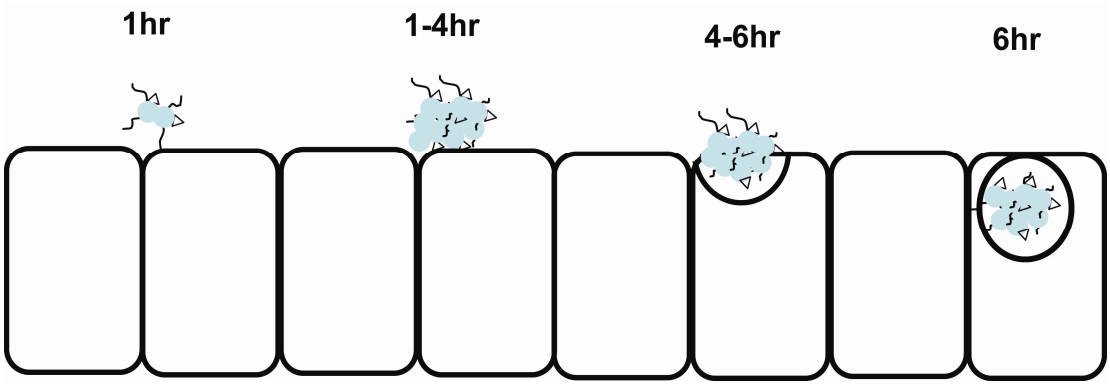

Figure 1. Gonococcal infection model of genital epithelial cells. Initial adherence of the gonococci occurs via pili binding to the epithelial cell surface. The pili retract to allow for a more intimate adherence via the adhesins, Opa and LOS. Gonococci multiply on epithelial cell surface for several hours to form microcolonies. By 4 h invasion begins to occur and is maximal by 6 h.

1.4 ErbB Receptor Family

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK). It belongs to the ErbB family of four closely related RTK's, named after the oncogene *v-erbB* of the avian erythroblastosis virus, a homologue of EGFR (158). The four ErbB family members are EGFR/ErbB1/HER1, ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4. The ErbB receptors contain an extracellular ligand binding domain, a transmembrane domain, an intracellular tyrosine kinase domain, and an intracellular hydrophilic tail that contains many tyrosine residues that can be phosphorylated (116). The ErbB receptors are activated by dimerization. Ligand binding induces a conformation shift of the extracellular domain, which exposes a dimerization site that noncovalently binds to this same region of another ligand bound ErbB receptor. The ErbB receptors are able to form homo- and heterodimers. Dimerization places the kinase domains in proximity to the hydrophilic tails, which are then trans-autophosphorylated on tyrosine residues. The phosphotyrosines serves as docking sites for proteins containing either SH2 (Src homology 2) or PTB (phosphotyrosine binding) sites, initiating signals that can lead to proliferation, migration, differentiation, cell survival, and/or adhesion.

The signals transduced via ErbB receptors are regulated by many factors. These include the type (growth factors), form (soluble or membrane associated), source (autocrine or paracrine) and concentration of ErbB ligands. These factors then direct the dimerization, either hetero- or homodimerization. ErbB2 and ErbB3 are unable to homodimerize thus allowing for a total of 10 distinct dimerization states. ErbB3 lacks the tyrosine kinase domain. ErbB2 lacks the ligand binding domain and

cannot homodimerize with itself, although ErbB2 homodimers can be stabilized through oncogenic mutation or over expression (17, 133, 148).

There is a hierarchy of dimerization, which is dependent upon the availability of ligand and ErbB receptor (142). Although ErbB2 has no known ligand and typically does not form homodimers, it is the preferred dimerization partner of the other three ErbB receptors because its dimerization site is constitutively exposed (142). ErbB2 has been shown to increase the binding affinity of the dimerizing ErbB partner for ligands (74, 142) and decrease the turnover rate of phosphorylated ErbB receptors, which significantly prolongs their signaling (56, 66).

There are eleven peptide ligands for the ErbB receptors. These ligands fall into four groups dependent upon which ErbB receptor(s) they bind (55, 73). The first group binds only EGFR, including EGF, transforming growth factor-α (TGF-α), and amphiregulin (AR). The second group binds to both EGFR and ErbB4, and includes heparin binding epidermal growth factor-like growth factor (HB-EGF), epiregulin, and betacellulin (BT). The third group contains only epigen, which binds to ErbB1, ErbB3, and ErbB4. The final group is the neuregulins (NRG), NRG-1, NRG-2, NRG-3 and NRG-4, which bind to either or both ErbB3 and ErbB4. Additionally NRG-1 and NRG-2 have two isomers each, α and β, which are created by differential splicing.

All of the ErbB ligands are expressed on the cell surface as transmembrane proteins that are cleaved by matrix metalloproteinases (MMP) and/or ADAMS (a disintegrin and metalloproteinase) to release the mature peptides (55, 115, 121, 134). These peptide ligands are shed into the extracellular matrix where they are able to

signal in an autocrine or paracrine manner. Additionally, both EGF and TGF-α are expressed systemically and are able to signal in an endocrine manner. The transmembrane, pro-forms of HB-EGF, AR, TGF-α and BT also have been shown to function in a juxtacrine manner (129, 139).

Activation of ErbB receptors via juxtacrine ligand binding results in a different response than autocrine/paracrine activation. For example, wound healing is governed (activated) by the switch from juxtacrine to autocrine/paracrine signaling via HB-EGF binding to ErbB1/4. (129) Juxtacrine ligation of HB-EGF is integral to cell layer integrity. When there is injury to the cell layer, the loss of pro-HB-EGF binding to neighboring cells expressing EGFR or ErbB4 induces metalloproteinase expression, leading to cleavage and release of HB-EGF. This then shifts the balance toward autocrine/paracrine signaling of ErbB1/4 by binding to soluble HB-EGF. ErbB1/4 binding to soluble HB-EGF induces wound healing by promoting cell proliferation and migration. The migration of new cells into the wound and up against other cells restores juxtacrine engagement of pro-HB-EGF with ErbB1/4, shifting cells back toward juxtacrine signaling.

Each of the ErbB receptors has several potential autophosphorylation sites. In addition, EGFR and ErbB2 also have several tyrosine residues that can be phosphorylated by Src kinase (140). Phosphorylated tyrosines in the cytoplasmic tail of ErbB serve as docking sites for a variety of signaling molecules. Different ErbB ligands lead to different dimerization possibilities of the four ErbB family members, differential tyrosine phosphorylation of their cytoplasmic tails, and docking of a

variety of signaling molecules, which allow for extensive and fine tuned signaling through ErbB receptors.

Signal transduction emanating from EGFR and ErbB2 can be induced through transactivation by various G-protein-coupled receptors (GCPR). GPCR activation by its ligands endothelin I (28), thrombin (28), lysophosphatidic acid (28), bradykinin (162), angiotensin II, carbachol (67), bombesin and IL-8 (62, 146) all have been shown to induce EGFR phosphorylation with subsequent activation of the Mitogen Activated Protein Kinase (MAPK) signaling pathway. Although GPCR transactivation of EGFR was initially thought to be a ligand independent event, increasingly studies are suggesting that GPCR activates metalloproteinases that promote the shedding of membrane anchored EGF-like ligands (105). GPCR-induced activation of the metalloproteinases is Ca^{2+} dependent. Dependent upon ligand and cell type, PKC, calmodulin-dependent kinase II, and proline rich tyrosine kinase 2 (PYK2), all calcium dependent kinases, are implicated in GCPR-mediated transactivation of EGFR (105). Activation of Src kinase and PI3K also have been shown to be important for EGFR transactivation in some cell lines (117).

1.5 EGFR Signaling Cascades

Ligand binding to EGFR induces dimerization and phosphorylation of tyrosine residues in the cytoplasmic tail (Fig. 2). EGFR has six tyrosine residues that can be trans-autophosphorylated and four tyrosine residues that can be phosphorylated by Src. Which tyrosine residues are phosphorylated is dependent upon the ligand type, ligand concentration and dimerization partner. Proteins that

contain SH2 domains bind to the phosphotyrosines. PLCγ, Grb2, Gab1 and Shc are major SH2 domain containing proteins that bind to phosphorylated EGFR (Fig.1). The prototypical signaling pathway from EGFR is the MAPK/ERK (extracellular signal-regulated kinase) pathway. Activation of MAPK/ERK pathway is initiated by the docking of Grb2 and/or Grb2/Shc to phosphotyrosines in the cytoplasmic tail of EGFR. Grb2 activates SOS, the GEF of the small G-protein Ras. Ras activation activates Raf (MAPK kinase kinase), which phosphorylates and activates MEK1/2 (MAPK kinase), which phosphorylates and activates ERK1/2 (p44/p42 MAPK). ERK1/2 can activate multiple transcription factors, leading to expression of genes that are required for cell proliferation and control of cell fates.

EGFR activation also leads to the activation of phospholipase-gamma (PLCγ) PLCγ cleaves phosphoinositol 4,5 bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol triphosphate (IP_3) (19). IP_3 causes sarcoplasmic reticulum induced Ca^{2+} release with the subsequent activation of several Ca kinases. DAG and Ca^{2+} together activate PKC.

EGFR activation induces activation of PI3K through Gab1 (78). Gab1 binds to phosphorylated EGFR through its SH2 domain, and the p85 subunit of PI3K binds to phosphorylated Gab1 through its SH2 domain. PI3K activates the AKT/PKB (protein kinase B) pathway by generating PIP_3 (phosphatidylinositol-3,4,5-triphosphate). The AKT/PKB pathway is one of the major signaling pathways promoting cell growth and suppressing apoptosis (35).

EGFR activates Src kinase via the adaptor protein Shc (123) which binds to activated EGFR. Src, a nonreceptor tyrosine kinase, has numerous substrates,

including EGFR. Phosphorylation of EGFR by Src is thought to potentiate its signaling.

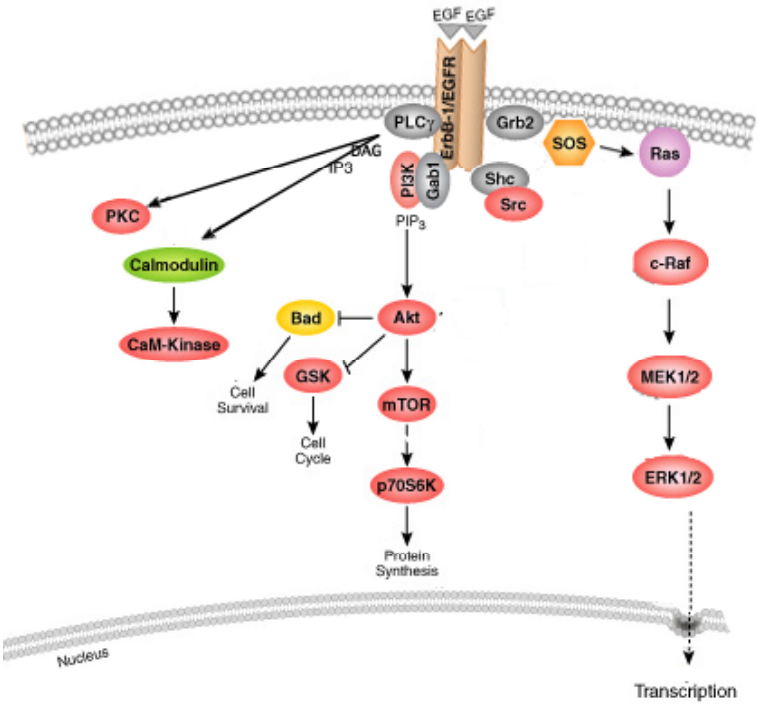

Figure 2. **Major signaling pathways induced via EGF binding to EGF receptor.** Modified from Cellsignal.com.

1.6 EGFR Regulation of Actin Dynamics

The interaction of gonococci with epithelial cells induces actin rearrangement in the host cells. This actin rearrangement has been shown to be important for the invasion of gonococci into epithelial cells of the female reproductive tract (114, 127). Actin cytoskeleton dynamics is controlled by actin regulators (31). These actin regulators, under the influence of intracellular and extracellular signals, can enhance actin polymerization and depolymerization. The induction of many signaling pathways has been shown to influence actin dynamics, including those activated by EGFR signaling.

The EGFR downstream signaling pathways, PI3K and PLCγ, can regulate the activity of cofilin. Cofilin (cofilin-1) is a member of the cofilin family of actin binding proteins that contains the highly homologous cofilin-1, cofilin-2 and muscle cofilin, along with actin-depolymerizing factor. The activity of cofilin shifts dependent upon the concentration gradient of cofilin within different regions of the cell (145). At low concentrations, cofilin binding to actin favors actin severing, resulting in actin depolymerization. Slightly higher cofilin concentrations increase cofilin binding to F-actin and stabilize actin filaments. The highest cofilin concentrations favor cofilin nucleation of actin with stabilization of the newly formed filaments. Cofilin binding to actin can be regulated by its phosphorylation by LIMK (LIM Kinase), which prevents its binding to actin. Cofilin binds to PIP_2 and $PIP_{3'}$ the product of PI3K, sequestering cofilin to the plasma membrane. When PIP_2 is cleaved by PLCγ, it releases active cofilin to bind F-actin.

Signaling through activated EGFR can regulate villin and gelsolin-mediated regulation of actin dynamics by activating PLCγ, calcium flux and Src. Gelsolin and villin are two actin binding proteins that belong to the gelsolin family that regulate actin cytoskeleton dynamics. Gelsolin is found across many cell types, whereas villin is restricted to epithelial cells, primarily of the gastrointestinal and urogenital tracts. Gelsolin has the ability to sever, cap, and nucleate actin filaments. (159) Under high (micromolar) calcium concentrations, gelsolin severs and caps actin filaments, leading to actin depolymerization (100, 103), or controversially under some circumstances may lead to actin nucleation by triggering the Arp2/3 complex (23).

Villin is highly homologous to gelsolin and retains its ability to sever, cap and nucleate actin, but has an additional head group that allows it to bundle F-actin by cross linking it. The bundling of F-actin via villin induces the formation of microvilli on the apical side of epithelial cells. Villin's functions are regulated by phosphorylation, calcium concentration and binding to PIP_2. At low calcium concentrations villin caps actin filaments, while at high (micromolar) calcium concentrations it nucleates actin in addition to capping F-actin. The binding of villin to PIP_2 in the membrane prevents its actin capping activity, but enhances its F-actin bundling activity. However, villin's bundling activity is not influenced by calcium, but is enhanced by PIP_2 binding. Villin can be phosphorylated by Src (70). This phosphorylation decreases villin's ability to bind to F-actin, but enhances its ability to bind to active PLCγ (70, 99). Furthermore, phosphorylation of villin inhibits its bundling and nucleating activities, and under moderate Ca^{2+} concentrations promotes its actin severing activity. EGFR triggered activated Src phosphorylates villin,

causing it to switch from PIP_2 to PLCγ binding and freeing local PIP_2 to be cleaved by PLCγ, which induces a calcium flux. The calcium flux shifts phosphorylated villin from bundling F-actin to the severing of it and activates the F-actin capping and severing activities of gelsolin.

1.7 Rationale

This study was designed to gain a better understanding of the signaling events that *N. gonorrhoeae* induce that are essential for gonococcal invasion of genital epithelial cells of the female reproductive tract. Women bear the brunt of the serious sequelae of this disease. Since women tend to be asymptomatic, infection often becomes chronic leading to outcomes including DGI, PID and infertility. 50% of the women who are infected are young, under age 24. Infertility of young women of child bearing age is a serious health concern. Because of this, research in the Song and Stein labs has focused on understanding gonorrhea in women. Because *N. gonorrhoeae* is a pathogen that is exclusive to humans, there is no animal model for the disease. This has forced researchers to establish cell lines and tissues of the female reproductive tract as models for the disease. Two cells lines, HEC-1-B, a human epithelial endometrial adenocarcinoma cell line, and ME180, a human cervical epidermal carcinoma cell line, have been used extensively in the *N. gonorrhoeae* field and were used for this study. Men shed *N. gonorrhoeae* that express Opa, pili and lacto-*N*-neotetraose LOS, therefore I used MKC, a strain that produces the lacto-*N*-neotetraose LOS, and selected Pil^+ Opa^+ variants.

EGFR is one of the proteins that is found in the cortical plaques that form beneath adherent *N. gonorrhoeae* on the surface of genital epithelial cells. (87). ErbB2 was found to be recruited to the site of *N. meningitidis* microcolonies on endothelial cells and signaling through ErbB2 was found to be important for invasion (59). The work in *N. meningitidis* implies that EGFR and possibly other ErbB receptors could be important for gonococcal infection. Bacterial pathogens are known to hijack host cell-signaling pathways and use them to their advantage in order to invade into and survive within host cells. Recent studies have shown that *Pseudomonas aeruginosa* activates EGFR in order to prevent epithelial cell apoptosis (161). This led to the hypothesis that *N. gonorrhoeae* activates ErbB receptor(s) for their invasion into genital epithelial cells.

1.7.1 Aim 1

This aim was designed to address the hypothesis that *N. gonorrhoeae* activates ErbB receptor(s) for their invasion into genital epithelial cells. I show that *N. gonorrhoeae* transactivate EGFR and ErbB2 for their invasion into genital epithelial cells. The transactivation of EGFR is accomplished by the activation of heparin bound MMP(s) that cleave HB-EGF.

1.7.2 Aim 2

This aim was designed to determine which signaling cascades *N. gonorrhoeae* induced transactivation of EGFR activates and which EGFR signaling cascades contribute to gonococcal invasion. I examined ERK1/2, PI3K and PLCγ

induction in genital epithelial cells after gonococcal infection. I show that neither PI3K nor ERK1/2, contributes to gonococcal invasion. I show that gonococcal infection induces PLCγ activation and that Ca^{2+} release from intracellular stores is important for gonococcal invasion. I discuss the possibility that Ca^{2+} release is due to EGFR induced PLCγ activation, and additional experiments needed to determine this.

Chapter 2: *Neisseria gonorrhoeae*-induced transactivation of EGFR is required for gonococcal invasion

2.1 Introduction

Neisseria gonorrhoeae is an obligate pathogen of humans and has no other natural host. Infection of genital epithelial cells by *N. gonorrhoeae* is a sequential process, consisting of adherence, invasion, intracellular survival, and exocytosis. These events are initiated and mediated by multiple interactions of gonococcal surface molecules with genital epithelial cells. Initial contact of gonococci to epithelial cells is mediated by their type IV pili (110). Pili retraction brings the bacteria closer to host cells, giving the opportunity for more intimate interactions with host cells mediated by other gonococcal surface molecules, such as opacity protein (Opa) and lipooligosaccharide (LOS) (88). Opas have been shown to bind to cell surface heparin sulfate proteoglycans (HSPG) or carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) and direct the invasion of gonococci into epithelial cells (144, 147). Gonococcal LOS is required for the efficient invasion of *N. gonorrhoeae* into the epithelial cells in the absence of Opa (131). All three of these surface molecules of *N. gonorrhoeae* have the ability to undergo phase variation and genetically vary their surface structures, which allows them to adapt to different host environments and evade host immune protection.

The interactions of gonococcal surface molecules with the epithelial cell surface activate signaling cascades in the host cells and trigger the reorganization of

the cytoskeleton, allowing the entry of the bacteria into host cells and transmigration across the host epithelium (51). Pili retraction from adherent gonococci on the epithelial cell surface activates Ca^{2+} flux (6), PI3K/Akt pathway (79), and MAP kinase ERK (60), leading to actin cytoskeletal rearrangement. The interaction of Opa with HSPG activates phosphatidylcholine-specific phospholipase C (PLC) and the acid sphingomyelinase (50). Opa also can trigger integrin-mediated protein kinase C (PKC) activation through binding to the serum-derived extracellular matrix proteins, fibronectin and vitronectin (30). It has long been reported that the adherence of *N. gonorrhoeae* to A431 cells, an epidermoid carcinoma cell line that expresses a high level of epidermal growth factor receptor (EGFR), induces co-clustering of EGFR, CD44v3, ICAM-1, and F-actin under bacterial adherent sites (87). EGFR, one of the common surface receptors that are essential for epithelial cell survival and proliferation, can activate signaling cascades, including PI3K, PLCγ, Ca^{2+} flux, PKC, and MAP kinases. However, whether EGFR has a role in gonococcal infection and whether and how *N. gonorrhoeae* activate EGFR to support their infectivity remain to be elucidated.

Bacterial pathogens are known to hijack host cell-signaling pathways and use them to their advantage in order to invade into and survive within host cells. Recent studies have shown that EGFR is a common signaling receptor that is manipulated by pathogens for these purposes. Both *Pseudomonas aeruginosa* and *Helicobacter pylori* activate EGFR in order to prevent epithelial cell apoptosis (157, 161). *Haemophilus influenzae* activation of EGFR negatively regulates TLR2 expression in infected host cells (90). *Pasteurella multocida* activation of EGFR stimulates proliferation of

fibroblasts (126). Activation of ErbB2, a related family member of EGFR, is crucial to *N. meningitidis* invasion of endothelial cells (59). *H. pylori* and *P. aeruginosa* both have been shown to transactivate EGFR via inducing the shedding of the EGFR ligand, heparin-binding EGF-like growth factor (HB-EGF) from the plasma membrane, but the mechanism by which these bacteria induce the cleavage of cell surface HB-EGF is largely unknown.

EGFR (ErbB1) belongs to the ErbB family of four closely related receptor tyrosine kinases. These receptors bind differentially to 13 peptide ligands. All of the ligands initially are expressed at the plasma membrane as transmembrane proteins. These precursor proteins are shed from the plasma membrane by proteolytic cleavage that is mediated by members of the matrix metalloproteinase (MMP) family and/or ADAM (a disintegrin and metalloproteinase) family (58). After these processed ligands bind to ErbB receptors, the receptors either homo- or heterodimerize. While ErbB2 does not bind to any ligand, it is able to heterodimerize with the other family members and is their preferred dimerization partner. The cytoplasmic tail of each of the ErbBs contains a tyrosine kinase that trans-autophosphorylates the cytoplasmic tail of its dimerization partner. The phosphotyrosines then serve as docking sites for Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains containing molecules and induce signaling cascades. Depending on the concentration of ligands, the density of the receptor, or the nature of the dimers formed, activation of the ErbB family of receptors results in diverse outcomes, including cell proliferation, survival, migration, and/or differentiation.

In this study, I examine the role of ErbB family receptors in gonococcal adherence to and invasion into genital epithelial cells. Our results show that the interaction of gonococci with genital epithelial cells induces both the tyrosine phosphorylation of EGFR and ErbB2 and their recruitment to the sites of gonococcal attachment. The kinase activity of EGFR is necessary for gonococcal invasion into epithelial cells. Furthermore, the activation of EGFR is not induced by direct interaction of the gonococci with the receptor, but by transactivation via stimulating the gene expression and surface cleavage of EGFR ligands.

2.2 Materials and Methods

2.2.1 Bacterial strains and epithelial cell lines

Neisseria gonorrhoeae strain $MS11_{MKC}$ (MKC) was maintained on gonococcal media base (GCK) with 1% Kellogg's supplement (152). Piliated (Pil^+), Opa-expressing (Opa^+) variants were selected by their light refracting properties using a dissecting light microscope. The concentration of bacteria in suspension was determined spectrophotometrically and verified by viable plate count. Gonococci were killed by incubation with 100 μg/ml gentamicin sulfate at 37°C for 2 h, followed by overnight at 4°C. Before use, the killed gonococci were washed three times in serum free Eagle's MEM. HEC-1-B cells, a human endometrial adenocarcinoma cell line (ATCC# HTB-113), were maintained in Eagle's MEM supplemented with 10% fetal bovine serum (FBS). ME180 cells, a human cervical epidermal carcinoma cell line (ATCC# HTB-33), were maintained in RPMI1640 supplemented with 10% FBS. For establishing polarized epithelial cells, HEC-1-B

cells were seeded at 4×10^4 into 6.5 mm diameter, 3 μm pore size transwell filters (Corning, Lowell, MA) and incubated at 37°C with 5% CO_2, changing the media every other day. Polarization was monitored by transepithelial resistance (TER) readings daily. The cells were allowed to grow for 7-9 days until the maximum TER values (~400 Ω) were reached.

2.2.2 Inhibitors and antibodies

AG1478, an EGFR kinase inhibitor, AG825, an ErbB2 kinase inhibitor, and anti-EGFR mAbs that were used for confocal microscopy and prevention of EGFR ligand binding were purchased from Calbiochem (San Diego, CA). Anti-EGFR antibody that was used for western blot and anti-ErbB2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-tubulin antibody and heparin were purchased from Sigma (St. Louis MO). Anti-phosphotyrosine mAb (4G10) was purchased from Millipore (Temecula, CA). Anti-ZO-1 was purchased from BD Biosciences (San Jose, CA). Anti-HB-EGF-biotin conjugate was purchased from R&D Systems (Minneapolis, MN).

2.2.3 Bacterial adherence and invasion assays

Epithelial cells (5×10^4/well) were seeded in 96-well plates and incubated at 37°C in 5% CO_2. After 24 h, cells were cultured in serum-free medium overnight. Cells were pre-incubated with AG1478 and AG825 for 2 h or anti-EGFR mAb for 30 min. Next, cells were incubated with MKC P^+O^+ at an MOI of 5 for 2 h for adherence assays and 6 h for invasion assays at 37°C. For adherence assays, cells were washed

with PBS and then lysed in 1% saponin, and appropriate dilutions were plated on GCK medium. For invasion assays, cells were washed and then incubated with 50 µg/ml gentamicin for 1.5 h at 37°C. After extensively washing to remove remaining gentamicin, bacteria that had invaded were quantified by lysing the epithelial cells with 1% saponin and serially plating the cell lysates on GCK plates. The significance of differences was assessed using the Student's t-test for independent population means.

For the heparin wash treatment, the epithelial cells were incubated with 5 mg/ml heparin in serum-free media at 37°C two times for 15 min each and three times for 1 min. The cells were washed with serum-free media four times to remove any remaining heparin before proceeding with the adherence or invasion assays.

2.2.4 Immunofluorescence microscopy

Epithelial cells were seeded at 2×10^5 onto coverslips in 24-well dishes, cultured for 24 h, and then serum-starved overnight. Cells were incubated with MKC P^+O^+ at an MOI of 5 for 4-6 h, washed, and fixed with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Ft. Washington, PA). Then, cells were stained with anti-ErbB2, anti-EGFR and anti-*N. gonorrhoeae* antibodies (13). The polarized cells were fixed prior to immunostaining using the pH shift method. The cells were first fixed with 4% PFA in 80 mM Pipes, pH 6.5, 150 mM NaCl, 5 mM EGTA, 2 mM $MgCl_2$ for 10 min and then shifted to 4% PFA in 100 mM NaBorate, 150 mM NaCl for 10 min. The cells were permeabilized and blocked in PB solution (DMEM, 10% FBS, 10 mM Hepes pH 7.6, 10 mM glycine, 0.05% saponin) and stained with

primary and secondary antibodies in PB. After post-fixing with 2% PFA, cells were mounted and analyzed using a Zeiss LSM 510 laser scanning confocal microscope. For Z-stack images, a series of images from the top to bottom of the cells were taken at 0.5 μm steps.

2.2.5 Immunoblotting

Epithelial cells were seeded at 1×10^6 in 6-well dishes. After 24 h, the cells were serum-starved overnight and incubated with MKC P^+O^+ at an MOI of 5 for up to 6 h. The cells then were washed with ice-cold PBS and lysed in 75 μl RIPA buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 2 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1× proteinase inhibitor cocktail (Sigma)). Cell lysates were mixed 1:1 with nondenaturing loading buffer (for detection of HB-EGF) or denaturing loading buffer for all other proteins. Lysates were separated through SDS-polyacrylamide gels (Bio-Rad, Hercules, CA), analyzed by western blot, and visualized using Western Lightning chemiluminescence substrate (Perkin Elmer, Boston, MA). Images were acquire and digitized directly using Fujifilm LAS-3000 (Valhalla, NY) or acquired with x-ray film. Blots were stripped with Restore western blot stripping solution (Pierce, Rockford, IL) and reprobed with anti-β-tubulin antibody. The blots were quantified by densitometry using MultiGauge software from Fujifilm.

2.2.6 Immunoprecipitation

Epithelial cells were seeded at 1×10^6 in 6-well dishes. After 24 h, the cells were serum-starved overnight and incubated with MKC P^+O^+ at an MOI of 5 for up to 6 h. The cells then were washed with ice-cold PBS and lysed in 1% Triton-X 100 lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1× proteinase inhibitor cocktail). The lysates were sonicated and centrifuged at 4°C. The supernatants were subjected to immunoprecipitation using anti-phosphotyrosine mAb and Protein G conjugated sepharose beads (GE Healthcare, Piscataway, NJ). The immunoprecipitates were analyzed using SDS-PAGE and western blot, probing for EGFR or ErbB2 using specific antibodies.

2.2.7 Real Time PCR

HEC-1-B cells that were grown to ~90% confluence were serum starved overnight and incubated with MKC P^+O^+ at an MOI of 5 for up to 8 h. Total RNA was extracted with Trizol (Invitrogen). RNA was converted to cDNA with Superscript III (Invitrogen) using oligo dT primers according to the manufacturer's protocol. The cDNA was amplified using 1X SYBR Green master mix (Applied Biosystems, Foster City, CA) using the following conditions: 50°C for 2 min and then 95°C for 10 sec, followed by 46 cycles of 95°C for 15 sec, 58°C for 15 sec and 72°C for 30 sec. The products were denatured at 95°C for 15 sec, annealed at 58°C for 30 sec and then subjected to a slow dissociation by ramping from 58°C to 95°C at 2% of the normal ramp rate in order to insure that only one PCR product was

amplified. The primers for the EGFR ligands have been previously published (Table 1, appendix) (132). Beta-actin was amplified as an internal standard using QuantumRNA beta-Actin primers (Ambion, Austin, TX).

Table 1 PCR Primers for EGFR Ligands

Ligand	bp	primer sequences
Amphiregulin	266	5' GGCTCAGGCCATTATGC 3' 5' ACCTGTTCAACTCTGACTGA 3'
Betacellulin	251	5' CTGCAAAGTGCCTTGCTCA 3' 5' TGACTAGTAATCCTGGTGAC 3'
EGF	101	5' AGCAATTGGTGGTGGATG 3' 5' ACTCTTTGCAAAAGTTGTC 3'
Epiregulin	238	5' CAAAGTGTAGCTCTGACATG 3' 5' CTGTACCATCTGCAGAAATA 3'
HB-EGF	126	5' GTGCCTAGACTGTTACTTTG 3' 5' GAAATGTAGACAGACATTAAAT 3'
TGFα	528	5' GCCCGCCCGTAAAATGGTCCCCTC 3' 5' CACCTGGCCAAACTCCTCCTCTGGG 3'

2.3 Results

2.3.1 EGFR and ErbB2 are recruited to adherent sites of gonococci in genital epithelial cells.

Previous studies have shown that *N. gonorrhoeae* and *N. meningitidis* recruit EGFR (87) and ErbB2 (59), respectively, to the bacterial adherent sites in epithelial or endothelial cells. To examine how these two receptors are involved in the interaction of gonococci with genital epithelial cells, I compared the cellular distribution of EGFR and ErbB2 in human endometrial epithelial cells (HEC-1-B) and human cervical epithelial cells (ME180) before and after incubation with live and killed *N. gonorrhoeae* strain $MS11_{MKC}$ (MKC). The epithelial cells grown on glass coverslips were inoculated with live or gentamicin-killed MKC for 5 h. The cells then were fixed and stained for EGFR, ErbB2 and the bacteria without permeabilization of the epithelial cells. In uninfected cells, both EGFR and ErbB2 were found evenly distributed on the surface of HEC-1-B and ME180 cells (Fig. 3A-D and data not shown). After incubation with MKC, both EGFR and ErbB2 accumulated and surrounded the gonococci at the surfaces of HEC-1-B (Fig. 3E-H) and ME180 cells (Fig. 3Q-T). Gentamicin-killed MKC adhered to the surface of both cell lines as diplococci, (Fig. 3I and data not shown). Neither EGFR nor ErbB2 accumulated or localized around the adherent gentamicin-killed MKC (Fig. 3I-L). This indicates that *N. gonorrhoeae* induces the recruitment of EGFR and ErbB2 to the site of bacterial adherence, and that this recruitment requires the viability of gonococci. However, since the gonococci do not appear to colocalize with EGFR and ErbB2 (Fig. 3E-H and 3Q-T), this suggests that gonococci do not bind directly to these two receptors.

EGFR and ErbB2 have been reported to localize predominately at the basolateral surface (75, 151), while gonococci initiate infection at the apical surface of polarized epithelial cells. All previous findings that EGFR and ErbB2 are recruited to gonococcal adherent sites are based on unpolarized epithelial cells. In order to investigate if basolaterally located EGFR and ErbB2 are recruited to the adherent gonococci on the apical surface, I polarized HEC-1-B cells by growing them on transwell filters, before infecting them with gonococci from the apical side. Polarization of the HEC-1-B cells was monitored by transepithelial resistance (TER) and confirmed by distinct cellular distribution of zonula occludens (ZO)-1, a marker protein of the tight junction that divides the apical and basolateral plasma membrane (Fig. 4A-B). The distribution of EGFR and ErbB2 was analyzed using confocal microscopy. In uninfected polarized HEC-I-B cells, both EGFR and ErbB2 were found predominately at the lateral surface below the tight junction (Fig. 4C and E, yellow arrows), but not at the apical surface of polarized HEC-1-B cells (Fig. 4C and E, white arrows), as reported previously in other types of polarized epithelial cells (75, 151). This further confirms that these HEC-1-B cells were polarized. After incubation with MKC, EGFR and ErbB2 were found at both the apical and basolateral surfaces (Fig. 4D and F). Particularly, EGFR and ErbB2 were accumulated under gonococci at the apical surface (Fig. 4D and F, white arrows). Gonococci had no significant effect on the TER of polarized HEC-1-B cells (data not shown). This result indicates that gonococcal infection induces the translocation of both EGFR and ErbB2 from the basolateral to apical surface and recruits them to the site of adherent gonococci at the apical surface.

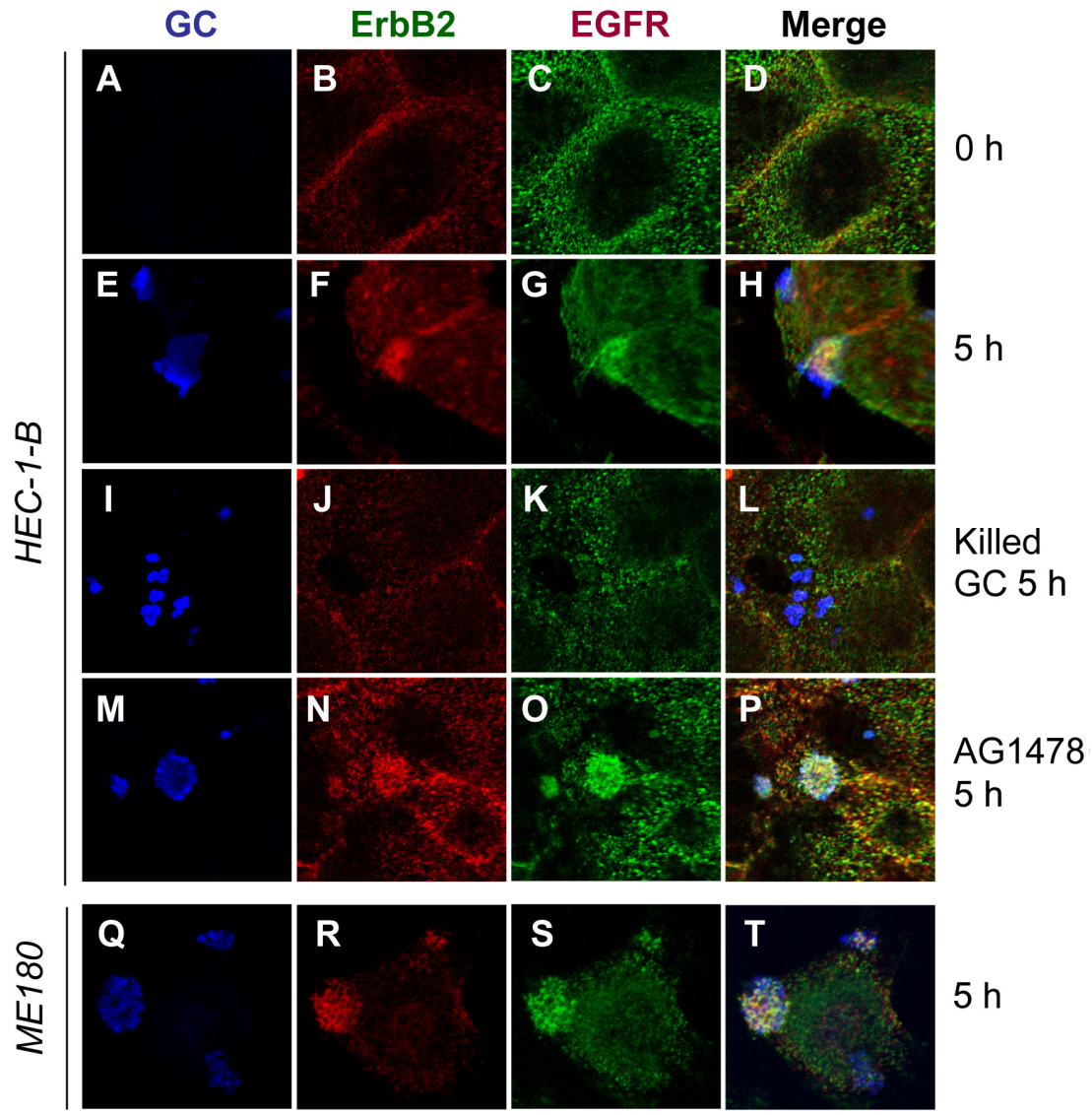

Figure. 3. Live, but not killed, *N. gonorrhoeae* causes redistribution of EGFR and ErbB2 at the epithelial cell surface. HEC-1-B (A-P) or ME180 (Q-T) cells were pretreated with (M-P) or without EGFR inhibitor AG1478 and then incubated with live or gentamicin-killed MKC Pil^+ Opa^+ for 5 h. Uninfected HEC-1-B cells (A-D) served as a control. The cells then were fixed and stained with gonococcal antiserum, anti-EGFR mAb, anti-ErbB2 mAb, and corresponding secondary antibodies. Optical sections were acquired using a confocal microscope (Zeiss LSM 510). Shown are representative images of single optical sections from three independent experiments. Bar, 5 μm.

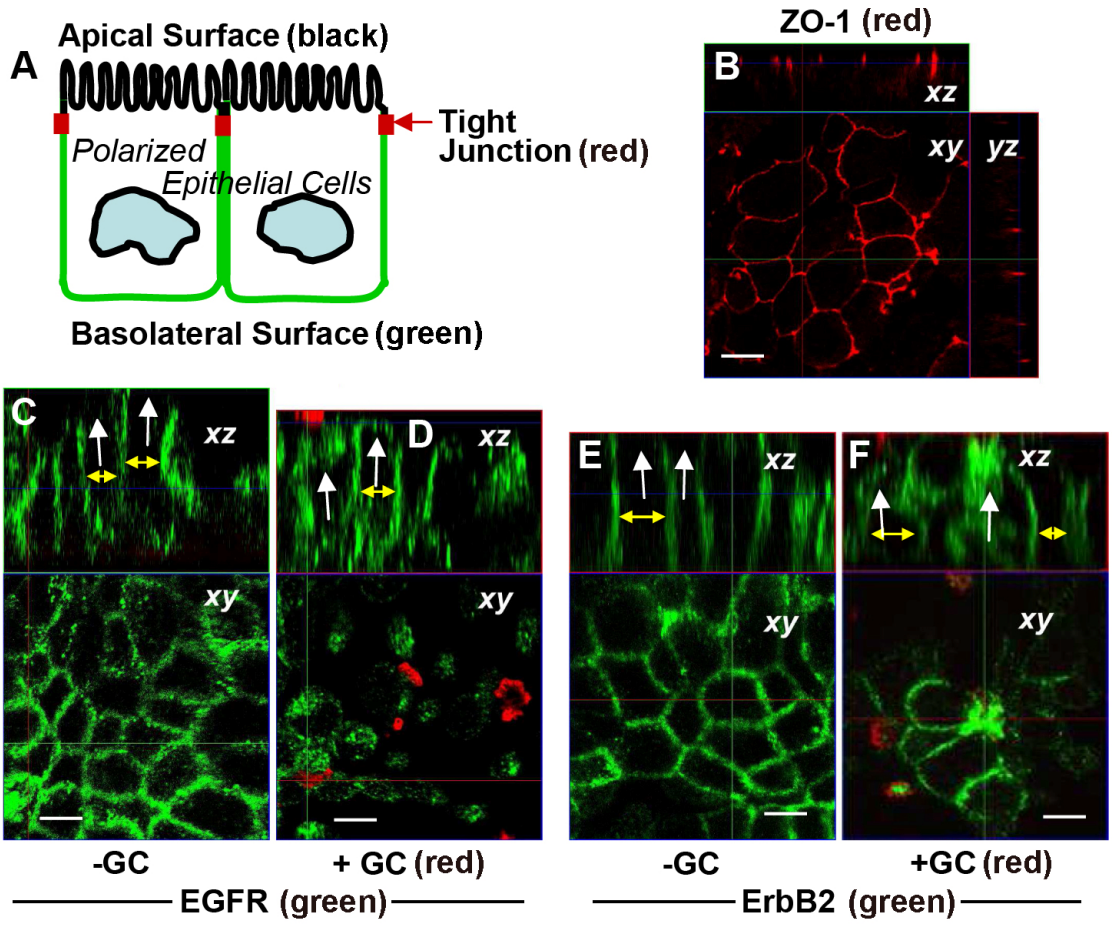

Figure. 4. *N. gonorrhoeae* recruits EGFR and ErbB2 from the basolateral to the apical surface beneath bacterial adherent sites in polarized epithelial cells. (A) A diagram shows the tight junction (red) dividing the apical (black) and basolateral (green) surfaces. (B-F) HEC-1-B cells were grown on transwells until transepithelial resistance peaked. Polarized HEC-1-B cells were incubated with (D and F) or without MKC Pil^+ Opa^+ (B, C and E) in the apical chamber for 5 h. Then cells were fixed and stained for gonococci, EGFR (C and D), ErbB2 (E and F), and/or the tight junction marker ZO1 (B). Series of images in Z-axes of cells were acquired at 1.0 (B) or 0.5 μm (C-F) per optical section using a Zeiss LSM 510 confocal microscope. Shown are representative images of xy, xz and yz optical sections from three independent experiments. Yellow arrows point to the lateral staining of EGFR and ErbB2. White arrows point to the apical surfaces and EGFR or ErbB2 staining that concentrates beneath the gonococci adhered to the apical surface of the HEC-1-B cells. Bar, 5 μm.

2.3.2 Kinase inhibitors of ErbB receptors alter *N. gonorrhoeae* invasion into epithelial cells.

In order to investigate the role of EGFR and ErbB2 in the invasion of *N. gonorrhoeae* into human epithelial cells, I performed gentamicin protection assays using inhibitors specific for the tyrosine kinases of the two ErbB family receptors. HEC-1-B or ME180 cells were treated with AG1478, an EGFR kinase inhibitor, or AG825, an ErbB2 kinase inhibitor, prior to their incubation with MKC. As shown in Fig. 5, inhibition of EGFR kinase activity with AG1478 significantly reduced invasion of MKC into both HEC-1-B (Fig. 5A) and ME180 cells (Fig. 5C). In ME180 cells there was a dose dependent decrease in the invasion level of MKC (80% at 5 μM) (Fig. 5C), whereas the invasion of MKC into HEC-1-B cells was more sensitive to the inhibitor and showed significant reductions at nanomolar concentrations (Fig. 5A). In contrast, inhibition of ErbB2 kinase activity with AG825 dramatically increased the invasion of MKC, up to 6-fold, in HEC-1-B cells (Fig. 5B), but had no significant effect on MKC invasion into ME180 cells (Fig. 5D). In contrast to their effects on gonococcal invasion, both inhibitors had no significant effect on adherence of MKC to HEC-1-B and ME180 cells (Fig. 5E-F) and the recruitment of EGFR and ErbB2 to the site of bacterial attachment (Fig. 3M-P and data not shown). Furthermore, there were no detectable effects of the inhibitors on bacterial viability and growth (data not shown). Different sensitivities of gonococcal invasion to the inhibitors in two different cell lines implicate differential expression levels of EGFR and ErbB2 in HEC-1-B and ME180 cells. Indeed, I found that ME180 cells, which required a much higher concentration of the EGFR inhibitor to reduce gonococcal

invasion, expressed a 17-fold higher protein level of EGFR and a 5-fold higher protein level of ErbB2 than HEC-1-B cells (Fig. 5G). These results suggest that the kinase activity of EGFR is required for efficient invasion of gonococci into endometrial and cervical epithelial cells, but the kinase activity of ErbB2 has a negative regulatory role in gonococcal invasion. Neither EGFR nor ErbB2 are essential for gonococcal adherence to epithelial cells.

2.3.3 *N. gonorrhoeae* infection induces the phosphorylation of EGFR and ErbB2.

The effect of EGFR and ErbB2 kinase inhibitors on the invasion of gonococci into epithelial cells suggests the involvement of these receptors in the gonococcal invasion process. Therefore, I examined whether gonococcal infection induces activation of the two ErbB family receptors. The activation of EGFR and ErbB2 was monitored by their tyrosine phosphorylation. Lysates were prepared from HEC-1-B cells that had been incubated with MKC for up to 6 h and were subjected to immunoprecipitation with a monoclonal antibody (mAb) specific for phosphotyrosine. The immunoprecipitates were analyzed using western blot, probing for EGFR and ErbB2. As shown in Fig. 6, gonococci induced the tyrosine phosphorylation of both EGFR and ErbB2. By 3 h post gonococcal inoculation, the phosphorylation levels of EGFR had doubled, as compared to uninfected control cells. The phosphorylated EGFR levels continued to increase until 4 h post infection, reaching a 3-fold increase over uninfected levels, and remained elevated at 6 h post inoculation. The phosphorylation levels of ErbB2 followed the same temporal

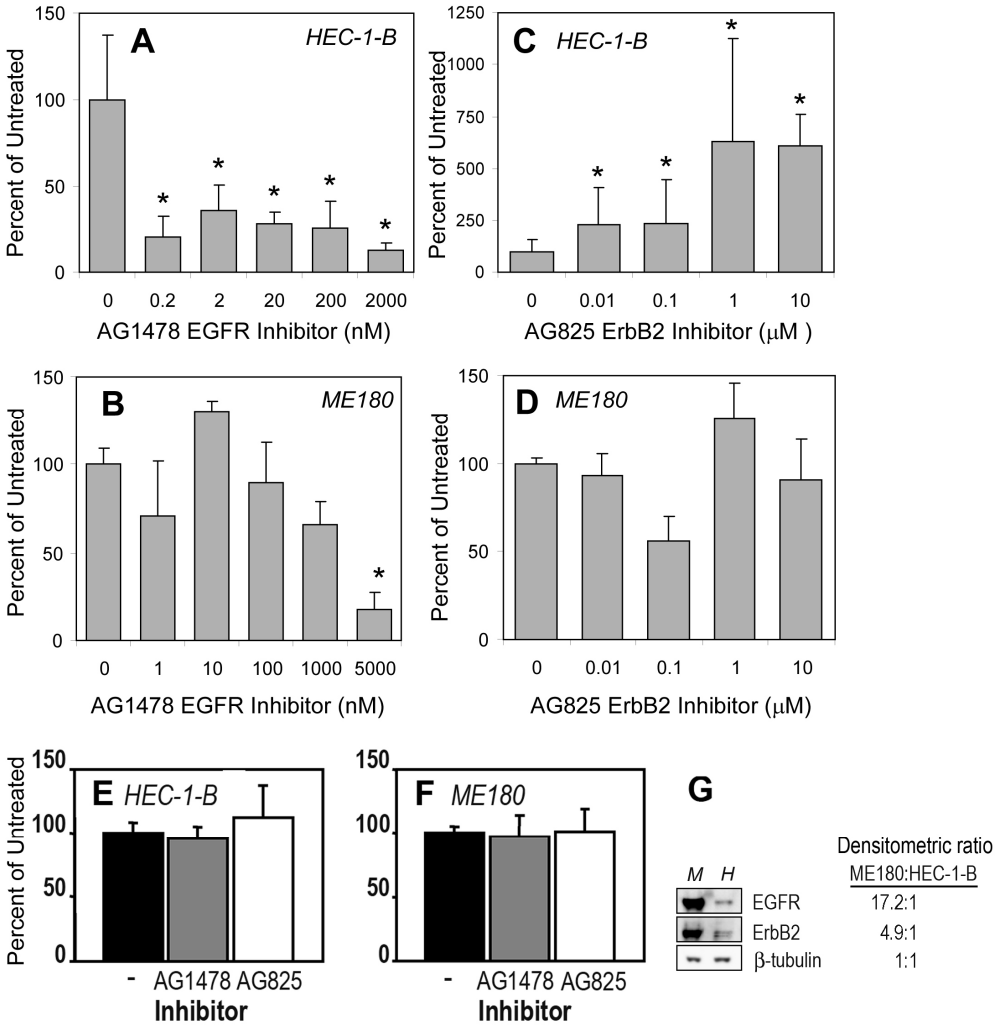

Figure. 5. ErbB kinase inhibitors alter gonococcal invasion. (A-D) To quantify invaded bacteria, HEC-1-B (A and B) or ME180 (C and D) were preincubated with either the EGFR kinase inhibitor AG1478 (A and C) or the ErbB2 kinase inhibitor AG825 (B and D) before the addition of MKC Pil^+ Opa^+. After incubating with the bacteria for 6 h, the epithelial cells were treated with gentamicin, washed and lysed to determine the number of invaded (gentamicin resistant) bacteria. (E-F) To quantify the number of adherent bacteria, HEC-1-B (E) and ME180 (F) cells were pretreated with the inhibitors (5 μM) and incubated with MKC for 2 h. Cells were washed and lysed to quantify the bacteria. The data are plotted as percentages of the gonococci invaded into (A-D) or adhered to (E and F) untreated cells. Shown are the mean percentages (± SD) from three independent experiments with six replicates per experiment. *$P < 0.05$ (as compared with no inhibitor). (G) Equal amounts of ME180 and HEC-1-B cell lysates were analyzed by SDS-PAGE and western blot, probing for EGFR and ErbB2. The blots were stripped and probed for β-tubulin as normalization controls. The blots were quantified by densitometry. Shown are representative blots and ratios of EGFR and ErbB2 expression between ME180 (*M*) and HEC-1-B cells (*H*).

pattern as those of EGFR, but its increase was more subtle. Phosphorylation levels of ErbB2 reached their maximum level at 4 h, but returned to near control levels by 6 h post inoculation (Fig. 6).

2.3.4 Anti-EGFR antibody inhibits *N. gonorrhoeae* invasion, but not their adherence to epithelial cells.

Our findings that gonococci induce the activation of EGFR and ErbB2 tyrosine kinases and that the inhibitor of EGFR kinase reduced gonococcal invasion indicate that the activation of EGFR kinase is important for gonococcal invasion of epithelial cells. I hypothesized that gonococci either activate these receptors by binding directly to EGFR and/or ErbB2, or by increasing the levels of the receptor's ligands. In order to investigate these possibilities, I preincubated ME180 cells with a mAb specific for the extracellular ligand binding domain of EGFR, which blocks the binding of ligands to EGFR (122). Adherence and gentamicin protection invasion assays then were performed. The treatment of anti-EGFR mAb had no influence on the ability of gonococci to adhere to ME180 cells (Fig. 7A). Microscopic studies revealed no differences in the size or number of gonococcal clusters formed on the surface of ME180 cells that were treated with or without anti-EGFR mAb (Fig. 7C). Anti-EGFR mAb at a concentration of 10 μg/ml, however, inhibited 50% of the gonococcal invasion as compared to a control mAb (Fig. 7B). These results suggest that the binding of ligand(s) to EGFR is involved in gonococcal invasion.

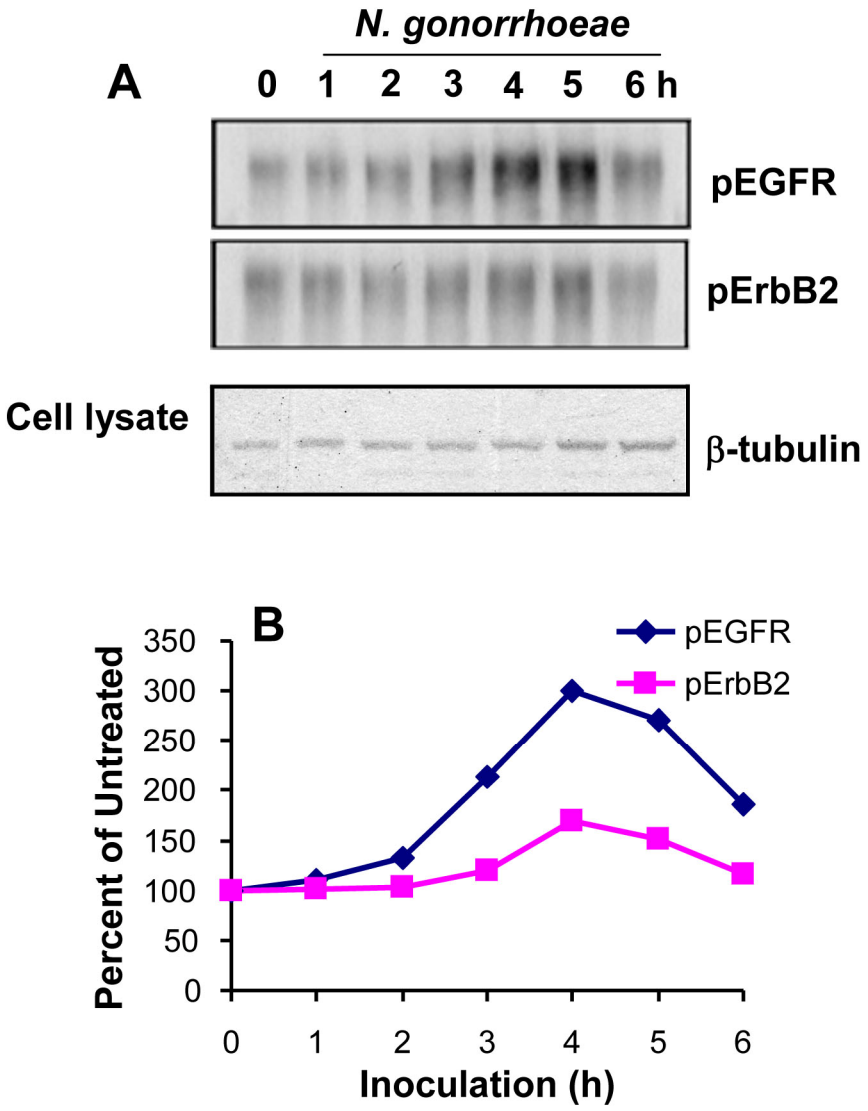

Figure. 6. *N. gonorrhoeae* infection induces the tyrosine phosphorylation of EGFR and ErbB2. HEC-1-B cells were incubated with MKC Pil$^+$ Opa$^+$ for up to 6 h. The cells were lysed and subjected to immunoprecipitation with anti-phosphotyrosine mAb 4G10. The cell lysates were analyzed by SDS-PAGE and western blot, probing for EGFR. ErbB2 was probed after stripping. β-tubulin in cell lysates was analyzed as normalization controls. The blots were quantified by densitometry, and the data are plotted as percentages of uninfected epithelial cell controls. Shown are representative blots (A) and the average percentages (B) from three independent experiments.

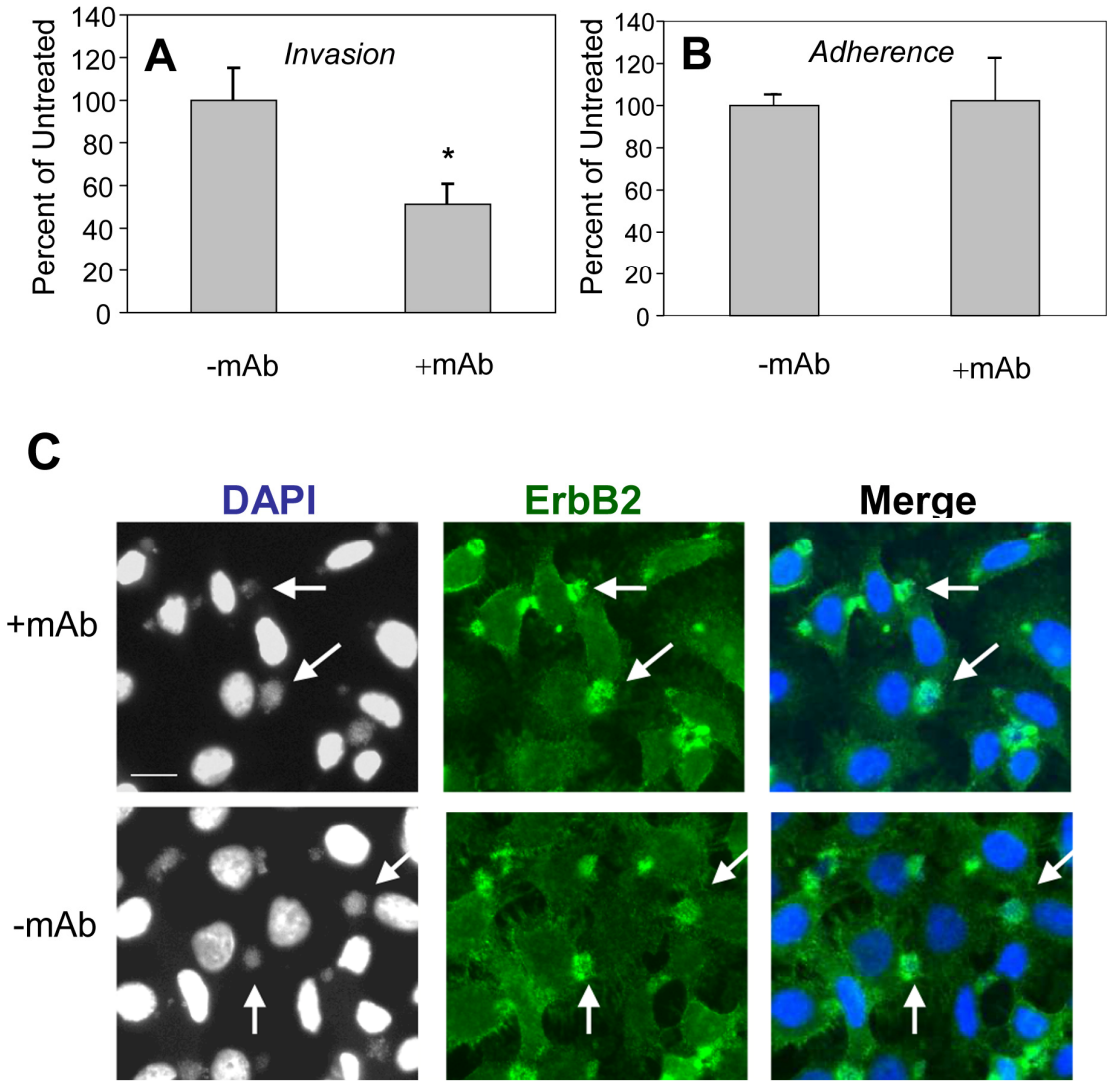

Figure. 7. Interference with EGFR ligand binding reduces gonococcal invasion. (A-B) ME180 cells were pretreated with either 10 μg/ml anti-EGFR mAb that prevents EGFR ligands from binding to EGFR or an isotype control mAb and incubated with MKC Pil^+ Opa^+ for 6 h. The number of epithelial cell-associated bacteria (A) or gentamicin-resistant invaded bacteria (B) was determined. The results are plotted as a percent of the bacteria adhered to and invaded into cells treated with the control mAb. Shown are mean values (± SD) from three independent experiments with replicates of six per experiment. *$P < .05$ (as compared to untreated). (C) ME180 cells that were incubated with anti-EGFR mAb and the bacteria as described above were fixed and stained with DAPI for visualization of nucleic acids and anti-ErbB2 mAb. Images were acquired using a fluorescence microscope. Arrows point to gonococcal clusters. Bar, 5 μm.

2.3.5 *N. gonorrhoeae* infection increases the transcription of a subset of EGFR ligands.

To test whether gonococci can transactivate EGFR and ErbB2 by increasing the expression of the ligands, I quantified mRNA levels of all six ligands that EGFR binds in HEC-1-B cells using real time-PCR. The transcription levels were normalized against the mRNA level of actin. The mRNA levels for heparin binding epidermal growth factor-like growth factor (HB-EGF) and amphiregulin dramatically increased after gonococcal infection (Fig. 8). HB-EGF mRNA transcripts had the largest increase, reaching 30-fold that of the levels in uninfected cells (Fig. 8). Amphiregulin transcripts increased 4.5-fold as compared to uninfected control cells. TGF-α transcripts steadily increased over time and had doubled that of uninfected cells by 8 h. The remaining three ligands, EGF, epiregulin, and betacellulin were either down-regulated or only marginally increased after infection, in comparison with the levels in uninfected cells. This indicates that gonococcal infection induces the transcription of a subset of EGFR ligands.

2.3.6 Inhibition of EGFR ligand cleavage inhibits *N. gonorrhoeae* invasion without altering their adherence.

The ligands for EGFR are expressed initially as transmembrane precursors and are shed from the plasma membrane after proteolytic cleavage by members of the MMP and ADAM families when needed (58). Many MMPs, including MMP-1, -2, -7, -9, and -13, bind to the heparan sulfate moieties that decorate cell surface heparan sulfate proteoglycans (HSPG) and/or are in the extracellular matrix (160). MMP-7 can be removed from rat uterus tissue by washing with heparin. It is presumed that other

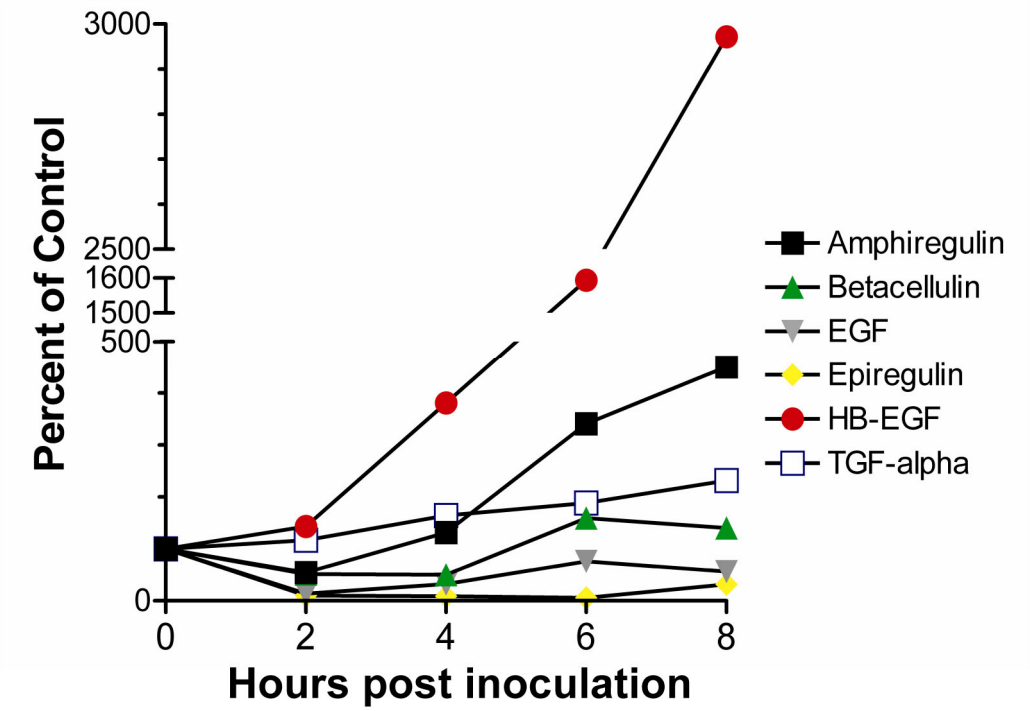

Figure. 8. *N. gonorrhoeae* infection increases transcription of a subset of EGFR ligands. HEC-1-B cells were incubated with MKC Pil^+ Opa^+ at a MOI of 5 for up to 8 h. Total RNA was extracted and reverse transcribed. The mRNA levels of six EGFR ligands were quantified by real-time PCR. β-actin was used as an internal control for normalization. Shown are representative results from two independent experiments.

heparan sulfate bound MMPs also can be removed in this manner (160), suggesting that heparin washes can be used to deplete heparan sulfate bound MMPs at the cell surface in order to prevent the cleavage of HB-EGF and other ErbB ligands. Previous studies have shown that including heparin during the incubation of gonococci with epithelial cells inhibits gonococcal adherence, due to inhibition of Opa binding to HSPG on the surface of epithelial cells. I have made a similar observation (data not shown). In order to determine the effect of the heparin washes on gonococcal invasion, free heparin was removed by extensive washes with serum free media after the heparin washes and before adherence and invasion analyses. After removing free heparin before adherence analysis, heparin washes had no significant effect on the adherence of gonococci to either HEC-1-B or ME180 cells (Fig. 9A). The heparin washes, however, inhibited the invasion of gonococci into both HEC-1-B and ME180 cells by 75% (Fig. 9A). This suggests that the cleavage of EGFR ligands is important for gonococcal invasion into epithelial cells.

To confirm that the heparin washes removed heparin sulfate-associated MMPs, consequently preventing the shedding of EGFR ligands, I determined the levels of cleaved, soluble HB-EGF (sHB-EGF). HEC-1-B cells that were subjected to either the heparin wash or medium wash were incubated with MKC for varying lengths of time. The HEC-1-B/MKC co-culture media was analyzed for sHB-EGF by ELISA. There was no detectable sHB-EGF in the co-culture media from HEC-1-B cells that were subjected to either the medium wash or the heparin wash (data not shown). Because the sHB-EGF is often found associated with heparin sulfate moieties on the cell surface but not found in the supernatant (156), I looked for cell-

associated sHB-EGF. HEC-1-B cells that were subjected to either the heparin wash or medium wash were incubated with MKC for varying lengths of time and lysed. The cell lysates were analyzed using non-reducing SDS-PAGE, and sHB-EGF was detected by western blotting using a biotinylated anti-HB-EGF antibody. In the lysates generated from cells subjected to the medium wash, gonococcal infection dramatically increased the amount of sHB-EGF. The levels of sHB-EGF increased with time and peaked at 4 h (Fig. 9B and C). This is consistent with the finding that gonococcal infection increases the transcripts of HB-EGF. Importantly, the heparin wash significantly reduced gonococci-induced production of sHB-EGF (Fig. 9B and C). This further supports our hypothesis that gonococci activate EGFR by inducing the production of EGFR ligands.

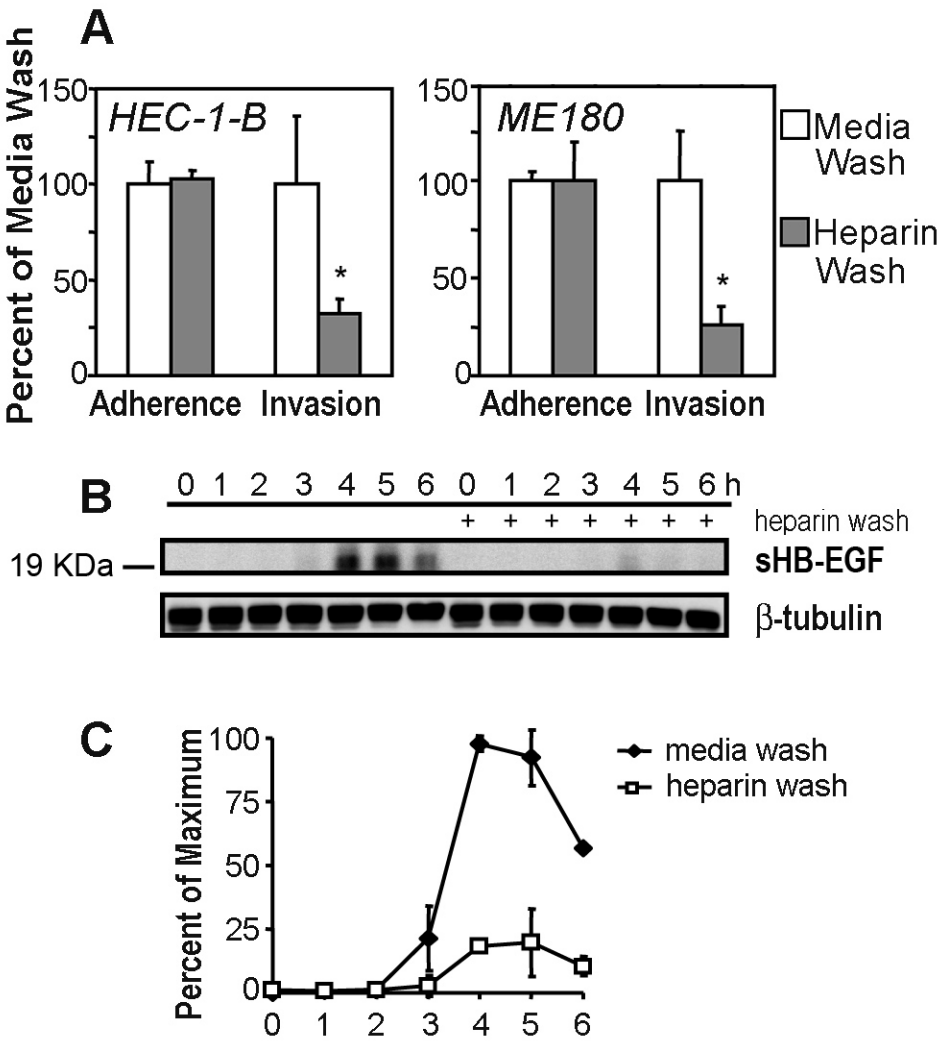

Figure. 9. Inhibition of EGFR ligand cleavage from epithelial cells by heparin washes reduces gonococcal invasion. (A) HEC-1-B and ME180 cells were washed with 5 mg/ml heparin to remove heparin-bound MMPs, and then were washed with serum-free media to remove all traces of free heparin. Heparin washed or media washed epithelial cells were incubated with MKC Pil$^+$ Opa$^+$ for 6 h. The number of epithelial cell-associated (adherent) and gentamicin-resistant (invasive) bacteria was determined. The results are plotted as a percent of the media washed control. Shown are the mean values ± SD generated from three independent experiments with replicates of six per experiment. (B) Lysates were prepared from media washed or heparin washed HEC-1-B cells that had been incubated with MKC Pil$^+$ Opa$^+$ for up to 6 h. The lysates were subjected to western blot, probing for HB-EGF. The blots were stripped and reblotted for β-tubulin, which served as a loading control. The blots were quantified by densitometry. The data was plotted as a percentage of the maximal amount of sHB-EGF in the cells exposed to the bacteria (C). Shown are representative blots (B) and the mean values (± SD) of three independent experiments (C). *$P < 0.05$ (as compared to media washed).

2.4 Discussion

In this study I investigated the role of EGFR, a common signaling receptor on the epithelial cell surface, in the invasion of *N. gonorrhoeae* into genital epithelial cells. Our results demonstrate that *N. gonorrhoeae* induces the activation of EGFR and that this activation is required for gonococcal invasion into epithelial cells. Our results further demonstrate that gonococci activate EGFR by increasing the gene expression and shedding of EGFR ligands, rather than interacting directly with EGFR. This uncovers a mechanism by which *N. gonorrhoeae* activates EGFR for their invasion.

Gonococci-induced EGFR activation is demonstrated by the tyrosine phosphorylation of EGFR and its dimerization partner ErbB2 and their recruitment to the site of gonococcal adherence. The recruitment of EGFR to gonococcal adherent sites has been observed previously (87). However, all earlier studies have used unpolarized epithelial cells, and therefore it was unclear whether such recruitment would occur in vivo, where genital epithelial cells are polarized with distinct apical and basolateral surfaces. *N. gonorrhoeae* establishes adherence at the apical surface of polarized epithelial cells, while EGFR predominately is expressed at the basolateral surfaces (75, 151). Here I show that EGFR and ErbB2, which are preferentially expressed on the lateral surface of polarized HEC-1-B cells, were translocated to the apical surface upon gonococcal adherence. This supports the notion that *N. gonorrhoeae* can recruit EGFR and ErbB2 in both polarized and unpolarized epithelial cells.

In addition, I found that the recruitment of both EGFR and ErbB2 is dependent upon the viability of gonococci, as gentamicin-killed gonococci are unable to induce the recruitment. The mechanistic reason for the inability of gentamicin-killed gonococci to recruit EGFR and ErbB2 is unclear. A major difference between live and killed gonococci is that while the killed bacteria are able to adhere to epithelial cells, they are unable to form microcolonies on or invade into epithelial cells (13). The formation of microcolonies may be essential to induce the redistribution of EGFR and ErbB2 simply by the physical impact of the microcolony on the epithelial cell surface, which could induce intracellular signaling. Pil^- gonococci that have all *opa* genes deleted do not form the typical microcolony; instead they attach to the surface of HEC-1-B and ME180 cells primarily as individual or small clusters of diplococci, appearing similar to killed gonococci that express both pili and Opa (unpublished data). In the absence of both pili and Opa, gonococci invade at least two logs less well than gonococci expressing either structure individually (138). This lends credence to the idea that microcolony formation is important for the invasion of gonococci into epithelial cells. A second possibility is killed gonococci are unable to synthesize new proteins and surface molecules, even though initially expressing the same surface structures as the live gonococci. The requirement of newly synthesized proteins and other molecules for gonococcal invasion has been suggested by previous observations that inhibition of bacterial protein synthesis with chloramphenicol inhibits gonococcal invasion (53), and that pre-incubation of the bacteria with fixed HEC-1-B cells, which potentially alters the expression of bacterial molecules, increases gonococcal invasion ability

(20). The third possibility is that killed gonococci would be unable to retract their pili, as this is an ATP driven event. Pilus retraction has been shown to induce signaling and to be important for the invasion process (88).

Our finding that the EGFR kinase inhibitor significantly reduced gonococcal invasion indicates an important role of EGFR activation in gonococcal invasion. Our data further showed that while gonococci induce the activation of both EGFR and ErbB2, blocking EGFR and ErbB2 kinases have different effects on gonococcal invasion. Prevention of EGFR kinase activation inhibits gonococcal invasion into epithelial cells. However prevention of ErbB2 kinase activity either had no effect (ME180 cells) or significantly increased (HEC-1-B) the invasive ability of gonococci.

EGFR and ErbB2 are two of four members of the ErbB family of receptor tyrosine kinases. Upon ligand binding, dimerized ErbB receptors phosphorylate each other. While ErbB2 does not bind any ligand, it is the preferred dimerization partner of EGFR because its ectodomain is locked in the active conformation for dimerization (47, 142). ME180 cells express 17-fold more EGFR and 5-fold more ErbB2 than HEC-1-B cells. These differences in expression levels provide an explanation for the different sensitivities of HEC-1-B and ME180 cells to the EGFR and ErbB2 kinase inhibitors. In addition, the differences in EGFR and ErbB2 expression levels would change the molecular ratios of EGFR to ErbB2, consequently altering the nature of ErbB dimers formed on the surface of the two cell lines in response to ligand binding. HEC-1-B cells have a higher ErbB2:EGFR ratio, thus are expected to generate more ErbB2:EGFR heterodimers than ME180 cells. ME180 cells on the other hand have a higher EGFR:ErbB2 ratio, and are expected to generate

more EGFR:EGFR homodimers than HEC-1-B cells. The two different dimers formed by ligand binding, EGFR:EGFR and EGFR:ErbB2, could activate different signaling cascades because their cytoplasmic tails contain different numbers of tyrosine phosphorylation sites and bind to different signaling molecules (158). While both EGFR and ErbB2 are essential for epithelial cell survival and proliferation, activation of ErbB2 has been shown to disrupt apical-basal polarity and tight junctions by ErbB2's direct association with the Par polarity complex (2). Thus, while both EGFR and ErbB2 are activated by gonococci, the two receptors could play roles in different steps of the infection. Our finding of enhanced gonococcal invasion in the absence of ErbB2 kinase suggests that the kinase activity of ErbB2 is not essential and may be inhibitory to gonococcal invasion, but it does not exclude possible roles for ErbB2 in other cellular processes of gonococcal infection, such as disrupting the tight junction for gonococcal transmigration across the epithelium. Furthermore, the expression levels of different ErbB family receptors could be varied at different genital tissue locations, and their expression levels can be further regulated by sex hormones that control the menstrual cycle (24, 84). As an opportunistic pathogen, the ability of *N. gonorrhoeae* to activate multiple members of ErbB receptors may allow the bacteria to establish infection at different locations of the genital tissue and different stages of the menstrual cycle using different mechanisms.

While the recruitment of EGFR to *N. gonorrhoeae* has been reported in the past, how the bacteria activate EGFR was unknown. Gonococci either may bind directly to EGFR or induce the expression and secretion of EGFR ligands thereby

transactivating it. The results from this study argue against a direct interaction of gonococci with EGFR as the mechanism for EGFR activation. First, killed gonococci failed to recruit EGFR and ErbB2, even through they can adhere to the epithelial cell surface. Second, confocal microscopic studies revealed that EGFR and ErbB2 were adjacent to, but did not appear to colocalize with the bacteria. Our finding that gonococci not only induce HB-EGF transcription, but also its shedding supports a ligand driven activation of EGFR by *N. gonorrhoeae*. EGFR ligands are expressed by many cell types, including epithelial and endothelial cells, and function in an endocrine, paracrine, autocrine or juxtacrine fashion. These ligands are expressed on the cell surface as transmembrane precursor proteins and are shed from the surface of cells by zinc metalloproteinases, either of the MMP or ADAM families. This study found that removal of several MMPs from the epithelial cell surface by sequential heparin washes inhibited not only the shedding of HB-EGF, but also the invasion of gonococci into epithelial cells. These results demonstrate that gonococci activate EGFR by inducing the expression and surface cleavage of EGFR ligands, a transactivation mechanism.

Transactivation of EGFR has been shown to occur under many circumstances (28, 67, 146, 162). HB-EGF ectodomain shedding is a major pathway used by the epithelia to activate wound healing (156). In polarized cells, transmembrane HB-EGF binds to EGFR on adjacent cells in a juxtacrine manner preventing proliferation. The cell-cell junction disruption caused by a wound causes the ectodomain shedding of HB-EGF, inducing cell proliferation. Recently pathogens have been shown to activate EGFR by activation of HB-EGF shedding. Both *P. aeruginosa* and *H. pylori* have

been shown to transactivate EGFR in corneal and gastric epithelial cells respectively via HB-EGF shedding (157, 161), and this activation was able to inhibit apoptosis of the epithelial cells.

In this study, I used heparin washes to remove MMPs that are responsible for cleaving membrane-associated EGFR ligands. Heparin previously has been shown to interfere with the Opa-mediated binding of gonococci to the epithelial cell surface, consequently preventing gonococci from adhering to epithelial cells (144). In our study, in order to examine the effect of the heparin washes on gonococcal invasion, I removed the remaining heparin from the cells by multiple washes after the heparin wash and before the addition of the gonococci. As I hypothesized, under such treatment conditions I only detected the effect of the heparin washes on the gonococci's ability to invade into, but not their ability to adhere to the epithelial cells.

As an in vitro study, I have used cancer epithelial cell lines that have originated from genital tissue to study the role of EGFR in gonococcal invasion. Over expression of the ErbB family of receptors is common in epithelial cell cancers. The results generated from an epithelial cell line that expresses much higher levels of ErbB receptors than normal epithelial cells may not reflect what occurs in vivo. To address this potential issue, I used two different cell lines that express different levels of EGFR and ErbB2. While HEC-1-B cells express much lower levels of EGFR and ErbB2 than ME180 cells, the two cell lines behaved similarly in most of the analyses. The higher sensitivity of HEC-1-B cells to EGFR and ErbB2 inhibitors in gonococcal invasion than ME180 cells further argue against a significant impact of EGFR and ErbB2 expression levels on our conclusion.

Taken together, the results of this study not only demonstrate that EGFR activation is required for *N. gonorrhoeae* invasion, but also reveal the mechanism by which *N. gonorrhoeae* activates EGFR, transactivation by increasing the gene expression and surface shedding of EGFR ligands. EGFR is a key surface receptor on epithelial cells, and its signal transduction function is essential for epithelial cell survival and proliferation. This study demonstrates that *N. gonorrhoeae* has the capability to co-opt host signaling through an indispensable receptor for their invasion. Activation of EGFR leads to many different outcomes depending on cell types and their microenvironment. Thus, hijacking the EGFR signaling pathway could be a common mechanism for pathogens to drive their invasion and intracellular survival. Further studies are required to understand how gonococci are able to induce the expression and surface cleavage of EGFR ligands and how EGFR signaling mediates gonococcal invasion.

Chapter 3: ErbB receptor mediated signaling regulates *Neisseria gonorrhoeae* invasion of genital epithelial cells

3.1 Introduction

Many pathogenic bacteria, including *Shigella*, *Salmonella*, *Yersinia* and *Listeria*, are able to make their entry into host cells by the induction of signaling events that leads to the actin cytoskeleton reorganization and pathogen phagocytosis (32). *Neisseria gonorrhoeae*, the causative agent of gonorrhea, also has been shown to be dependent upon actin for its invasion into epithelial cells (114, 127). To have adapted to ever varying host environment, *N. gonorrhoeae* has evolved many means to invade host epithelial cells. OpaA/30 mediates gonococcal invasion by binding to HSPG on the cell surface, activating phosphatidylcholine-specific phospholipase C and acidic sphingomyelinase (50). In the absence of Opa, pili retraction can induce the uptake of gonococci (87). Opsonization of LOS by complement factor C3b and subsequent conversion to C3bi induces the complement receptor 3 to internalize bound gonococci (38). Although *N. gonorrhoeae* has many means by which they can invade epithelial cells, all involve the actin cytoskeleton. Previous studies from several labs have shown that disruption of the actin cytoskeleton with cytochalasin B or D prevents invasion of *N. gonorrhoeae* into genital epithelial cells (114, 127).

N. gonorrhoeae adherence to epithelial cells induces the accumulation of F-actin beneath the bacterial microcolony, elongation of host cell microvilli to surround the microcolony, and cortical plaque formation, all of which are dependent upon host cytoskeletal rearrangement (34, 51, 86). The cortical plaques contain ezrin, a protein

that links the actin cytoskeleton with the membrane, along with the transmembrane proteins, EGFR (ErbB1), CD44v3 and ICAM-1, and unknown phosphorylated host proteins (36, 67, 68). In Chapter 2, I showed that ErbB2, as well as EGFR, is enriched beneath gonococcal microcolonies.

EGFR and ErbB2 belong to a family of four closely related receptor tyrosine kinases. The ErbB receptor family contains an extracellular ligand binding domain, a transmembrane domain, an intracellular tyrosine kinase domain, and an intracellular hydrophilic domain that contains multiple tyrosine residues that can be phosphorylated (116, 158). There are eleven peptide ligands that bind differentially to the ErbB receptors. Upon binding to ligand, the receptors undergo either homo- or hetero-dimerization, which leads to trans-autophosphorylation of each other on tyrosine residues in the dimer. The phosphotyrosines serve as docking sites for proteins containing either SH2 (Src homology 2) or PTB (phosphotyrosine binding) domain. Different proteins dock on different phosphorylated tyrosines, initiating different signaling cascades that can lead to diverse outcomes, including proliferation, migration, differentiation, cell survival or adhesion. However, the exact role of EGFR and ErbB2 signaling in gonococcal invasion is not known.

EGFR activation potentially induces rearrangement of the host actin cytoskeleton that is essential for the invasion of gonococci into epithelial cells. Ligand binding to EGFR leads to the activation of PI3K, PLCγ and MAP kinase signaling pathways, all of which are known to be able to regulate actin dynamics (19, 70, 72, 119, 120). Actin cytoskeleton dynamics is under the control of a large number of actin binding proteins, which are regulated by signaling cascades, such as

phosphatidylinositide production and Ca^{2+} flux. PI3K and PLCγ are two key signaling molecules that control phosphatidylinositide metabolism. Phosphatidylinositides regulate the cellular location and actin binding activities of actin binding proteins (119). PLCγ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol (1,4,5)P_3 (IP_3) and diacylglyceride (DAG). IP_3 induces the release of Ca^{2+} from intracellular stores, which could trigger Ca^{++} influx from the extracellular pool (19). MAP kinase ERK signaling pathway regulates actin cytoskeleton dynamics by down-regulating the expression of ROCKI (Rho-associated kinase) and/or ROCKII (102, 120) and positively regulating Rnd3 expression (72). ROCK is an upstream negative-regulator of the actin binding protein cofilin, while Rnd3 is a negative-regulator of ROCK.

In Chapter 2, I showed that gonococci transactivate EGFR and ErbB2 by increasing the expression and inducing the cleavage of HB-EGF, one of the ligands for EGFR and that the transactivation of EGFR was important for gonococcal invasion of genital epithelial cells. In this chapter I examine the activation of PI3K/AKT, PLCγ, and ERK, three signaling pathways induced by EGFR activation, in order to determine how EGFR transactivation induces gonococcal invasion into human endometrial and cervical epithelial cells, HEC-1-B and ME180. My studies show that all three signaling pathways are induced by gonococcal adherence. Additionally, my data suggests that PLCγ activation by EGFR supports the invasion of epithelial cells, while ERK and PI3K are not essential for gonococcal invasion.

3.2 Materials and Methods

3.2.1 Bacterial strains and epithelial cell lines

Neisseria gonorrhoeae strain $MS11_{MKC}$ (MKC) was maintained on gonococcal media base (GCK) with 1% Kellogg's supplement (152). Piliated (Pil^+), Opa-expressing (Opa^+) variants were selected by their light refracting properties using a dissecting light microscope. The concentration of bacteria in suspension was determined spectrophotometrically and verified by viable plate count. Gonococci were killed by incubation with 100 μg/ml gentamicin sulfate at 37°C for 2 h, followed by overnight at 4°C. Before use, the killed gonococci were washed three times in serum free Eagle's MEM. HEC-1-B cells, a human endometrial adenocarcinoma cell line (ATCC# HTB-113), were maintained in Eagle's MEM supplemented with 10% fetal bovine serum (FBS). ME180 cells, a human cervical epidermal carcinoma cell line (ATCC# HTB-33), were maintained in RPMI1640 supplemented with 10% FBS.

3.2.2 Inhibitors and antibodies

AG1478, an EGFR kinase inhibitor, AG825, an ErbB2 kinase inhibitor, LY294002, wortmannin, BAPTMA/AM, 2-APB and U0126 were purchased from Calbiochem (San Diego, CA). Anti-EGFR (1005) and anti-ErbB2 (C-18) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-tubulin antibody (SAP 4G5), rabbit polyclonal anti-PLCγ1 antibody, and 100× proteinase inhibitor cocktail were purchased from Sigma (St. Louis MO). Anti-phosphotyrosine

mAb (4G10) was purchased from Millipore (Temecula, CA). Anti-pERK (E10), anti-pAKT (D94), and rabbit polyclonal anti-AKT, anti-pERK and anti ERK antibodies were purchased from Cell Signaling Technology (Danvers, MA).

3.2.3 Bacterial invasion assays

Epithelial cells (5×10^4/well) were seeded in 96-well plates and incubated at 37°C in 5% CO_2. After 24 h, cells were cultured in serum-free medium overnight. Cells were pre-incubated with AG1478 and AG825 for 2 h, wortmannin, LY294002 and U0126 for 1 h or BAPTMA/AM and 2-APB for 30 min. Next, cells were incubated with MKC P^+O^+ at an MOI of 5 for 6 h at 37°C. For invasion assays, cells were washed with serum-free medium and then incubated with 50 µg/ml gentamicin for 1.5 h at 37°C. After extensively washing to remove the remaining gentamicin, bacteria that had invaded were quantified by lysing the epithelial cells with 1% saponin and plating the cell lysates on GCK plates. The significance of differences was assessed using the Student's t-test for independent population means.

3.2.4 Immunoblotting

Epithelial cells were seeded at 1×10^6 in 6-well dishes. After 24 h, the cells were serum-starved overnight and incubated with MKC P^+O^+ at an MOI of 5 for up to 6 h. The cells then were washed with ice-cold PBS and lysed in 75 µl RIPA buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 2 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1× proteinase inhibitor cocktail (Sigma). Cell lysates were mixed 1:1 with denaturing

loading buffer. Lysates were separated through SDS-polyacrylamide gels (Bio-Rad, Hercules, CA), analyzed by western blot, and visualized using Western Lightning chemiluminescence substrate (Perkin Elmer, Boston, MA). Images were acquire and digitized directly using Fujifilm LAS-3000 (Valhalla, NY) or acquired with x-ray film. Blots were stripped with Restore western blot stripping solution (Pierce, Rockford, IL) and reprobed with anti-β-tubulin antibody. The blots were quantified by densitometry using MultiGauge software from Fujifilm.

3.2.5 Immunoprecipitation

Epithelial cells were seeded at 1×10^6 in 6-well dishes. After 24 h, the cells were serum-starved overnight and incubated with MKC P^+O^+ at an MOI of 5 for up to 6 h. The cells then were washed with ice-cold PBS and lysed in 1% Triton-X 100 lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1× proteinase inhibitor cocktail). The lysates were sonicated and centrifuged at 4°C. The supernatants were subjected to immunoprecipitation using anti-phosphotyrosine mAb and Protein G conjugated sepharose beads (GE Healthcare, Piscataway, NJ). The immunoprecipitates were analyzed using SDS-PAGE and western blot, probing for PLCγ using a specific antibody.

3.3 Results

3.3.1 *N. gonorrhoeae* activates the MAP kinase ERK via transactivation of EGFR.

I have shown in Chapter 2 that gonococci transactivate EGFR and that this transactivation is necessary for their invasion into epithelial cells. EGFR activation can induce multiple signaling pathways, including the MAP kinase ERK. To analyze the activation of the MAP kinase ERK in response to gonococcal infection by its phosphorylation, lysates were prepared from HEC-1-B endometrial epithelial cells and ME180 cervical epithelial cells that had been incubated with live or killed MKC P^+O^+ for up to 7 h. The lysates were analyzed using western blot, probing for phosphorylated ERK (pERK). As shown in Fig. 1 gonococci induced the phosphorylation of ERK in both cell lines. HEC-1-B cells have a certain level of constitutive ERK phosphorylation (Fig. 10B, 0 h) in the absence of the bacteria, while a basal level of pERK was almost undetectable in ME180 cells (Fig 10A, 0 h). After the addition of live MKC, levels of pERK increased steadily for 4 h and remained augmented throughout the assay (6 or 7 h). In contrast, epithelial cells that were incubated with killed MKC showed very little increase (HEC-1-B) or no increase (ME180) in pERK. This suggests that induction of ERK activation by gonococci is dependent on the viability of the bacteria, as only the live Pil^+ Opa^+ gonococci induced a sustained increase in pERK.

To investigate whether gonococci-induced activation of ERK was due to the transactivation of EGFR or ErbB2 by the bacteria, I examined the effect of EGFR

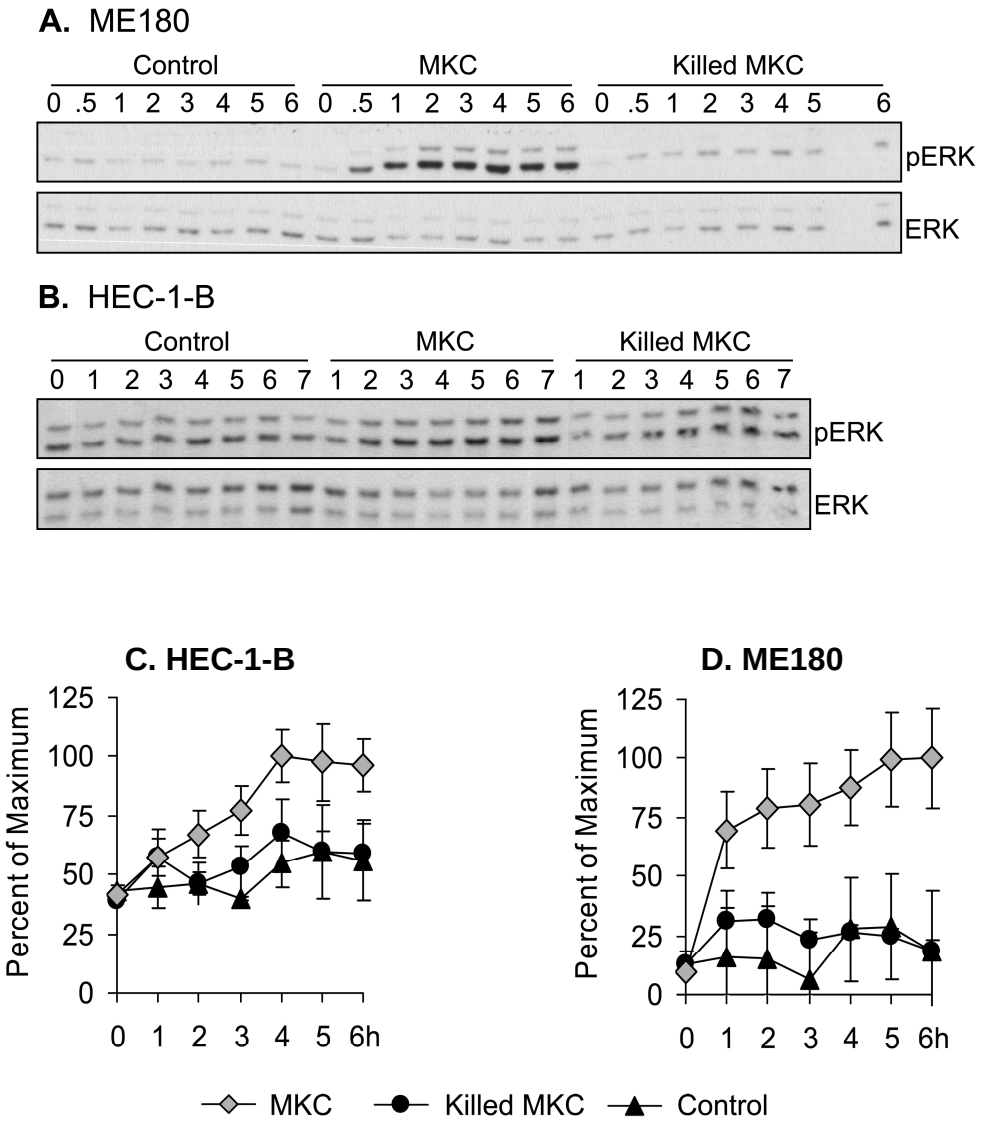

Figure 10. *N. gonorrhoeae* infection activates MAP Kinase ERK. ME180 (A) or HEC-1-B (B) cells were infected with either live or gentamicin-killed MKC Pil^+ Opa^+ at an MOI of 5 or incubated with serum-free media as the control for up to 6 h. At each hour the cells were lysed and an aliquot was analyzed by SDS-PAGE and western blot, probing for pERK. The blots were stripped and reprobed for total ERK as loading controls. Shown are representative blots from at least three independent experiments. Densitometry of pERK activation in HEC-1-B (C) and ME180 (D). Data is shown as the mean of the independent experiments ± SEM.

and ErbB2 kinase inhibitors on gonococcal induced ERK activation. HEC-1-B and ME180 cells were preincubated with AG1478, an EGFR kinase inhibitor, or AG825, an ErbB2 kinase inhibitor, before the addition of MKC P^+O^+. As shown in Fig. 11, the EGFR inhibitor AG1478 was able to abolish most of the constitutive ERK phosphorylation and gonococcal induction of ERK phosphorylation in both HEC-1-B and ME180 cells. In contrast to the EGFR kinase inhibitor, the ErbB2 kinase inhibitor AG825 had no effect on the gonococcal induced activation of ERK in either cell line. Taken together the data here suggests that *N. gonorrhoeae* activates the ERK signaling pathway via their transactivion of EGFR kinase.

3.3.2 *N. gonorrhoeae* activates PI3K by transactivation of EGFR.

The PI3K signaling pathway is one of the major downstream signaling pathways of EGFR and ErbB2. PI3K has been shown by others to be activated in response to gonococcal inoculation (158). I investigated whether PI3K activation was dependent on gonococci induced transactivation of EGFR. I assessed the activation of the PI3K pathway in HEC-1-B cells by phosphorylation of AKT, a downstream effector of PI3K. AKT is activated by binding to PIP_3 followed by its subsequent phosphorylation by PDK1 and PDK2 (phosphoinositide dependent kinase). As shown in Fig. 12, uninfected HEC-1-B cells have a certain level of intrinsic phosphorylated AKT (pAKT). After incubation with MKC Pil^+ Opa^+, the levels of pAKT increased steadily for the first 2 h and remained at the heightened level up to 6 h post inoculation. Recently it has been suggested that gonococci induced AKT phosphorylation is dependent on myosin light chain kinase (MLCK),

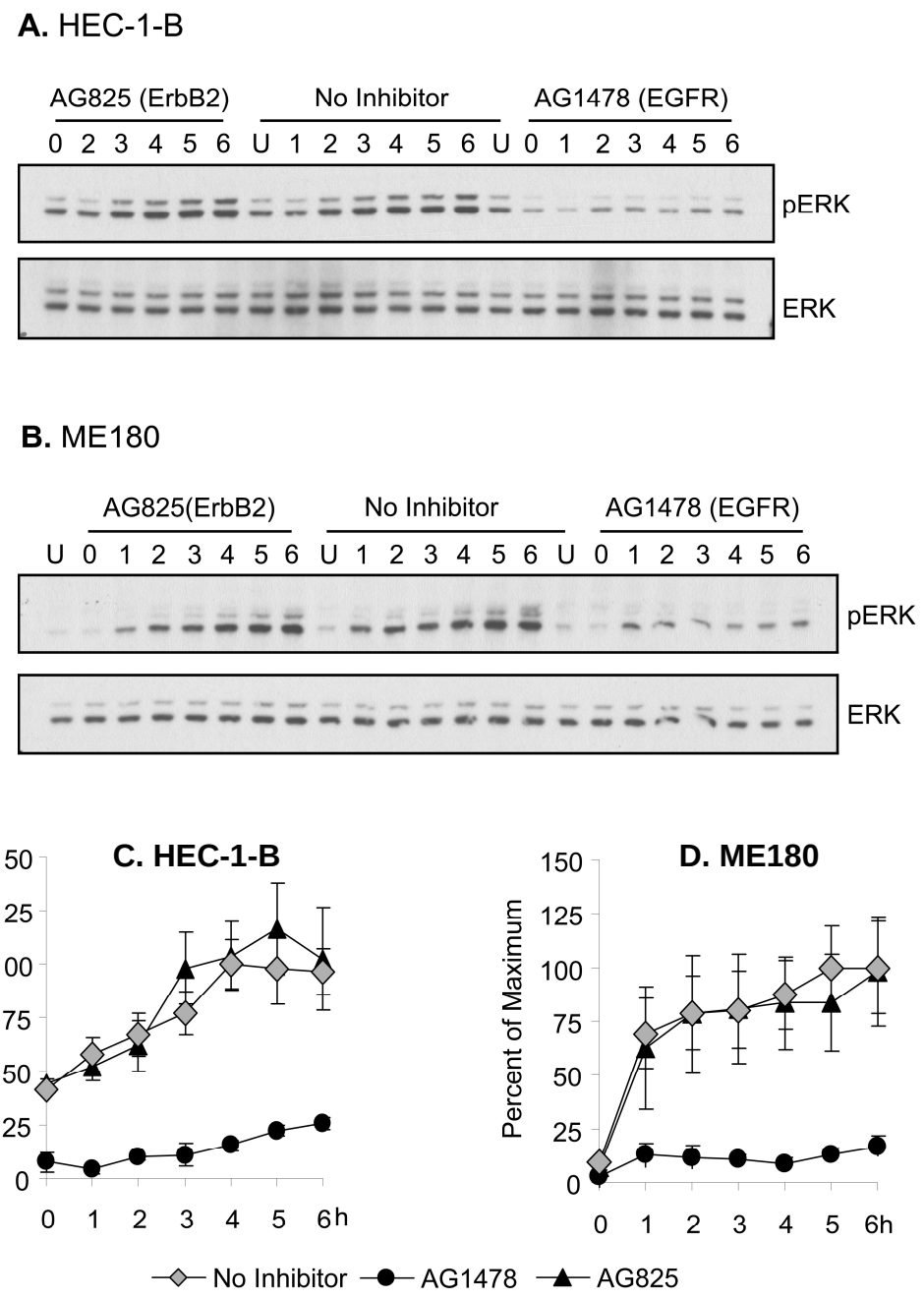

Figure 11. *N. gonorrhoeae*-induced ERK activation depends on signaling through EGFR. HEC-1-B (A) or ME180 (B) cells were preincubated with either AG825 (5 μM), an ErbB2 inhibitor, or AG1478 (5 μM), an EGFR inhibitor, for 2 h prior to the addition of MKC Pil^+ Opa^+ at an MOI of 5 or with serum-free media as the control for up to 6 h. U indicates the untreated sample in the absence of gonococci. At each hour the cells were lysed and an aliquot of the cell lysate was analyzed by SDS-PAGE and western blot, probing for pERK. The blots were stripped and reprobed for total ERK as loading controls. Shown are representative blots from at least three independent experiments. Densitometry of pERK activation in HEC-1-B (C) and ME180 (D). Data is shown as the mean of the independent experiments ± SEM. 100% is the maximum pERK level in no inhibitor treated cells.

instead of PI3K activation (36). In order to determine if the phosphorylation of AKT was dependent on PI3K activation, I preincubated HEC-1-B and ME180 cells with the PI3K inhibitors LY294002 and wortmannin before the inoculation with MKC. Although wortmannin is considered to be a PI3K inhibitor (IC_{50} 1-10 nM), it also can inhibit PI4K (IC_{50} 200 nM) and MLCK (IC_{50} 200 nM) at higher concentrations (3, 40, 96). LY294002, however, has not been found to have any other inhibitory activities. I find that in PI3K inhibitor-treated HEC-1-B cells, there is no detectable level of pAKT before or after the inoculation of the bacteria with use of either inhibitor (Fig.12). The concentration of wortmannin that inhibited gonococcal induced AKT phosphorylation was 50 nM, well below the published IC_{50} for MLCK and PI4K. This suggests that activation of AKT in HEC-1-B cells is dependent on PI3K activation.

In order to investigate whether gonococci induced AKT activation requires EGFR or ErbB2 mediated signaling, HEC-1-B and ME180 cells were incubated with EGFR and ErbB2 kinase inhibitors, AG1478 or AG825, respectively. The EGFR inhibitor abolished most of the constitutive pAKT levels in both cell lines. In the presence of the EGFR kinase inhibitor, gonococcal inoculation failed to increase the levels of pAKT in HEC-1-B and ME180 cells (Fig. 13). The ErbB2 inhibitor, however, had no effect on both the constitutive pAKT and gonococci induced pAKT in HEC-1-B and ME180 cells. These results suggest that gonococci induce PI3K-dependent AKT activation in epithelial cells and this activation is due to the gonococcal induced transactivation of EGFR, but not ErbB2.

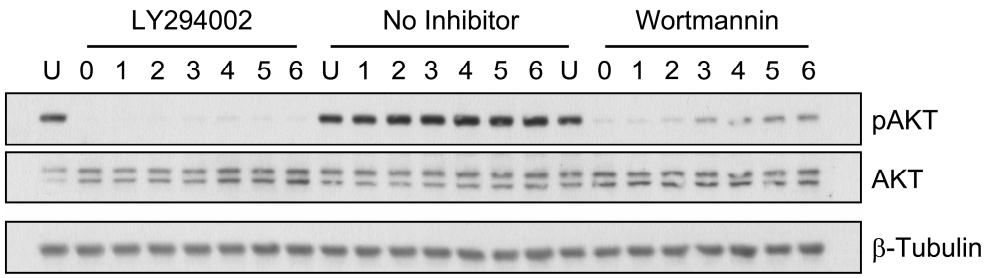

Figure 12. *N. gonorrhoeae* infection induces PI3K-dependent activation of AKT. HEC-1-B cells were preincubated with either LY294002 (5 μM) or wortmannin (50 nM), two PI3K inhibitors, for 30 min prior to the addition of MKC Pil^+ Opa^+ at an MOI of 5 or with serum-free media as the control for up to 6 h. U indicates the untreated sample in the absence of gonococci. At each hour the cells were lysed and an aliquot of the cell lysate was analyzed by SDS-PAGE and western blot, probing for pAKT. The blots were stripped and reprobed for total AKT and β-tubulin as loading controls. Shown is a representative blot from three independent experiments.

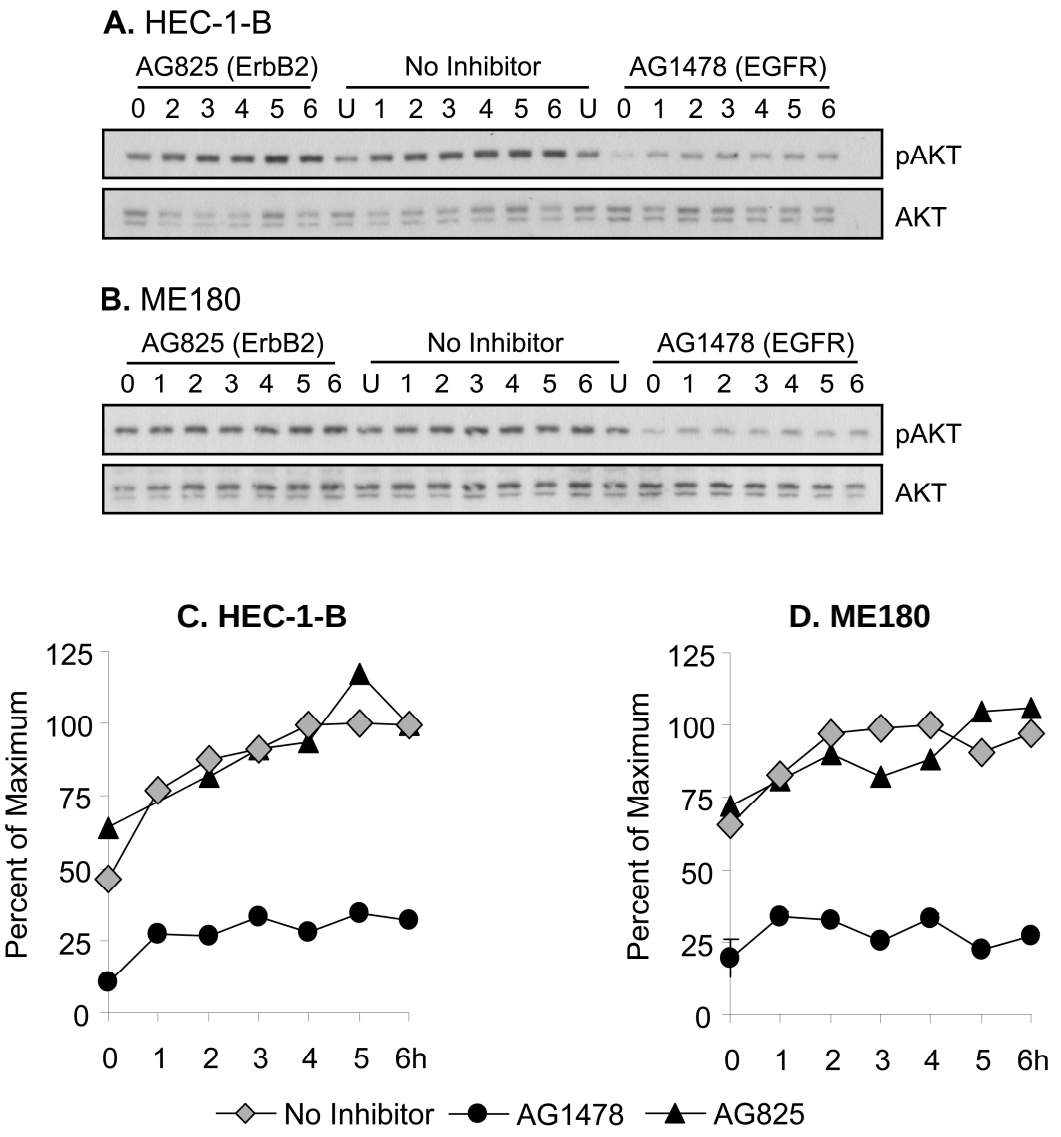

Figure 13. *N. gonorrhoeae* infection activates AKT via EGFR. HEC-1-B (A) or ME180 (B) cells were preincubated with either 5 μM AG825, an ErbB2 inhibitor, or (5 μM) AG1478, an EGFR inhibitor, for 2 h prior to the addition of MKC Pil^+ Opa^+ at an MOI of 5 or with serum-free media as the control for up to 6 h. U indicates the untreated sample in the absence of gonococci. At each hour the cells were lysed and an aliquot of the cell lysate was analyzed by SDS-PAGE and western blot, probing for pAKT. The blots were stripped and reprobed for total AKT as loading controls. Shown are representative blots from at least three independent experiments. Densitometry of pAKT activation in blot A HEC-1-B (C) and blot B ME180 (D). 100% is the maximum pAKT level in no inhibitor treated cells.

3.3.3 The expression of pili or Opa is required for *N. gonorrhoeae* to induce ERK and AKT activation.

Others and we previously have shown that expression of Opa and pili together enhances the gonococci's ability to invade ME180 cells (39, 104). When gonococci express only Opa or pili individually, gonococcal invasion is reduced to ~50% of what is seen with gonococci expressing both (138). It has been reported recently that in the absence of Opa, gonococci induce ERK activation through PilT induced retraction of their pili (46). Based on these observations, here I ask whether Opa and pili play a role in the activation of ERK and AKT. I incubated ME180 cells with different variants of *N. gonorrhoeae* MKC, including P^+O^+, P^-O^+, $P^+\Delta O$, $P^-\Delta O$ or killed P^+O^+, and examined the effect of differential expression of pili and Opa on the gonococci's ability to induce the activation of ERK and AKT. Neither gentamicin killed MKC P^+O^+, which cannot invade (11), nor MKC $P^-\Delta O$, which invade 100-fold less well than P^+O^+ (138), were able to induce ERK or AKT phosphorylation up to 6 h post-inoculation (Fig. 14). The two MKC variants that express either pili or Opa individually were able to induce activation of ERK and AKT in ME180 cells. Although P^+O^+, P^-O^+ and $P^+\Delta O$ gonococci activated ERK to comparable levels in ME180 cells, the timing of the pERK increase was slightly varied between the three (Fig. 14). In ME180 cells incubated with the P^+O^+ variant, ERK phosphorylation increased steadily over the 6 h. In cells incubated with the $P^+\Delta O$ variant, ERK phosphorylation rose more rapidly than that in cells incubated with the P^+O^+ variant, while inoculation of the P^-O^+ variant increased ERK phosphorylation later than both the P^+O^+ and P^+O^- variants (Fig. 14).

The effects of Opa and/or pili expression on the induction of pAKT mirrored those of pERK induction. Neither the killed MKC P^+O^+ nor the $P^-\Delta O$ variant altered pAKT levels in ME180 cells (Fig. 14). MKC P^+O^+, P^-O^+, and $P^+\Delta O$ variants all induced AKT phosphorylation, but in different temporal patterns. The $P^+\Delta O$ variant activated AKT earliest, followed by the P^+O^+ and then P^-O^+ variant, which is similar to the pattern of ERK activation induced by the different variants of gonococci. These results together suggest that gonococcal induced ERK and AKT activation is dependent upon the expression of either pili or Opa, and expression of pili and Opa regulates the timing of ERK and AKT activation.

3.3.4 *N. gonorrhoeae* activates PLCγ.

Another major downstream signaling pathway of EGFR and ErbB2 is PLCγ. Activation of PLCγ via EGFR has been shown to be important for actin-dependent cell motility and wound healing (21) and is induced by HB-EGF. Because I had difficulty in obtaining antibodies against activated (phosphorylated) PLCγ, I first assessed whether gonococci could induce phosphorylation of a protein around the molecular weight of PLCγ (155 kDa). Lysates were prepared from HEC-1-B cells that had been incubated with MKC Pil^+ Opa^+ for up to 6 h. The lysates were analyzed using western blot, probing for phosphorylated tyrosine (pY). As shown in Fig. 15A, gonococci induced the phosphorylation of a doublet of proteins of 155 kDa in range. Both of the phosphorylated bands in the doublet increased in intensity over the 6 h of incubation with the bacteria. This is consistent with a previous report that HB-EGF

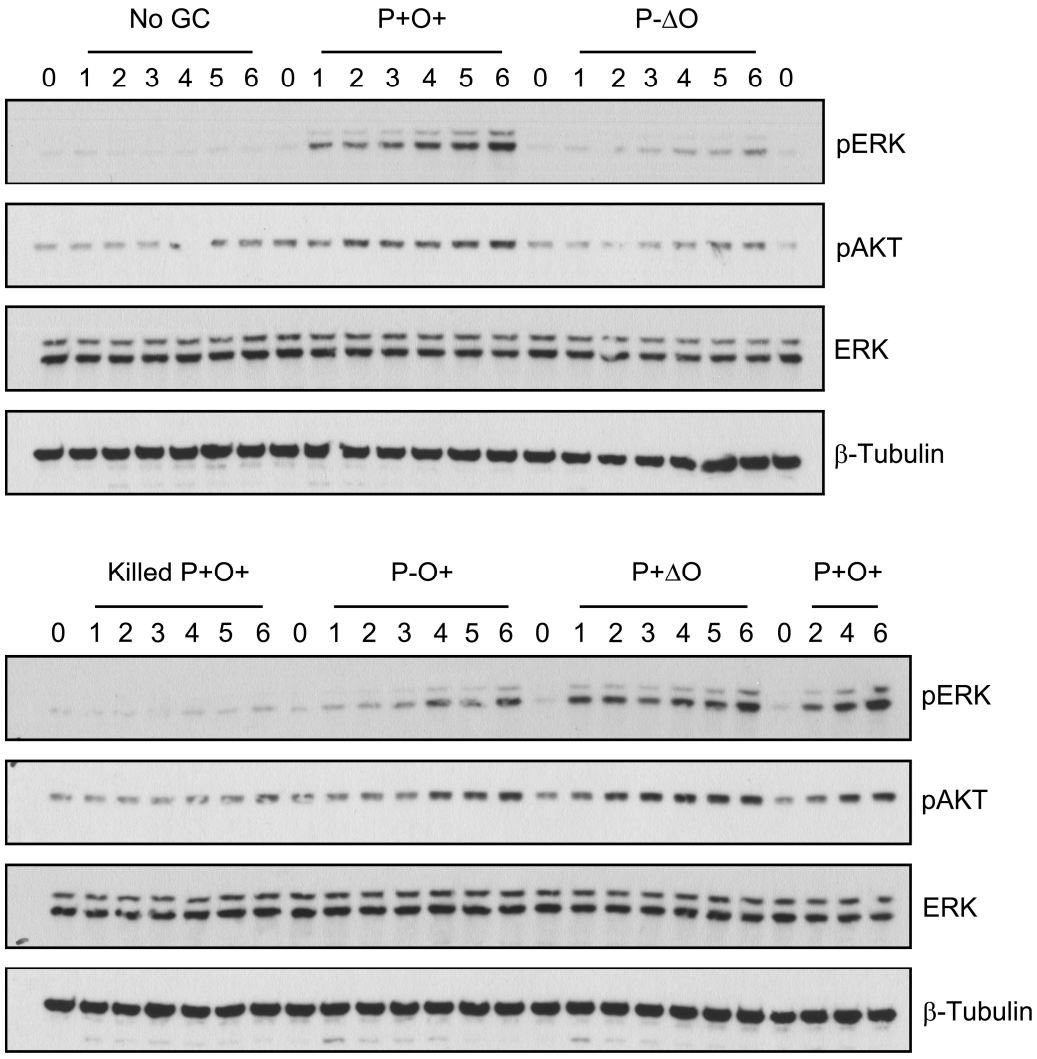

Figure 14. *N. gonorrhoeae* must express either pili or Opa to activate ERK and AKT. ME180 cells were infected with either live MKC variants expressing P^+O^+, P^-O^+, $P^+\Delta O$ or $P^-\Delta O$ or gentamicin-killed MKC P^+O^+ at an MOI of 5 or with serum-free media as the control for up to 6 h. At each hour the cells were lysed and an aliquot of the cell lysate was analyzed by SDS-PAGE and western blot, probing for pERK. The blots were stripped and reprobed for pAKT, followed by total ERK and β-tubulin as loading controls. Shown are representative blots from three independent experiments.

induces the phosphorylation of a doublet with an observed size of 135 and 150 kDa in NIH 3T3 epithelial cells (14). To determine whether phosphorylation of these proteins is dependent on gonococcal transactivation of EGFR, HEC-1-B cells were incubated with the EGFR kinase inhibitor AG1478 prior to being inoculated with MKC P^+O^+. The EGFR inhibitor reduced the constitutive phosphorylation level of the lower band in the doublet and blocked gonococcal induced the phosphorylation of both bands in the doublet (Fig. 15C).

As another measure of determining whether PLCγ is activated in response to gonococci, lysates were prepared from HEC-1-B cells that either had or had not been incubated with MKC for 4 h and were subjected to immunoprecipitation with a monoclonal antibody (mAb) specific for phosphotyrosine. The immunoprecipitates were analyzed using western blot, probing for PLCγ. As shown in Fig. 15D, gonococci induced the tyrosine phosphorylation of PLCγ. These results confirm that gonococci induce PLCγ activation in epithelial cells and suggest that PLCγ activation may be due to the gonococcal induced transactivation of EGFR.

3.3.5 Ca^{2+} flux, but not ERK and PI3K activation, is essential for gonococcal invasion.

To investigate whether signaling cascades initiated by gonococci induced transactivation of EGFR are involved in gonococcal invasion, I examined the effects of inhibitors specific for ERK, PI3K, and Ca^{++} signaling, the downstream effect of PLCγ activation, using gentamicin protection assays. I used BAPTA/AM and 2-APB to inhibit Ca^{2+} flux. BAPTA/AM, a cell permeable calcium chelator, is

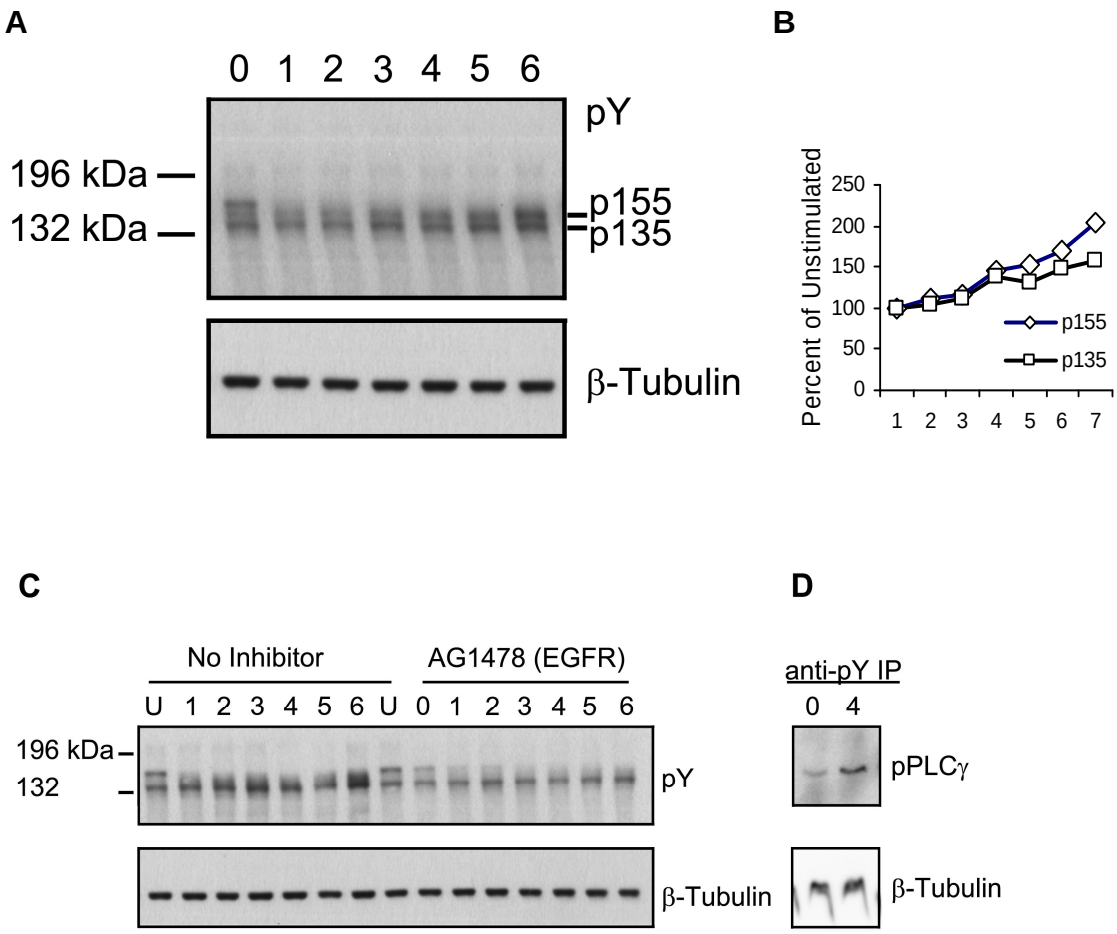

Figure 15. *N. gonorrhoeae* infection activates PLCγ. HEC-1-B cells were infected with either MKC Pil$^+$ Opa$^+$ at an MOI of 5 or incubated with serum-free media as the uninfected control (U) for up to 6 h. (A, C and D). At each hour the cells were lysed and an aliquot was analyzed by SDS-PAGE and western blot, probing for phosphotyrosine (A and C). The blots were stripped and reprobed for β-tubulin as loading controls. (B) Densitometry analysis was done on blot A for the two bands that are present after the addition of gonococci. The lower band is p135 and the upper band is p155. (D) After the addition of MKC, the cells were lysed at each hour and the cell lysates were subjected to immunoprecipitation with anti-phosphotyrosine mAb 4G10. The cell lysates were analyzed by SDS-PAGE and western blot, probing for PLCγ.

hydrolyzed by esterases once inside the cell and becomes trapped inside as the active calcium chelator BAPTA. 2-APB is an inhibitor of IP_3-induced Ca^{2+} release. HEC-1-B cells were incubated with the two calcium flux inhibitors before inoculating the cells with gonococci. Chelation of intracellular Ca^{2+} by BAPTA/AM reduced invasion of MKC P^+O^+ in a dose dependant manner (70% at 100 μm) (Fig. 16A), and 2-APB inhibited gonococcal invasion of HEC-1-B cells by greater than 90% (Fig. 16B). To investigate whether PI3K activation is important in gonococcal invasion, I used two inhibitors, wortmannin and LY294002. Wortmannin is able to inhibit 90% of gonococcal invasion into HEC-1-B cells at concentration as low as 50 nM (Fig. 16C). LY294002, however, had no significant effect on gonococcal invasion (Fig. 16D). Since wortmannin, but not LY294002, is known to inhibit other signaling pathways in addition to PI3K, our results suggest that wortmannin inhibits gonococcal invasion by interfering with one of the other signaling pathways, but not by inhibiting PI3K. To study ERK's role in gonococcal invasion, HEC-1-B cells were preincubated with U0126, an inhibitor of ERK activation, before the inoculation of the bacteria. U0126 has no effect on gonococcal invasion at concentrations near its IC_{50}, but increases the amount of gonococcal invasion into HEC-1-B cells at very high doses of U0126 (>5 μM, approximately 100× its IC_{50}).

Taken together, these results do not support a role for ERK or PI3K signaling in gonococcal invasion of epithelial cells, but rather suggest that Ca^{2+} signaling is important for gonococcal invasion into HEC-1-B cells and that the Ca^{2+} signaling is likely to be induced by PLCγ activation.

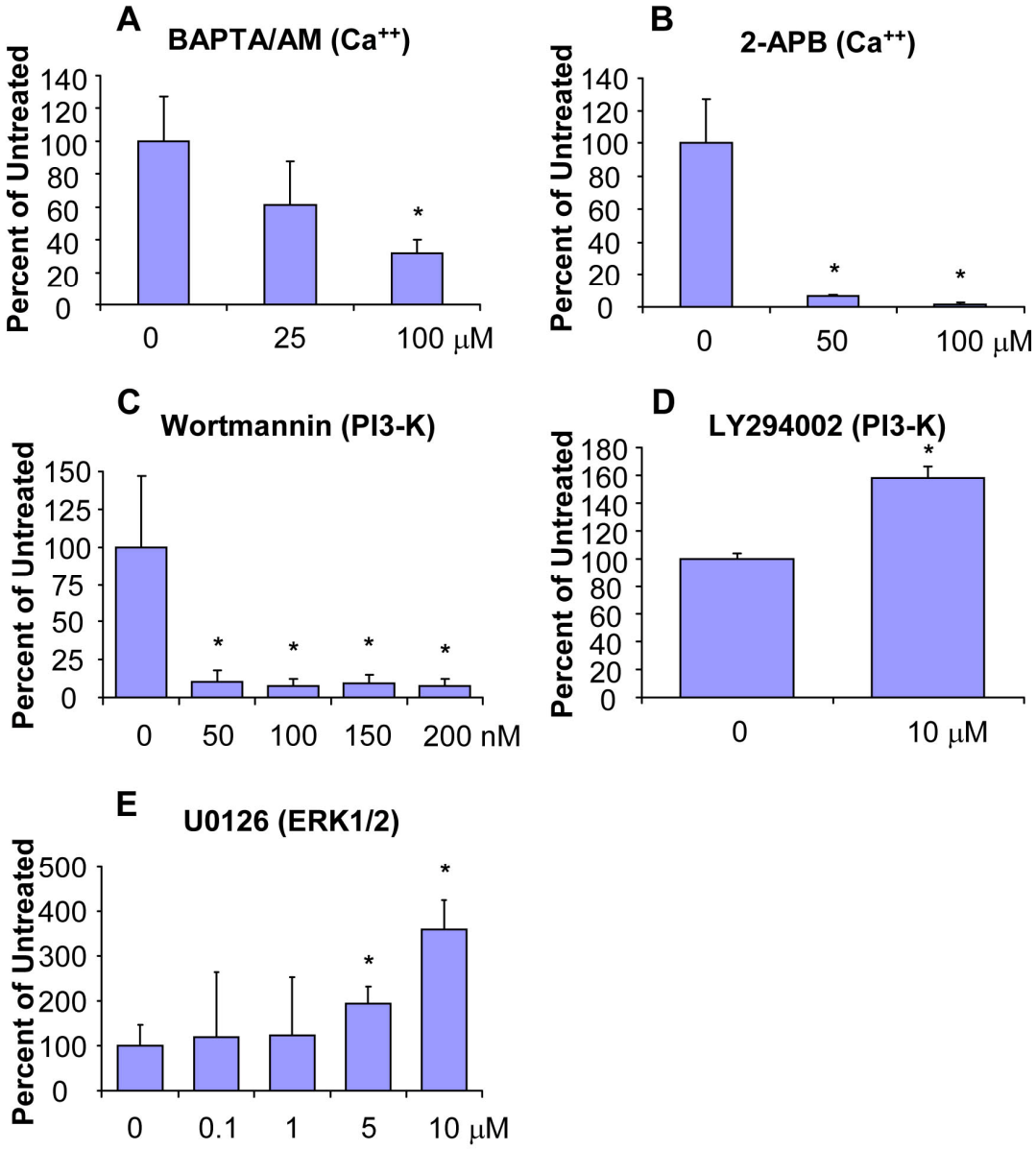

Figure 16. The effects of Calcium flux, PI3K, and ERK inhibitors on gonococcal invasion. To quantify invaded bacteria, HEC-1-B cells were preincubated with either the inhibitors as indicated before the addition of MKC Pil^+ Opa^+. After incubating with the bacteria for 6 h, the epithelial cells were treated with gentamicin, washed, and lysed to determine the number of gentamicin resistant bacteria as invaded bacteria. Shown are the mean percentages (± SD) from three independent experiments with six replicates per experiment. *$P < 0.05$ (as compared with no inhibitor).

3.4 Discussion

Epithelial cells, unlike phagocytic cells of the immune system, are designed to create a physical barrier for pathogens, but not engulf bacteria by phagocytosis. *N. gonorrhoeae* has devised means by which it can manipulate the host epithelial cells to induce its uptake. The invasion of gonococci into epithelial cells of the female reproductive tract has been shown to be an actin-dependent process (114, 127). I showed in Chapter 2 that gonococci transactivate EGFR and that the kinase activity of EGFR was important for gonococcal invasion. One of the known effects of EGFR activation is regulation of actin dynamics.

EGFR, a receptor tyrosine kinase, is activated by ligand binding to its extracellular domain. Ligand binding induces EGFR dimerization with either itself or another ErbB family member and trans-autophosphorylates intracellular tyrosine residues in the dimer, leading to the activation of multiple signaling pathways inside the cell. This leads to diverse outcomes dependent upon the dimerization partner, the ligand, and the timing and duration of the ligand binding. ERK, PI3K/AKT and PLCγ, three major signal transduction pathways downstream of EGFR, all have been implicated to be able to regulate actin organization. I show here that all three pathways are activated in genital epithelial cells by gonococcal inoculation. The activation of these three pathways in HEC-1-B and ME180 cells by gonococci has two characteristics. First, the activation of both ERK and PI3K/AKT was sustained for at least 6 hr. Second, activation of ERK and AKT was induced through EGFR. In the previous chapter I showed that gonococci induce the transcription and shedding of HB-EGF and that gonococcal-induced shedding of the growth factor HB-EGF

activates EGFR. Thus, HB-EGF mediated transactivation of EGFR leads to the sustained activation of ERK and AKT.

In this study, I show that activation of ERK and AKT is induced by *N. gonorrhoeae* that express either pili or Opa, but not by the bacteria that lose both surface structures. This suggests a role for both pili and Opa in gonococci-induced EGFR transactivation. There are two possible explanations for the role of pili and Opa in this process. First, the expression of either pili or Opa allows the bacteria to form microcolonies on the epithelial cell surface. Mechanical stresses generated by the microcolonies on the surface membrane of epithelial cells might trigger EGFR transactivation and the activation of EGFR downstream signal molecules The second possibility is that the direct interaction of pili or Opa with host cells or indirect interaction of pili and Opa with host cells through other bacterial surface molecules induces the transactivation of EGFR, and ERK and AKT activation. The results from my studies support the idea that the formation of microcolonies is important for the transactivation of EGFR leading to ERK and AKT signaling, as killed gonococci that express both pili and Opa adhere to the epithelial cell surface, but are unable to form microcolonies and induce ERK and AKT activation. Furthermore, the hypothesis of microcolony-dependent EGFR transactivation does not exclude the possible involvement of other gonococcal surface structures in addition to pili and Opa.

The results from this study suggests that *N. gonorrhoeae* induces the activation of PLCγ and that the Ca^{2+} signal induced by the PLCγ pathway is essential for the uptake of gonococci by epithelial cells. PLCγ induces a calcium flux by cleaving PIP_2 to produce IP_3 that induces the Ca^{2+} release from ER (19). This study

shows that chelation of intracellular Ca^{2+} and inhibition of intracellular Ca^{2+} release inhibits gonococcal invasion. PLCγ can activate PKC via its product DAG and Ca^{2+} flux. The PKC inhibitor, staurosporine, failed to inhibit gonococcal invasion into HEC-1-B cells, suggesting that PKC activation is not essential for gonococcal invasion into HEC-1-B cells (149). Therefore, gonococci induced PLCγ activation is likely to contribute to gonococcal invasion by causing the release of intracellular Ca^{2+} but not via activation of PKC.

N. gonorrhoeae induced Ca^{2+} fluxes have been reported previously (4, 5, 76, 95),. These studies suggested that the porin, PorB, and pilus retraction under the control of PilT contribute to the induction of Ca^{2+} flux. PorB, upon inserting itself into the membrane of host epithelial cells, induces a Ca^{2+} influx that occurs within 2 min (4, 95), while pilus retraction causes the release of Ca^{2+} from intracellular stores within 10 min (5, 76). It also has been shown that the pilus retraction induced Ca^{2+} flux is dependent upon the PorB Ca^{2+} influx preceding it (6). PLCγ activation is detected at a much later time after the initial PorB and pilus induced Ca^{2+} fluxes. This suggests that PLCγ induced Ca^{2+} flux may play a different role from the PorB/PilT induced Ca^{2+} fluxes in gonococcal invasion.

How these gonococci induced signaling pathways mediate the bacterial invasion is not known. These signaling pathways can potentially initiate and/or regulate the rearrangement of the host actin cytoskeleton that is essential for the invasion of gonococci into epithelial cells. Signaling cascades regulate actin cytoskeleton dynamics via actin regulatory proteins, including gelsolin and villin. The gelsolin family of actin binding proteins is Ca^{2+} regulated. Gelsolin, under high Ca^{2+}

concentrations, severs F-actin (159). The gelsolin family proteins, villin, advillin and supervillin, sever and promote depolymerization of F-actin under high Ca^{2+} concentrations. In addition, these three actin binding proteins are able to bundle F-actin, creating microvilli. Since gonococci are known to induce elongation of epithelial cell microvilli, any of these three microvilli inducing proteins could contribute to this process. Villin is particularly intriguing among the gelsolin family proteins, since its expression is restricted to epithelial cells, primarily of the gastrointestinal and urogenital tracts. In addition to having actin severing activity, villin, under high Ca^{2+} concentrations, also can promote actin nucleation by triggering the Arp2/3 complex.(99).

The results from this study show that both PI3K inhibitors, wortmannin and LY294002, inhibit gonococci-induced PI3K activation as assessed by AKT phosphorylation, but have opposing effects in gonococcal invasion. Wortmannin, but not LY294002, inhibits gonococcal invasion. A similar phenomenon has been reported previously for *N. gonorrhoeae* invasion of pex cells (36), and the activity of wortmannin in inhibiting uptake of gonococci by pex cells had been attributed to the inhibition of myosin light chain kinase instead of PI3K. Different from LY294002, wortmannin, at high concentrations (IC_{50} 200 - 300 nM), has been shown to inhibit MAP kinase, myosin light chain kinase, PI4K and phospholipase D. Although the lowest concentration of wortmannin (50 nM) used in this study would not be expected to affect these other enzymes, it is possible that it acted on one of them or another yet unknown kinase. Therefore, these results argue against a role for PI3K in gonococcal invasion.

Our finding that gonococcal invasion of epithelial cells via transactivation of EGFR is not dependent upon its ERK or PI3K/AKT activation suggests that the activation of these signaling pathways has another role in gonococcal disease. EGFR stimulation is known to produce cell survival and anti-apoptotic signals. Growth factor withdrawal conversely stimulates apoptosis (80). *Helicobacter pylori* and *Pseudomonas aeruginosa* both have been shown to transactivate EGFR via inducing the shedding of the EGFR ligand, HB-EGF, from the plasma membrane, leading to the prevention of apoptosis in the host epithelial cell (157, 161). This supports the possibility that EGFR signaling, in addition to its importance for invasion, also protects the host epithelial cell from apoptosis.

Apoptosis of bacterially infected host cells is one of the host defense mechanisms against a pathogen. Several intracellular pathogens have been shown to propagate intracellular replication via inhibiting apoptosis of their host cells. There is increasing evidence that *N. gonorrhoeae* is capable of inhibiting apoptosis of their host cell, which is associated with persistence of the infection. In the Fallopian tubes, *N. gonorrhoeae* prevents apoptosis of the epithelial cells that they invade, while inducing apoptosis of the ciliated cells that they do not invade. It has been reported by several groups that *N. gonorrhoeae* can protect epithelial cell lines from apoptosis in vitro. Wild type gonococci inhibited, whereas the noninvasive Δ*pilT* induced apoptosis of A431 cells (57). *N. gonorrhoeae* infection did not induce apoptosis of primary urethral epithelial cells (UEC) and End/E6E7 cells, a primary endocervical cell line that had been transformed with E6 and E7 proteins from HPV (human papiloma virus). (12, 43), but protected these cells from staurosporine induced

apoptosis. The anti-apoptosis function of gonococci was further supported by recent finding that P^+O^- gonococci downregulated the expression of the proapoptotic proteins BAD and BIM in T84 colorectal epithelial cells in an ERK dependent manner (60).

Gonococcal transactivation of EGFR induces sustained activation of both ERK and AKT. Sustained ERK activation is associated with transcriptional downregulation of the proapoptotic proteins BAD and BIM (111), ERK-induced phosphorylation of BAD, BIM and other BH3-only family proteins can trigger the ubiquitination and proteosomal degradation of them (41). AKT is known to induce the activation of NF-κB, an activator of anti-apoptotic genes. Gonococcal infection of UECs and End/E6E7 cells was found to increase the transcript expression of *bfl-1* and *c-IAP-2* in an NF-κB dependent manner (11, 43).

Based on these reports, I would postulate that in HEC-1-B and ME180, the two epithelial cell lines used in this study, gonococcal-induced sustained activation of ERK and AKT would downregulate proapoptotic proteins (BH3-only family proteins) and upregulate anti-apoptotic proteins, Bfl-1 and c-IAP, all contributing to host cell survival.

The results of this study demonstrate the requirement for Ca^{2+} flux in *N. gonorrhoeae* invasion, and also suggest that PLCγ activation induced by transactivation of EGFR contributes to this Ca^{2+} flux. This study also suggests that EGFR transactivation may have additional effects on the infection process by promoting the survival of intracellular gonococci by the prevention of host cell apoptosis via the ERK and AKT signaling pathways. EGFR signal transduction is

essential for epithelial cell survival and proliferation. By the activation of this one receptor, EGFR, gonococci have developed means by which they can ensure their survival. Future studies will further examine the relationship between PLCγ activation and *N. gonorrhoeae* induced transactivation of EGFR, the role of pili and Opa in gonococcal induced PLCγ activation, and the role of transactivation of EGFR in protection of host cells from apoptosis.

Chapter 4 Conclusions

4.1 Summary and General Discussion

Neisseria gonorrhoeae is an obligate parasite of humans that has no other natural host. It causes significant disease in the U.S. and worldwide, with gonorrhea being the second most commonly reported infectious disease in the US. Infected women are typically asymptomatic, thus allowing them to become chronically infected. Chronic infection heightens the risk of serious sequelae, which can include PID and DGI. Consequences of PID include scarring of the reproductive organs, resulting in chronic pelvic pain, ectoptic pregnancy and infertility. Additionally, the significance of this disease is exacerbated by the findings that gonococcal infection increases the risk of HIV transmission (42).

N. gonorrhoeae primarily infects epithelial cells of the genitourinary tract of both men and women. Productive infection by gonococci of genital epithelial cells consists of four sequential steps: adherence, invasion, intracellular survival, and exocytosis. Only a small subpopulation of adherent gonococci is able to invade epithelial cells. The establishment of this intracellular niche protects the bacteria from immune surveillance. Additionally the ability to adapt to a constantly changing environment in the female reproductive tract, as older cells are shed and newer epithelial cells differentiate, seems essential for the establishment of chronic infection in women.

The goal of this study was to gain a better understanding of the host signaling events that *N. gonorrhoeae* induces and that are essential for gonococcal invasion of genital epithelial cells of the female reproductive tract. This study was designed with an inside-out approach in order to investigate mechanisms that lead to gonococcal invasion of genital epithelial cells. Through this approach I found that gonococci induce the activation of EGFR and ErbB2 and recruitment of them from the basolateral surfaces to gonococcal clusters at the apical surface of the epithelial cells. Gonococci activate EGFR via inducing the expression and surface release of EGFR ligand. This gonococci-induced activation of EGFR kinase is important for gonococcal invasion. Activation of EGFR kinase leads to phosphorylation of EGFR and ErbB2, which induces signaling pathways, including PI3K, PLCγ, and MAP kinase ERK. Gonococcal variants that express either pili or Opa are able to activate ERK and PI3K/AKT in an EGFR-dependent manner. This suggests that pili and Opa-mediated invasion may converge at EGFR activation. Among these EGFR downstream signaling pathways, Ca^{2+} influx that is induced by PLCγ activation, but not PI3K and ERK, is required for gonococcal invasion.

Both pili and Opa proteins have been suggested to be both adhesins and invasins. Pili-induced invasion and Opa-induced invasion of epithelial cells have been described in multiple studies (30, 44, 51, 53, 77, 118, 125, 136). The role of pili and Opa in invasion has been suggested in studies using gonococcal variants that expressed either pili or Opa, without the other adhesion. In the absence of pili and Opa expression, gonococci invade very poorly, but also adhere very poorly. This clearly supports both pili and Opa proteins as adhesins, but does not provide evidence

that either is an invasin. Although it is possible that adhesins also can serve as invasins, the evidence that either pili or Opa is an invasin is lacking.

This study shows that activation of and signaling through EGFR is important for gonococcal invasion of genital epithelial cells. Furthermore, adherence to epithelial cells via either Opa or pili leads to EGFR activation, although neither is sufficient to trigger the activation of EGFR, since killed gonococci that express both pili and Opa do not activate EGFR and invade epithelial cells (13). It previously has been shown that gonococci cultured with fixed epithelial cells reduced the time needed for maximal invasion from six to two hours (20). This suggests that expression of invasion promoting factors is induced in the bacteria when grown with epithelial cells. Requirement for bacterial viability in addition to the expression of pili or Opa and enhanced invasion ability of gonococci preincubated with fixed epithelial cells support that additional bacterial factors are responsible for invasion.

Exactly what induces the recruitment of EGFR and ErbB2 to the gonococcal microcolonies is not clear. The clustering of EGFR does not appear to be an actin mediated event since cytochalasin D, an actin perturbing agent, prevents the formation of the actin cortical plaque beneath the microcolonies, but does not affect the recruitment of EGFR to the site of the microcolonies (87). It was suggested that this may be due to a cytochalasin D insensitive pool of actin, but the lack of F-actin localization with the EGFR surrounding the microcolonies argues against this. The EGFR kinase inhibitor AG1478 does not have an effect on EGFR or ErbB2 recruitment to the gonococcal microcolony. This implies that EGFR signaling is not required for EGFR and ErbB2 recruitment. The surface distribution of EGFR can be

regulated by ligand binding and dimerization formation, but EGFR and ErbB2 recruitment appears to precede the binding of ligand. Heparin washes of HEC-1-B cells prevent the release of cleaved soluble HB-EGF, but have no effect on EGFR and ErbB2 recruitment to the gonococcal microcolony. Taken together, these results suggest that EGFR and ErbB2 recruitment to the gonococcal microcolony is neither actin mediated nor dependent on the function of EGFR.

EGFR can activate multiple signaling pathways. Two of these pathways that have been shown to be important for gonococcal invasion are Src kinase and Ca^{2+} flux. I was not able to detect any changes in Src activation in HEC-1-B cells that were inoculated with gonococci. This study suggests that the gonococci induced transactivation of EGFR activates of PLCγ, and the expected Ca^{2+} flux activated by PLCγ may be the source of the Ca^{2+} flux necessary for gonococcal invasion.

EGFR signal transduction also activates ERK and AKT signal pathways. Neither of these pathways is vital for gonococcal invasion of epithelial cells. Both of these pathways are known to be important for cell survival and anti-apoptosis in general. A review of the *Neisseria* literature suggests that many prosurvival or anti-apoptotic genes are induced by gonococci through either ERK or PI3K/AKT signaling (11, 60, 79). Furthermore, an increasing number of studies imply that *N. gonorrhoeae* can prevent the apoptosis of the epithelial cells that they invade. These suggest that ERK and AKT signaling induced by gonococcal transactivation of EGFR may be important for the survival of the infected epithelial cells and intracellular gonococci. Thus gonococci-induced transactivation of EGFR has double functions in

gonococcal infection, inducing its invasion into epithelial cells and ensuring its intracellular survival by preventing apoptosis.

The data presented in this dissertation furthers the understanding of cellular mechanism by which gonococci infect genital epithelial cells. Based on the data presented here along with the published literature, I postulate a working model of *N. gonorrhoeae* invasion of genital epithelial cells (Fig. 17). When women are exposed to Pil^+ Opa^+ gonococci, initially pili adhere to the surface of epithelial cells. Through retraction of their pili, the gonococci move closer and establish intimate interaction with the epithelial cell surface via their Opa proteins and LOS. Gonococci form microcolonies on the epithelial cell surface over time through replication and the fusion of smaller gonococcal clusters. The interaction with epithelial cells induces the expression of new gonococcal invasion factor(s) in the bacteria. The induction of this new factor(s) and possibly the action of the microcolony trigger the activation of heparin-bound MMPs. The activated MMPs cleave EGFR ligands, including HB-EGF, that transactivate EGFR. EGFR activation induces several signaling cascades, including PLCγ-mediated Ca^{2+} flux. This Ca^{2+} flux induces actin cytoskeletal rearrangements through Ca^{2+}-responding actin regulators, resulting in the uptake of gonococci. EGFR transactivation also induces ERK and AKT signaling pathways that protect the infected epithelial cell from apoptosis, promoting *N. gonorrhoeae's* intracellular survival.

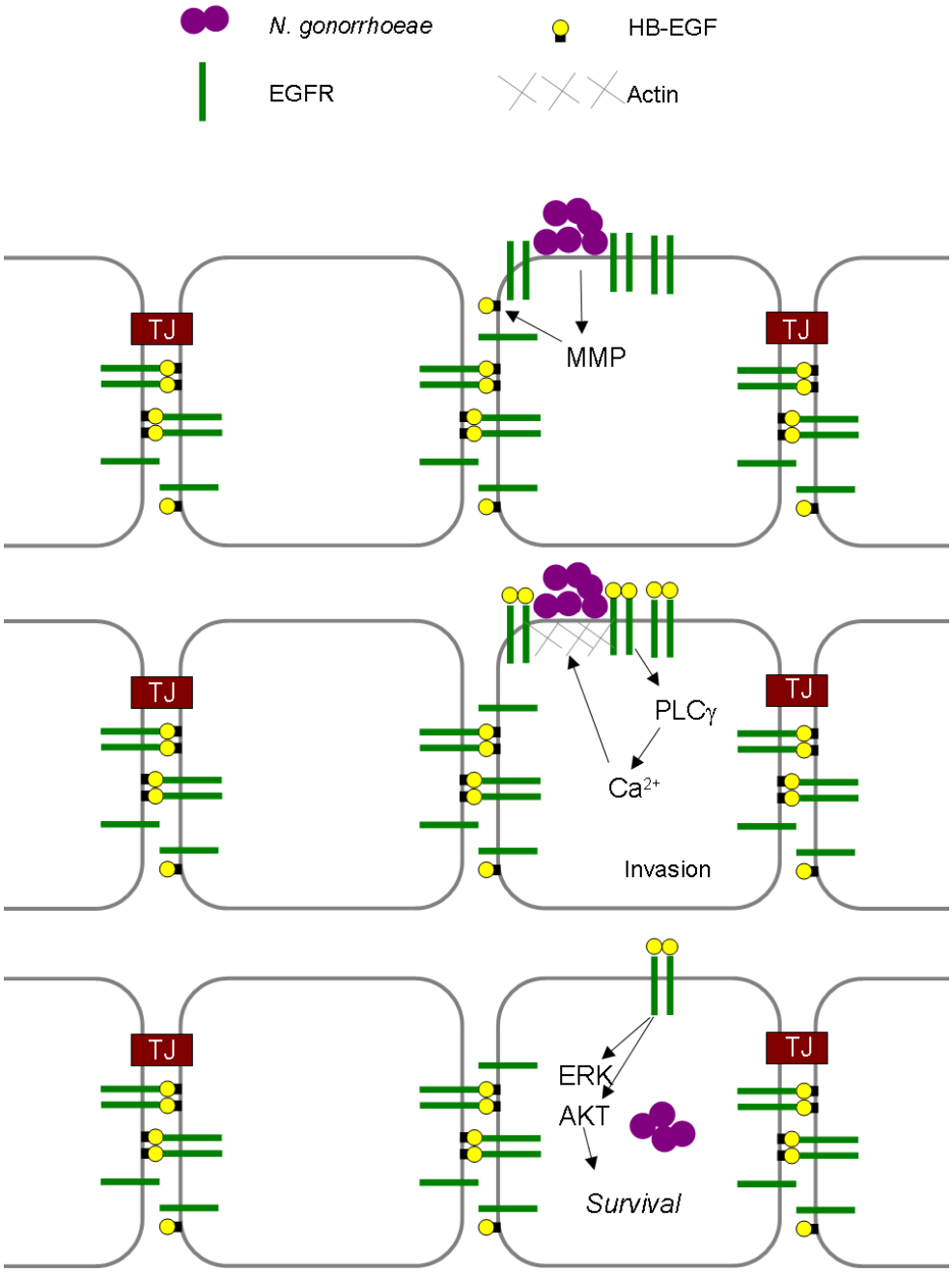

Figure 17. Working Model of *N. gonorrhoeae* invasion into genital epithelial cells. Gonococci that express either pili or Opa adhere to the surface of the epithelial cells forming microcolonies, causing the recruitment of EGFR and ErbB2. Heparin-bound MMPs become activated, cleaving HB-EGF to bind to its receptor EGFR and initiating downstream signaling events. PLCγ is activated downstream of EGFR inducing a Ca^{2+} flux that causes actin reorganization leading to the invasion of gonococci. ERK and AKT also are activated downstream of EGFR leading to survival of the gonococci within the host epithelial cell.

4.2 Future Directions

The study described here supports the idea that EGFR signaling is important for gonococcal invasion and intracellular survival. Further studies are required to understand how gonococci are able to induce the expression and surface cleavage of EGFR ligands and how EGFR signaling mediates gonococcal invasion. In addition, gonococcal invasins that are induced by interacting with host cells and required for gonococcal invasion should be identified.

This study suggests that PLCγ activation by gonococcal transactivation of EGFR is important for gonococcal invasion. Additional studies are required to definitively link PLCγ activation with *N. gonorrhoeae* induced transactivation of EGFR and the Ca^{2+} flux that is required for gonococcal invasion. The time course of gonococci-induced PLCγ activation, the effect of gonococcal viability on this activation, the dependency of this activation on the transactivation of EGFR, and the role of pili and Opa in gonococcal induced PLCγ activation should be examined. Phosphorylation of EGFR tyrosine 992 has been shown to be critical to the activation of PLCγ. The binding of PLCγ to EGFR pY992 via its SH2 domain leads to its phosphorylation and activation. We could test the effect of over expression of an available dominant-negative EGFR Y992 mutant in HEC-1-B and/or ME180 cells on gonococcal invasion(155). Mutating Y992 has been shown not to interfere with cell growth. It may be necessary to simultaneously knock-down the endogenous wt EGFR by siRNA in order to examine the effect the EGFR mutant, since the expression of EGFR in both HEC-1-B and ME180 cells is very high. There are two possible

problems with this approach. The first is that PLCγ also has been reported to bind to the pY1173 of EGFR, although activation of the ERK pathway via the adaptor SHC typically occurs via binding to this site. The second potential problem is that EGFR has been shown to activate PLCγ indirectly. PLCγ has been shown to be activated by PI3K by direct binding to its product PIP_3, at the plasma membrane. Since loss of PI3K activity with LY294002 does not inhibit invasion, it is possible that loss of EGFR to PLCγ signaling may enhance EGFR to PI3K signaling thus leading to some PLCγ activity.

This study did not address the role of Src kinase in gonococcal invasion, which has been shown to be important for gonococcal invasion. Since Src activation was not significantly altered after the incubation of gonococci with HEC-1-B cells, its activation is likely not induced by gonococci in our experimental system. Src kinase is able to phosphorylate EGFR at Y845 and Y1101 that are not the phosphorylation target of EGFR tyrosine kinase. If the Src kinase activity that is required for gonococcal invasion involves phosphorylation of EGFR, we could follow the phosphorylation of Y845 by western blot using a commercially available antibody. There are currently no commercially available antibodies to pEGFR Y1101.

LPA (lysophosphatidic acid) binding to its G-protein coupled receptors is known to activate Src and EGFR tyrosine kinases (27, 78, 105, 140). Gonococci express phospholipase D (PLD) an enzyme that hydrolyzes phosphatidylcholine to generate choline and phosphatidic acid (PA) (36). PA can then be converted to LPA by phospholipase A (PLA) within epithelial cells. PLD has been shown to be important for gonococcal invasion as ΔPLD mutants were unable to invade (39). The

addition of extracellular PLD to the media is able to compensate in the invasion of ΔPLD mutants. It is possible that gonococcal PLD is necessary for gonococcal mediated transactivation of EGFR. This hypothesis can be investigated by examining the effect of purified gonococcal PLD on the phosphorylation of EGFR and the effect of ΔPLD mutants on their ability to transactivate EGFR.

The studies described in this thesis are only a beginning, but should be helpful in furthering our understanding of how gonococci are able to induce the expression and surface cleavage of EGFR ligands and how EGFR signaling mediates gonococcal invasion. Additional directions of this work might entail understanding if and how ERK and AKT activation is able to prevent host cell apoptosis.

Appendix

A.1 MMP Inhibitors

In Chapter 2 I showed that inhibition of heparin-bound MMPs with heparin washes inhibits gonococcal invasion into HEC-1-B and ME180 cells. Loss of heparin-bound MMPs resulted in the prevention of the cleavage of membrane bound HB-EGF to the soluble form. Here I examined the role of MMP activation in gonococcal invasion by inhibiting MMP activity with the use of four MMP inhibitors. The MMP inhibitors, Batimastat, Marimastat, TAPI-1 and GM6001, vary in their specificity to various MMPs (Table 2). The inhibitor TAPI-1 in addition to being an MMP inhibitor also inhibits ADAM17/TACE (TNFα-converting enzyme). TACE has been shown in some cell lines to cleave membrane bound HB-EGF (134). The results of the MMP inhibition were mixed and fell into three categories based on the epithelial cell being invaded. The first category included heparin washes and Batimastat. Batimastat was able to significantly inhibit invasion of gonococci into both HEC-1-B and ME180 cells (Fig. 18 A and B). The second category included Marimastat and TAPI-1; these two inhibitors had no effect on the invasion of gonococci into HEC-1-B cells (Fig. 18 E and G), but significantly increased gonococcal invasion of ME180 cells (Fig. 18 F and H). GM6001 alone comprised the final category and had no effect on gonococcal invasion of HEC-1-B and ME180 cells. Because ME180 cells express CEACAM while HEC-1-B cells do not, I thought it was possible that MMP inhibition in ME180 cells might enhance invasion by Opa mediated mechanisms. To test this gentamicin protection assays were done in ME180

cells using TAPI-1 and MKC variants that expressed P^+O^+, $P^+\Delta O$ or P^-O^+. TAPI-1 doubled invasion of all three variants into ME180 cells (Fig. 18 I). Although the increase in invasion as compared to no inhibitor was statistically significant for all three variants, there was no difference in the TAPI-1 induced increase in invasion between the three gonococcal pili and Opa variants.

It is not clear why some of the MMP inhibitors can block gonococcal invasion while others have no effect or increase invasion. Marimastat was originally synthesized as a structural relative to Batimastat, but that had better solubility. The two inhibitors have the same MMP specificities with slight differences in their IC_{50}. As similar as Batimastat and Marimastat are, they have a quite marked difference in their ability to inhibit gonococcal invasion. Many of the MMP inhibitors that were used here have overlapping specificities, but no clear pattern emerges when trying to distinguish which MMPs may be important. It may be necessary to determine if the MMP inhibitors are preventing HB-EGF cleavage as is the case for heparin washes. Additionally, differences between invasion of ME180 and HEC-1-B cells in the presence of the different MMP inhibitors may have to do with which EGF ligand is being cleaved. Data from this work suggest that HEC-1-B cells cleave HB-EGF to transactivate EGFR. RT-PCR of gonococci stimulated HEC-1-B cells shows that mRNA for HB-EGF is upregulated 30-fold and amphiregulin is upregulated 5-fold. However, RT-PCR analysis of ME180 shows that gonococci upregulate amphiregulin 90-fold, with no significant change in HB-EGF expression (104).

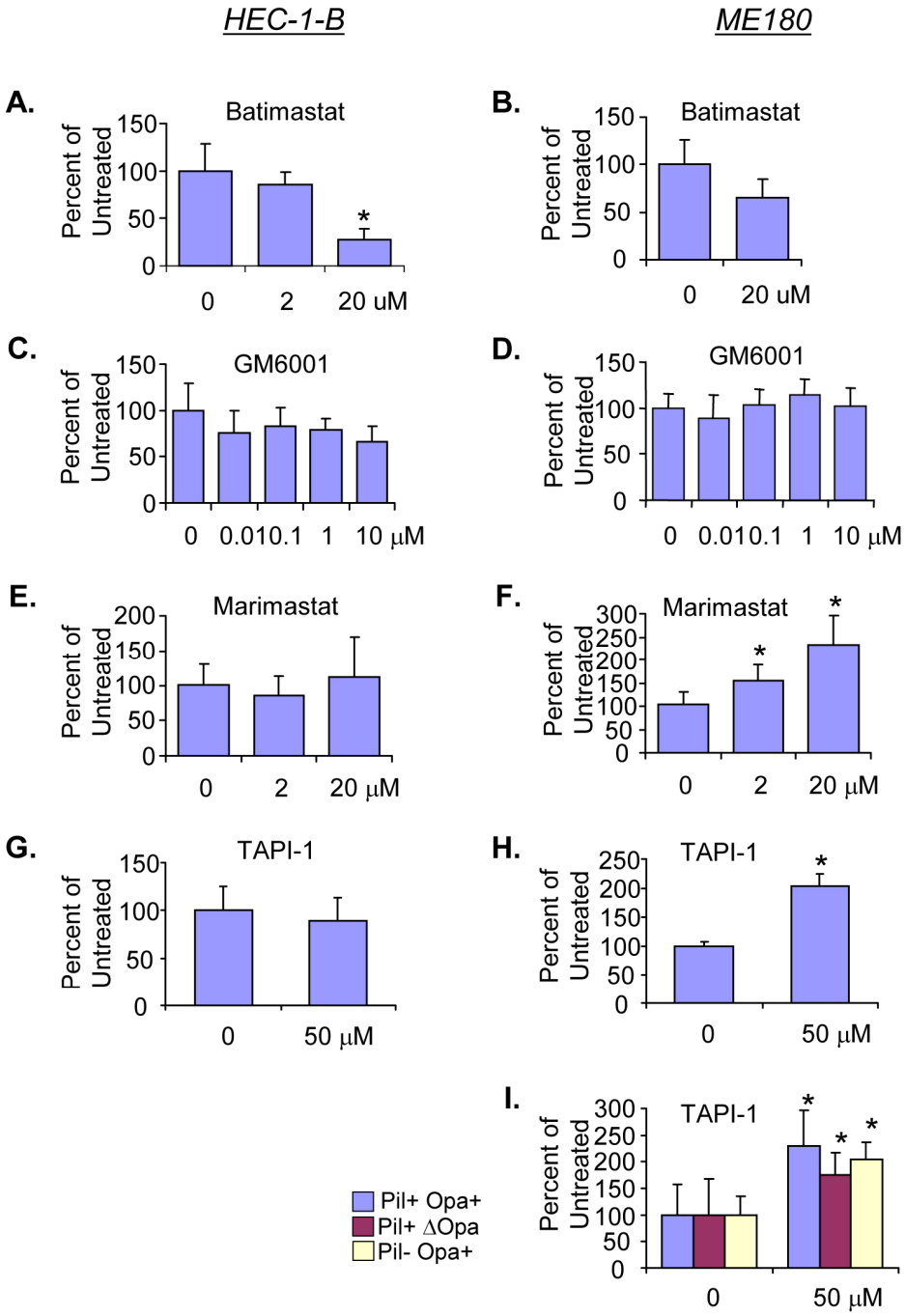

Figure 18. The effects of MMP and ADAM inhibitors on gonococcal invasion. To quantify invaded bacteria, HEC-1-B cells (A, C, E and G) or ME180 (B, D, F, H and I) were preincubated for 30 min with the inhibitors as indicated before the addition of MKC Pil^+ Opa^+ or MKC variants as indicated (I). After incubating with the bacteria for 6 h, the epithelial cells were treated with gentamicin, washed, and lysed to determine the number of gentamicin resistant bacteria as invaded bacteria. Shown are the mean percentages (± SD) from two independent experiments with six replicates per experiment. *$P < 0.01$ (as compared with no inhibitor).

Table 2. MMP Inhibitor data summary

Inhibitor	IC_{50}	HEC-1-B	ME180	Reference
Heparin		+++	+++	(160)
MMP-1				
MMP-2				
MMP-7				
MMP-9				
MMP-13				
Batimastat		+++	++	(29, 150)
MMP-1	3 nM			
MMP-2	4 nM			
MMP-9	4 nM			
MMP-7	6 nM			
MMP-3	20 nM			
Marimastat		N.C.	–	(113)
MMP-9	3 nM			
MMP-1	5 nM			
MMP-2	6 nM			
MMP-14	9 nM			
MMP-7	13 nM			
MMP-3	230 nM			
TAPI-1		N.C.	–	(26, 92)
TACE/ADAM17				
MMPs				
	3-10 μM			
GM6001*		N.C.	N.C.	(45, 46)
MMP-1	0.4 nM			
MMP-2	0.5 nM			
MMP-3	27 nM			
MMP-8	0.1 nM			
MMP-9	0.2 nM			

MMP inhibitors are listed with the specific MMPs that they inhibit along with the IC_{50} concentration for that particular MMP for the purified metalloproteinase. Heparin was purchased from Sigma, Batimastat and Marimastat were purchased form Tocris Bioscience (Ellisville, Missouri), and GM6001 was purchased form Calbiochem.

*Data for GM6001 is given as K_i.

+ Inhibition of gonococcal invasion.

– Increase in gonococcal invasion.

N.C. No change in gonococcal invasion.

A.2 Myosin Light Chain Kinase

Invasion of gonococci into pex (immortalized primary cervical) cells has been suggested to be influenced by myosin light chain kinase (MLCK) activity. 300 nM ML-7, an MLCK inhibitor, was able to inhibit 85% of gonococcal invasion into pex cells. ML-7 activity toward the inhibition of gonococcal invasion is partially due to the loss of gonococcal adherence (50%) at this concentration. MLCK is a CaMK (calmodulin kinase), which is a family of calmodulin binding kinases that are activated in response to Ca^{2+} fluxes. Since I have shown in Chapter 3 that Ca^{2+} fluxes are important for gonococcal invasion of epithelial cells, it seemed plausible that this was due to the activation of MLCK. HEC-1-B and ME180 cells were preincubated with 1 or 10 μM MLCK before the addition of MKC $P^{+}O^{+}$. As shown in Fig. 19, neither concentration was able to inhibit the invasion of gonococci into either HEC-1-B or ME180 cells. At very high concentrations (50 μM) MLCK inhibits cytokinesis (128). When invasion assays were performed using HEC-1-B and ME180 with 50 μM MLCK, the cells rounded up and lost cell-cell contact. After the multiple washes that are done for the gentamicin protection assays, most of the HEC-1-B and ME180 cells were washed away making it impossible to perform the assays at this very high concentration of ML-7. Cancer cells are known to be less sensitive to some inhibitors than primary cells. It is possible that this could explain the difference in sensitivity to ML-7. Since I used 33-fold more ML-7 with the HEC-1-B and ME180 cells than was used with pex cells, and since invasion was inhibited 85% in pex cells, I would have expected to be able to see some inhibition of invasion in HEC-1-B or ME180 cells

with ML-7. Although I never confirmed that 10 μM MLCK inhibited MLCK in HEC-1-B or ME180 cells, the rounding up of the epithelial cells seen at 50 μM MLCK, being phenotypically consistent with blockade of cytokinesis, suggests that ML-7 does affect MLCK in HEC-1-B and ME180 cells. Taken together this suggests that MLCK activity is not essential for gonococcal invasion into HEC-1-B and ME180 cells.

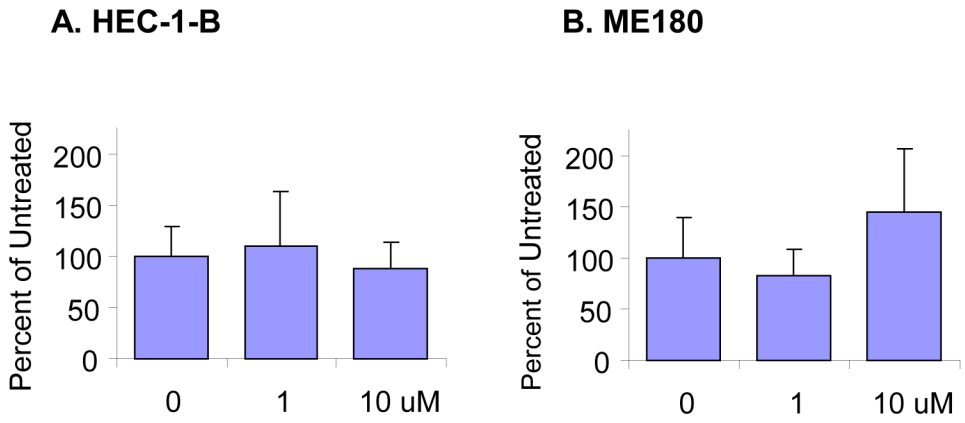

Figure 19. The effects of MLCK inhibition on gonococcal invasion. To quantify invaded bacteria, HEC-1-B cells (A) or ME180 (B) were preincubated for 30 min with the MLCK inhibitor, ML-7, at the indicated concentration before the addition of MKC Pil^+ Opa^+ at an MOI of 5. After incubating with the bacteria for 6 h, the epithelial cells were treated with gentamicin, washed, and lysed to determine the number of gentamicin resistant bacteria as invaded bacteria. Shown are the mean percentages (± SD) from three independent experiments with four or five replicates per experiment.

A.3 PLCγ.

Chapter 3 suggests that PLCγ activation may be responsible for the Ca^{2+} flux that is needed for gonococcal invasion into HEC-1-B and ME180 cells. U71322 is the only known inhibitor of PLCγ activation. U71322 inactivates itself quickly in the presence of cells by cross-linking to cell surface proteins, and is inactivated within 20 min (153). Because of this it has not been able to inhibit PLCγ activation in a number of studies. Because I did not know when PLCγ activation initially occurs in response to gonococci, I devised two strategies to test U71322's influence on gonococcal invasion. First, I added U71322 only once at a high dose at 0, 1, 2, 3 or 4 hours during the invasion assay with MKC $P^{+}O^{+}$. Second, I added multiple doses of U71322 hourly throughout the invasion assay from 2-4 hr. As shown in Fig 20, U71322 had no effect on the invasion of gonococci into HEC-1-B or ME180 cells under any of the conditions tested. Since it is unclear whether or not the conditions tested, either a single dose or multiple doses of U71322, was able to inhibit gonococcal induced PLCγ activation, the importance of PLCγ activation can not yet be definitively linked to gonococcal invasion of epithelial cells.

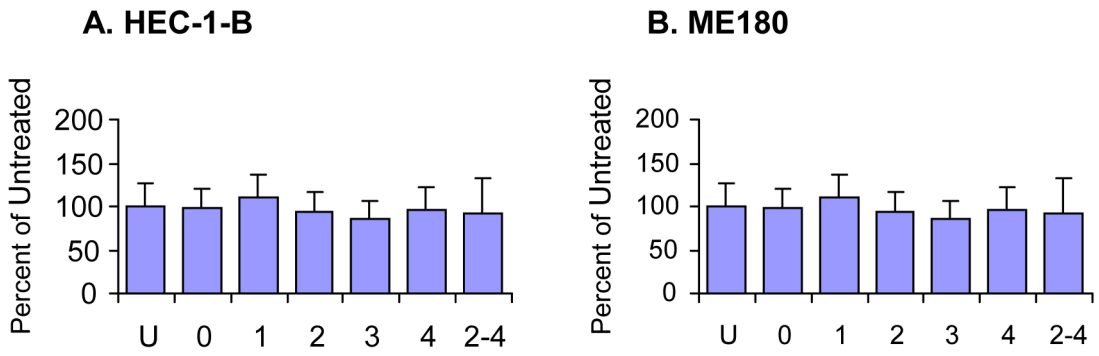

Figure 20. The effects of PLCγ inhibition on gonococcal invasion. MKC Pil^+ Opa^+ was added at a MOI of 5 at 0 h to HEC-1-B cells (A) or ME180 (B) in the presence or absence (U) of 1 μM PLCγ inhibitor, U71322,. The inhibitor was either added as a single dose at the hour indicated or as three 1 μM doses added hourly from 2- 4 hrs. To quantify invaded bacteria, after 6 h incubation with the bacteria, the epithelial cells were treated with gentamicin, washed, and lysed to determine the number of gentamicin resistant bacteria as invaded bacteria. Shown are the mean percentages (± SD) from three independent experiments with four or five replicates per experiment.

A.4 Tyrosine Kinase Inhibitor AG126

AG126 is a general tyrosine kinase inhibitor that has been shown to inhibit LPS induced TNFα synthesis and ERK phosphorylation. Exactly how AG126 is able to do this is not clear. AG126 was tested to see whether it could inhibit the invasion of MKC Pil^+ Opa^+ into HEC-1-B cells. AG126 significantly inhibits gonococcal invasion of HEC-1-B cells that is dose dependent (Fig. 21A). At 200 μM, the highest dose tested, AG126 inhibits 90% of gonococcal invasion. Since one of the downstream modes of action of AG126 is prevention of ERK activation, I determined whether AG126 is able to inhibit gonococcal induced ERK activation. HEC-1-B cells that had been preincubated with 200 μM AG126 were inoculated with MKC Pil^+ Opa^+ at an MOI of 5 for up to 6 h. Cells were lysed and were analyzed using western blot, probing for phosphorylated ERK. The tyrosine kinase inhibitor AG126 had no effect on the levels of activated ERK (pERK) in HEC-1-B cells inoculated with gonococci (Fig. 21B). The data presented in Chapter 3, suggests that pERK activation does not influence gonococcal invasion of epithelial cells. The data presented here does not oppose the pERK data presented earlier, since the mode of action of AG126 is not prevention of pERK activity. AG126 is synthesized from the naturally occurring tyrosine kinase inhibitor erbstatin (91).. Erbstatin has been shown to be an inhibitor of EGFR and ErbB2 activation. Thus it is possible that AG126 is able to inhibit gonococcal invasion of HEC-1-B cells by activity toward EGFR and ErbB2.

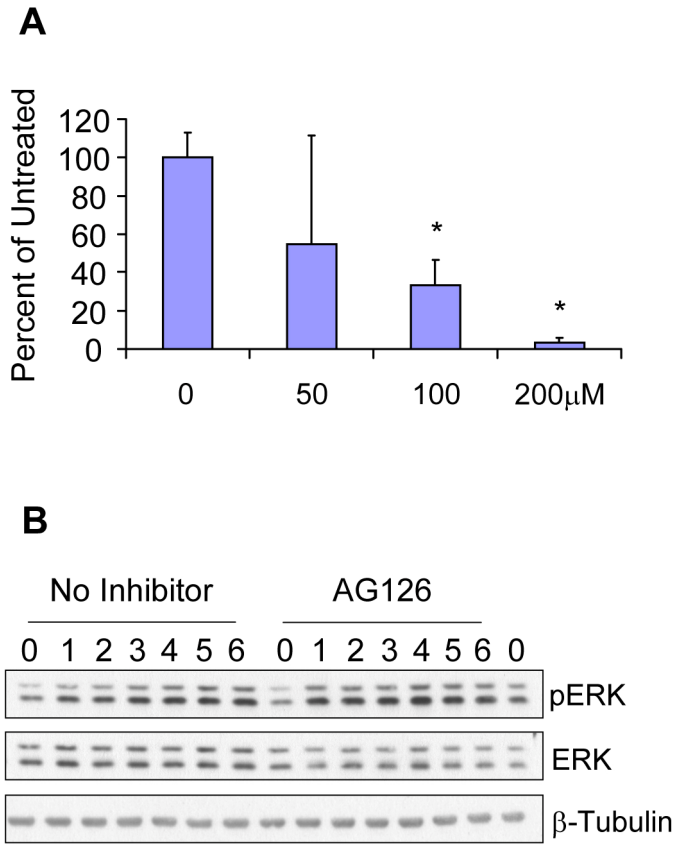

Figure 21. The effects tyrosine kinase inhibitor AG126 on gonococcal invasion. (A) To quantify invaded bacteria HEC-1-B cells were preincubated for 30 min with the tyrosine kinase inhibitor AG126, at the indicated concentrations before the addition of MKC Pil^+ Opa^+ at an MOI of 5. After incubating with the bacteria for 6 h, the epithelial cells were treated with gentamicin, washed, and lysed to determine the number of gentamicin resistant bacteria as invaded bacteria. Shown are the mean percentages (± SD) from three independent experiments with six replicates per experiment. *$P < 0.01$ (as compared with no inhibitor). (B) HEC-1-B cells were treated with 200 μM AG126 for 30 min, prior to the addition of MKC. At each hour the cells were lysed and an aliquot of the cell lysate was analyzed by SDS-PAGE and western blot, probing for pERK. The blots were stripped and reprobed for total ERK and β-tubulin as loading controls. Shown are representative blots from two independent experiments.

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