Mechanotransductive Signaling Mediated by Retraction of the *Neisseria gonorrhoeae* Type IV Pilus

by

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CERTIFICATE OF APPROVAL

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

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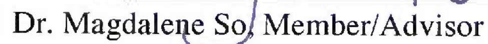

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TABLE OF CONTENTS

Table of Contents i

List of Tables v

List of Figures vi

Acknowledgements ix

Abstract x

Chapter 1: Introduction 1

Neisseria 2

Gonococcal Pathogenesis 3

Infection of the Male Urethra 3

Infection of the Female Genital Tract 5

Infections at Non-Genital Sites 6

Stages of Infection 6

Adhesion and Invasion 7

Intracellular Survival, Trancytosis and Exit 8

Type IV Pili 9

Structure 11

Assembly 12

The PilT ATPase 13

Twitching Motility and Pilus Retraction 14

Type IV Pilus Mediated Signaling 17

Signaling through Pilus Receptors 17

Other Pilus Stimulated Cellular Effects 19

Evidence of Retraction-Mediated Mechanical Stress 21

Mechanical Forces 22

Forces Generated by a Bacterial Microcolony 22

Physiological Forces Experienced by the Body 24

Thesis Overview 25

Chapter 2: Manuscript 1 – The *N. gonorrhoeae* Type IV Pilus Stimulates Mechanosensitive Pathways and Cytoprotection through a *pilT*-Dependent Mechanism 29

Summary 30

Introduction 31

Results 34

Discussion 48

Materials and Methods 54

MIAMI checklist – Protocol S1 61

Supplementary data – Table S1 69

Acknowledgements 71

Chapter 3: Manuscript 2 – ERK Activation by *Neisseria gonorrhoeae* Modulates Host Cell Pro-Apoptotic Proteins Bad and Bim 72

Summary 73

Introduction 74

Results 77

Discussion 90

Materials and Methods 95

Acknowledgements 99

Chapter 4: Discussion and Conclusions 100

Type IV Pilus Retraction Triggers Host-Cell Mechanotransduction 101

Host Cell Sensing of Mechanical Signals 103

Mechanosensitive Ion Channels 104

Integrins 106

G-Protein Coupled Receptors and Receptor Tyrosine Kinases 108

Mechanical Stress Induced Signaling 111

Calcium Signaling 111

Phosphoinositide-3 Kinase Signaling 114

Potential Cytoprotective Signaling Pathways Induced by Pilus Retraction 115

Overview of Apoptotic Signaling Pathways 116

Regulation of FLICE-Like Inhibitor Protein 120

Regulation of Forkhead Proteins 120

Regulation of IAP Proteins 122

Regulation by GIRE Genes 123

The Nature of Pilus Retraction and Microcolony-Induced Mechanical Force 124

Pilus Retraction: Random Events or a Coordinated Process 125

Pushing Forces and Pulling Forces 128

Conclusions 131

Appendix 1: Lutenizing Hormone Receptor: A Potential Mechano-sensor of Type IV Pilus Retraction 133

Summary 134

Introduction 135

Results 138

Discussion 145

Materials and Methods 146

Appendix 2: Force Generation by Type IV Pilus Retraction Causes Accumulation of Cytoskeletal Proteins 151

Summary 152

Introduction 153

Results 154

Discussion 161

Progress Towards Manuscript 163

Materials and Methods 164

Appendix 3: Mechanical Force Calculations 168

References 171

List of Tables

Chapter 1

1-1 Relative forces of physiological events 25

Chapter 2

2-1 Assays on demand (Taqman probes and primers) used for real-time quantitative RT-PCR in this study 56

S1 Supplementary References 69

Chapter 3

3-1 Bim and Bad mRNA levels following infection 83

Appendix 1

A1-1 LHr expression in cell lines used for *N. gonorrhoeae* infection 139

List of Figures

Chapter 1

1-1	Pathogenesis of uncomplicated gonorrhea of the male urethra	4
1-2	Steps in *N. gonorrhoeae* infection of the host epithelium	8
1-3	Structure of Type IV pili	10
1-4	Ultrastructure of the PilT ATPase	14
1-5	The grappling hook model of twitching motility	15
1-6	CD46-dependent host cell responses to *N. gonorrhoeae* adherence	18
1-7	Model of radial membrane tension	23

Chapter 2

2-1	Infection regulated, retraction enhanced epithelial cell genes	35
2-2	Real-time quantitative RT-PCR verification of microarray results and initial characterization of retraction enhanced genes	36
2-3	Levels of activated MAPK in infected cells and their involvement in retraction enhanced gene expression	38
2-4	Artificial force triggers MAPK phosphorylation and induces the expression of retraction enhanced genes	41
2-5	Pilus retraction during bacterial attachment promotes host cell cytoprotection	46
2-6	Model of the role of pilus retraction in promoting a cytoprotective environment during gonococcal infection of an epithelial cell	52

Chapter 3

3-1	Effect of ERK inhibition on infection-mediated cytoprotection	78

3-2 Phosphorylated Bad and total Bad levels in infected T84 cells 80

3-3 Levels of BH3-only proteins in infected and uninfected cells 82

3-4 Inhibition of Bad and Bim degradation and Bad phosphorylation by ERK and proteasome inhibitors 85

3-5 *N. gonorrhoeae* inhibition of cytochrome c release from the mitochondria 87

3-6 siRNA reduction of Bad and Bim levels and cytoprotection against STS-induced apoptosis 89

Chapter 4

4-1 Mechanisms for activation of ion channels by mechanical stimuli 105

4-2 Integrin and CD46 colocalize within cortical plaques 107

4-3 Schematic diagram of proposed receptors and G proteins as mechanosensors 109

4-4 Mechanisms of force-induced calcium signaling 112

4-5 An overview of the extrinsic and intrinsic apoptosis pathways 117

4-6 Potential cytoprotective signaling pathways induced by pilus retraction 119

4-7 Pilus retraction and motility from a single bacterium 125

4-8 Pilus retraction detector 128

Appendix 1

A1-1 Immunofluorescence detection of LHr in epithelial cells 139

A1-2 LHr localization during infection with *N. gonorrhoeae* 140

A1-3 Localization of myc-tagged LHr during infection with *N. gonorrhoeae* 141

A1-4 Localization of GFP-tagged β-arrestin-2 during infection with 142

N. gonorrhoeae

A1-5 Colocalization of myc-LHR and β-arrestin-2-GFP during infection with *N. gonorrhoeae* 144

Appendix 2

A2-1 Permanent magnet-induced clustering of actin beneath magnetic beads 154

A2-2 Electromagnet-induced clustering of actin beneath magnetic beads 155

A2-3 Qualitative evidence of force-intensity-dependent actin recruitment 156

A2-4 Actin recruitment as a function of relative force 157

A2-5 Actin recruitment as a function of force duration 158

A2-6 Model of pushing and pulling force 159

A2-7 Actin and ezrin recruitment require different types of force 160

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Abstract

Neisseria gonorrhoeae is the etiological agent of gonorrhea; the second-most reported sexually transmitted disease in the world. Infection by this bacterium is initiated by type IV pili, retractile fimbriate appendages that are thought to generate tension on the host cell membrane. The consequences of how such mechanical stress may affect the host cell and the infection process have not been investigated. In this dissertation I demonstrate that type IV pilus retraction modulates host epithelial cell gene expression. Approximately two-thirds of the identified genes are known to be induced specifically by mechanical stress, and the majority of these via activation of MAPK. Indeed, activation of ERK JNK, and P38 are all induced by *N. gonorrhoeae* infection, and enhanced by pilus retraction. Moreover, both MAPK activation and gene expression changes can be recapitulated by applying artificial force on the epithelial membrane using a magnet and magnetic beads, indicating that force alone is sufficient for such a response. I also show that infection induced and retraction enhanced ERK activation triggers cytoprotective signaling pathways within the host cell. This survival mechanism is mediated by ERK dependent down-regulation of two BH3-only proteins, Bad and Bim through a proteasomal dependent mechanism. Taken together, these data demonstrate a novel mechanism of host-pathogen signaling; mechanical force. Moreover, it provides a mechanism by which the bacterium can alter the homeostasis of the host cell, thus providing a safe niche for bacterial replication, and ensuring the host remains healthy enough to transmit the gonococcus. Importantly, a large number of pathogenic bacteria have been shown to harbor type IV pili, many of which have been shown to be

retractable. Thus the results described in this thesis have implications not only for Neisserial disease, but numerous other diseases as well.

Chapter 1

Introduction

Neisseria

The genus Neisseria is comprised of both pathogenic and commensal gram-negative diplococci. At least eighteen different commensal species have been isolated from humans and a variety of other animals, including dogs, cats, guinea pigs, sheep, rabbits, cattle, and monkeys (CDC, 1998). These bacteria are usually found as normal flora in the pharynx, but some can act as opportunistic pathogens in an immunocompromised host.

While numerous commensal Neisseria have been isolated, only two species are known to be consistently pathogenic in man: *Neisseria gonorrhoeae* (gonococcus), the causative agent of gonorrhea, and *Neisseria meningitidis* (meningococcus), a leading cause of bacterial meningitis. Interestingly, both the gonococcus and meningococcus can colonize asymptomatically, reminiscent of their commensal cousins. *N. meningitidis* is often considered normal flora of the throat, persisting in a carrier state in up to 30% of the population, while *N. gonorrhoeae* has recently been shown to be carried asymptomatically by approximately 5% of the population (Turner *et al.*, 2002). Given this propensity to act like a commensal, and the fact the Neisseriae are >85% identical at the genetic level, the pathogenic Neisseriae are often referred to as "accidental pathogens."

How the "pathogenic" Neisseria can be carried asymptomatically by one person, yet cause disease in another remains a mystery. There is no selective advantage for gonococci to sicken their host and diminish their chances for subsequent transmission via sexual contact. Nor is it advantageous for meningococci to kill their host, as is often the

outcome. By studying how the bacterium interacts with its host during the infection process, insight might be gained as to why some infections may cause symptomatic disease and some do not. Importantly, while the diseases caused by *N. gonorrhoeae* and *N. meningitidis* are quite different, the bacterial infection strategies are remarkably similar between the two organisms. Thus, discoveries found using one species can help us understand the pathogenesis of the other. The work presented in this thesis uses *N. gonorrhoeae* as a model for studying early events in the infection process, with results pertinent to the understanding of *N. meningitidis* infection, and commensal colonization as well.

Gonococcal Pathogenesis

Neisseria gonorrhoeae is the etiologic agent of the sexually transmitted disease (STD) gonorrhea. The origins of gonococcal disease date back to biblical times, making it one of the oldest recorded human diseases. Today, gonorrhea is the second most reported STD in the world, with over 60 million cases reported annually (Gerbase *et al.*, 1998). The organism is most often transmitted via direct sexual contact, with primary infections occurring at the mucosal epithelia of either the male urethra or the female uterine cervix.

Infection of the Male Urethra

Infection of the male urethra is usually (though not always) accompanied by acute urethritis, a condition that is characterized by purulent discharge and dysuria. This discharge is the result of a fulminate inflammatory response triggered by increased expression of IL-6, IL-8 and TNFα by the infected urethral epithelial cells (Figure 1-1B)

(Ramsey *et al.*, 1995). Increased levels of these cytokines attract polymorphonuclear leukocytes (PMNs) to the site of infection, and induce the subsequent shedding of urethral epithelial cells. High levels of PMNs together with the shed epithelium constitute the characteristic purulent discharge (Figure 1-1C).

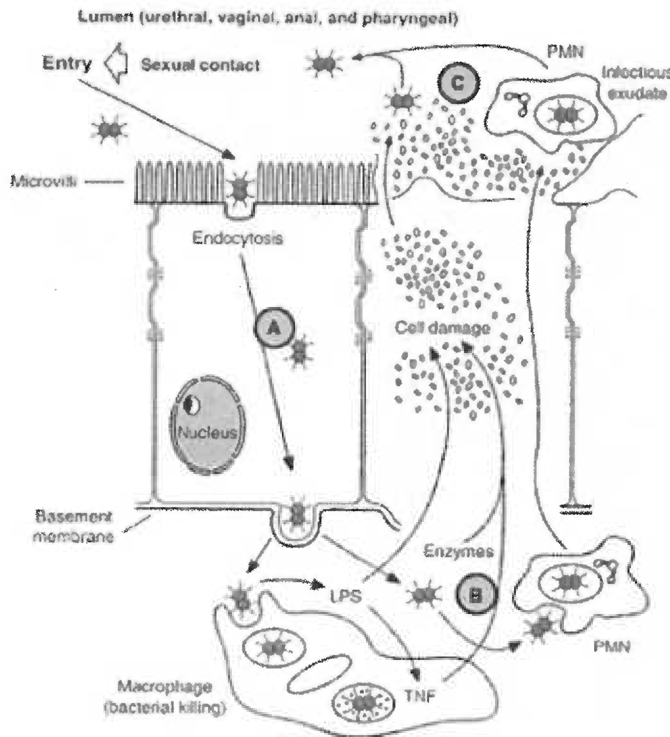

Figure 1-1 – Pathogenesis of uncomplicated gonorrhea of the male urethra (modified from Morse, 1996).

(A) Following infection, bacteria reside within cells for a period of 2-10 days.

(B) Infected epithelial cells release cytokines and chemokines which attract PMNs to the site of infection

(C) PMNs induce fulminant inflammation and shedding of epithelial cells. This is clinically apparent as a purulent discharge.

There is a prolonged incubation period between the time of inoculation and the appearance of clinical symptoms (Figure 1-1A) (Schneider *et al.*, 1995). In studies of infected male volunteers, purulent discharge and dysuria were not detected until at least 3 days post-inoculation (Cohen *et al.*, 1994; Cornelissen *et al.*, 1998). Only at later stages of infection, when the cellular immune system has become activated by cytokines, do symptoms appear. Together, these data suggest that the bacterium is able to adhere to and invade the host cell while inflicting little to no damage. Recent studies have discovered that gonococci variants capable of binding carcinoembryonic antigen-related cell

adhesion molecules (CEACAMs) on the host cell membrane actively prevent shedding of the infected epithelium (Muenzner *et al.*, 2005), even in the presence of elevated levels of cytokines and PMNs. Consistent with these observations, some infected men remain asymptomatic throughout the course of infection (Handsfield, 1990; Hook III and Handsfield, 1999; Sparling, 1999; Turner *et al.*, 2002).

Infection of the Female Genital Tract

Primary infections in females are targeted to the uterine cervix. Unlike the fulminate inflammatory response seen during infection of the male urethra, infection of the lower female genital tract is asymptomatic in 50-80% of cases (Densen *et al.*, 1982; Densen, 1989; Hook III and Handsfield, 1999; Sparling, 1999). If symptoms do occur, they are generally mild, and often mistaken to be associated with the menstrual cycle. Consistent with this, there is no increase in IL-6 or IL-8 levels during cervical infection (Hedges *et al.*, 1999) and PMN infiltration is usually absent. Due to their asymptomatic nature, many cases of gonococcal cervicitis are untreated, and up to 45% of infected women go on to develop ascending infections (Bolan *et al.*, 1999; Knapp and Rice, 1995). In the latter case, the gonococci infect the fallopian tubes and/or ovaries, which can ultimately lead to pelvic inflammatory disease (PID). The risk for gonococcal-mediated PID is significantly higher during menses, during which time hormonal changes alter the expression of gonococcal receptors on the mucosal epithelium (Bolan *et al.*, 1999; Edwards *et al.*, 2001; Hasty *et al.*, 1994; Reshef *et al.*, 1990). Of consequence, ascending infection in females can lead to sterility or serious complications during pregnancy.

Infections at Non-Genital Sites

While *N. gonorrhoeae* most often leads to infections of the urogenital epithelium, the bacterium can also infect other mucosal surfaces (Morse, 1996). Rectal infections accompany 30% of diagnosed cervical gonococcal infections in women. These are most often caused by auto-inoculation from cervical discharge. Infections of this area are also common in homosexual men, resulting from anal intercourse. The gonococcus is also capable of infecting the conjunctival epithelium, which can lead to corneal scaring or perforation. These ocular infections are most common in newborns, and are due to exposure to infected secretions in the birth canal. Finally, the initial gonococcal infection can progress to the bloodstream, causing disseminated gonococcal infection, or DGI. This can lead to serious complications in both men and women, including acute gonococcal arthritis, endocarditis, and meningitis (Cucurull and Espinoza, 1998; Masi and Eisenstein, 1981).

Stages of Infection

N. gonorrhoeae infects the human host exclusively; there is no animal model available to study the infection process. Human challenge studies are limited due to logistics. In consequence, most studies on gonococcal interactions with host cells are derived from those using primary cells, or transformed cell lines. In such studies, it has been found that *N. gonorrhoeae* infection of mucosal epithelial cells is a multi-step process that is dependent not only on the assortment of virulence factors expressed by the bacterium, but also by the type of cell infected and the receptors/signaling molecules present on/within the cell. The following is a general overview of the infection process.

Adhesion and Invasion

The initial interaction between the gonococcus and its target cell is mediated by Type IV pili (Tfp), fimbriate organelles ~6nm in diameter and several microns in length that extend from the bacterial surface (Figure 1-2A) (McGee *et al.*, 1981; Merz *et al.*, 1996; Mosleh *et al.*, 1997; Swanson, 1973). Subsequently, the bacteria interact both with the host epithelium and each other, forming organized clusters called microcolonies, each containing between 10-100 bacteria (Figure 1-2B). Microcolony formation induces numerous changes to the host cell, including microvillus elongation (Shaw and Falkow, 1988; Ward and Watt, 1972; Ward *et al.*, 1974) and the recruitment of cortical cytoskeletal proteins and signaling molecules beneath the site of bacterial attachment. These structures of rearranged proteins, termed cortical plaques, contain membrane receptors, cytoskeletal components, kinases, and phospho-proteins (Hoffmann *et al.*, 2001; Merz and So, 1997; Merz *et al.*, 1999). Cortical plaques are visible at early stages of microcolony formation (30 minutes post infection), and appear fully developed within 3 hours. Following this stage of initial attachment, other adhesin/receptor interactions may occur between the bacterium and host cell. Compared to some bacterial pathogens like Salmonella and Shigella, which invade almost immediately following adhesion, Neisseria spend a relatively long time on the surface of the host cell (~3-6hrs).

Approximately six hours after initial attachment, the gonococci begin to enter the cell. Pili are lost from the bacterial surface, and the bacteria disperse from the microcolony structure (Figure 1-2C). Individual bacteria then become intimately associated with the host cell membrane, often appearing as if the host and bacterial membranes have actually

fused (McGee *et al.*, 1981; Shaw and Falkow, 1988; Ward and Watt, 1972; Ward *et al.*, 1974). The gonococci are then engulfed by the host, by a process resembling macropinocytosis (Apicella *et al.*, 1996; McGee *et al.*, 1981; Shaw and Falkow, 1988; Ward *et al.*, 1974).

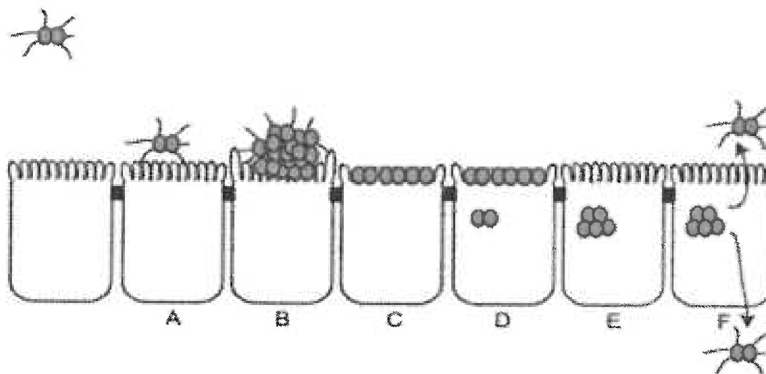

Figure 1-2 – Steps in *N. gonorrhoeae* infection of the host epithelium

(A) Initial contact involving Type IV pili
(B) Microcolony formation and microvilli elongation
(C) Shedding of pili and intimate association of bacteria with the host membrane
(D) Bacterial entry via macropinocytosis
(E) Intracellular survival and replication
(F) Trancytosis and exit basolaterally and perhaps apically

Intracellular Survival, Trancytosis and Exit

Once inside the host epithelium, gonococci not only survive but also replicate (Figure 1-2E) (Hopper *et al.*, 2000; Lin *et al.*, 1997). It is unclear whether the bacteria at this stage reside within a vacuole or remain free in the host cell cytosol, as evidence for both events has been described (Apicella *et al.*, 1996; Harvey *et al.*, 1997; Lin *et al.*, 1997; McGee *et al.*, 1983; Mosleh *et al.*, 1997; Pujol *et al.*, 1997; Shaw and Falkow, 1988; Stephens *et al.*, 1983; Wang *et al.*, 1998; Williams *et al.*, 1998). Approximately 24-36 hours post-infection, the gonococci transcytose across the epithelial monolayer, and exit the cell through the basolateral membrane (Figure 1-2F) (McGee *et al.*, 1981; Stephens *et al.*,

1983). An alternative exit route via the apical membrane has been postulated, and this route would provide a source of bacteria for re-infection of the epithelium and transmission to other hosts. Neither exit strategy appears to lower cellular integrity, thus adhesion, invasion and exocytosis of the bacteria do not significantly damage the host cell.

Type IV Pili

Type IV pili (Tfp) are fimbriate structures that initiate gonococcal adhesion to the host epithelium (see above). These organelles are present on numerous pathogenic and non-pathogenic gram-negative organisms including *N. gonorrhoeae, N. meningitidis, Haemophilus influenzae, Pseudomonas aeruginosa, Legionella pneumophila, Moraxella bovis, Eikenella corrodens, Myxococcus xanthus*, enteropathogenic and enterohemorrhagic *Escherichia coli, and Vibrio cholerae* (reviewed in Mattick, 2002). In addition to adhesion (Bieber *et al.*, 1998; Comolli *et al.*, 1999; Dorr *et al.*, 1998; Merz *et al.*, 1999; Nassif *et al.*, 1993; Pujol *et al.*, 1999; Swanson, 1973; Zolfaghar *et al.*, 2003), Tfp are also involved in other interactions between the bacterium and its environment, as evidenced by their role in motility (Brossay *et al.*, 1994; Swanson, 1973; Wall and Kaiser, 1999; Wu *et al.*, 1997), bacterial aggregation/biofilm formation (Bechet and Blondeau, 2003; Kirn *et al.*, 2000; O'Toole and Kolter, 1998; Swanson *et al.*, 1971), and horizontal gene transfer (Bradley, 1974; Dubnau, 1999; Karaolis *et al.*, 1999; Stone and Kwaik, 1999; Yoshida *et al.*, 1999). These diverse functions can all be accounted for by three basic Tfp processes: assembly/extension, substrate attachment and disassembly/ retraction.

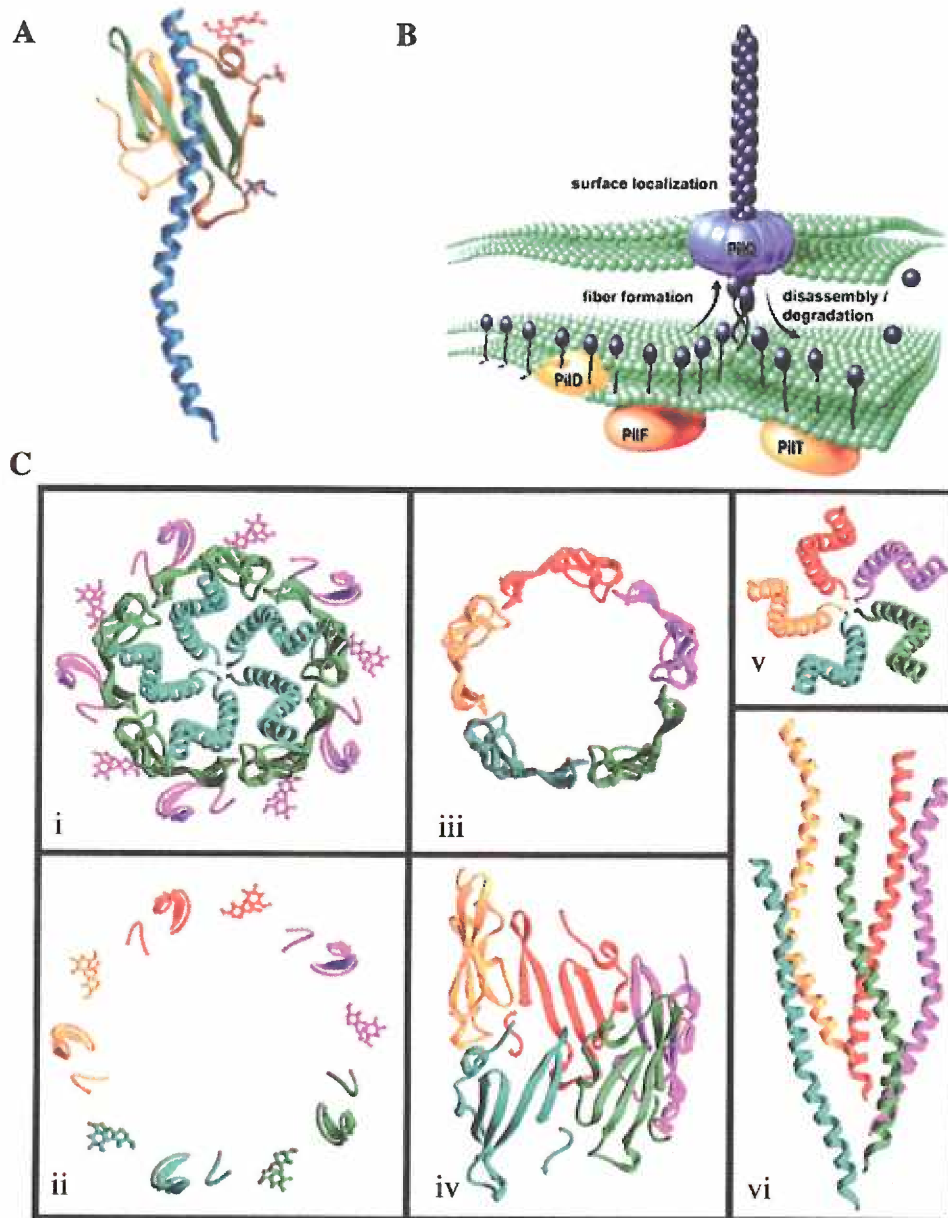

Figure 1-3 - Structure of Type IV pili

(A) Structure of a mature pilin subunit – demonstrating globular head and α-helical tail domains (Forest and Tainer, 1997)

(B) Prepilin subunits are processed by PilD, and incorporated into the growing pilus by PilF. The pilus fiber is extruded from the bacteria through the PilQ pore. Fiber disassembly occurs through the action of PilT. (Wolfgang *et al.*, 2000)

(C) Three-layer representation of the modeled *N. gonorrhoeae* pilus fiber (Forest and Tainer, 1997).

i. End-on view of three-layer model
ii. Outermost layer – the hypervariable region
iii. Central layer - β sheet (end-on view)
iv. Central layer - β sheet (side view)
v. Inner layer – α helical coiled coil (end-on)
vi. Inner layer - α helical coiled coil (side view)

Structure

Tfp are polymeric fibers composed primarily of pilin, an 18-22kDa polypeptide. The gonococcal pilin subunit, encoded by *pilE* (Meyer *et al.*, 1984), is synthesized as an immature precursor protein that undergoes post-translational modification. Modifications include cleavage to remove the signal peptide, O-glycosylation and phosphorylation (Forest *et al.*, 1999; Jennings *et al.*, 1998; Marceau *et al.*, 1998; Marceau and Nassif, 1999; Parge *et al.*, 1995; Stimson *et al.*, 1995; Virji *et al.*, 1993).

The three-dimensional structure of the mature gonococcal pilin has been resolved to the 2.6Å level (Parge *et al.*, 1995) (see Figure 1-3A). The pilin monomer consists of a C-terminal globular head composed of β-sheets and an N-terminal α-helical hydrophobic tail. Individual pilin subunits polymerize into a right-handed cylinder with five-fold symmetry about the helix axis (Forest and Tainer, 1997; Parge *et al.*, 1995). The hydrophobic tails of each monomer are packed into the core of the cylinder to form a helical coiled-coil bundle. Conversely, the globular heads face outwards, comprising the surface of the cylinder. These globular heads each contain a hypervariable loop, which allows for antigenic variation within the pilus structure (Seifert, 1996).

The structure of the type IV pilus has been more thoroughly examined by use of computational approach to model how the pilin subunits are packed within the fiber, and how they interact with one another (Forest and Tainer, 1997). This "helical fiber packing model" describes the pilus as a three-layered spiraling fiber (Figure 1-3C). Importantly, each layer has special properties, which together account for the structure and function of

the pilus fiber. The outermost layer contains the fully exposed hypervariable region (Figure 1-Cii). Importantly this region is loosely associated with the central layer by a few conserved interactions and is not an integral part of the pilus structure. Rather, this layer allows for extreme variation in amino-acid sequence, so as to allow for antigenic variation without disrupting normal pilus assembly and function. The central layer is composed of β-sheets that are arranged in such a way that any cross section of the pilus fiber would cut through 25 individual strands (Figure 1-Ciii and iv). Importantly, the β-sheets of one pilin monomer interact with the β-sheets of its neighbor through hydrogen bonding. This hydrogen bonding is hypothesized to provide the majority of the mechanical strength of the pilus fiber. The innermost layer of the pilus is a parallel coiled-coil composed of the hydrophobic α-helical pilin tails (Figure 1-Cv and vi). The nature of the hydrophobic packing in this layer is hypothesized to allow for flexibility of the pilus fiber.

Assembly

Tfp assembly is hypothesized to take place within the cytoplasmic membrane and periplasmic space (Fussenegger *et al.*, 1997; Hultgren *et al.*, 1993; Pugsley, 1993) (see Figure 1-3B). The process is dependent on the general prokaryotic secretory pathway and at least fifteen specialized proteins (Carbonnelle *et al.*, 2005; Duong and Wickner, 1997; Pugsley, 1993; Pugsley *et al.*, 1997). Individual precursor pilin subunits are first translocated from the bacterial cytoplasm to the inner membrane by a membrane precursor translocase (Pugsley, 1993). The pilin monomers are then targeted to PilD,

through recognition of an unconventional N-terminal secretory signal sequence (Freitag *et al.*, 1995; Nunn and Lory, 1991; Strom *et al.*, 1993). PilD, the prepilin peptidase/ transmethylase then cleaves and methylates the precursor pilin into a mature pilin subunit with an α-methylated phenylalanine N-terminal residue (N-met-Phe). Mature pilins are then incorporated into a growing pilus fiber through an undefined function of the bacterial ATPase PilF (Freitag *et al.*, 1995; Watson *et al.*, 1996). The pilus fiber crosses the outer membrane through PilQ, a large polymeric pore structure that is a member of the secretin family (Bitter *et al.*, 1998; Drake and Koomey, 1995). Once assembled, numerous proteins, including PilW, PilC and a conserved set of pilin-like proteins, are involved in stabilizing the fiber (Carbonnelle *et al.*, 2005; Morand *et al.*, 2004; Winther-Larsen *et al.*, 2005).

The PilT ATPase

Another bacterial ATPase, PilT, is hypothesized to mediate pilus disassembly, resulting in retraction of the pilus fiber (Merz *et al.*, 2000; Wolfgang *et al.*, 2000). The ultrastructure of purified *N. gonorrhoeae* PilT was recently identified by use of freeze-etch microscopy (Forest *et al.*, 2004). Figure 1-4 shows hexameric PilT rings measuring ~115Å in diameter, with a height of 70Å, and a central opening of 15-35Å. *In vivo*, the PilT hexamer is located at the cytoplasmic face of inner membrane, and is thought to encircle the base of the pilus fiber (Brossay *et al.*, 1994; Okamoto and Ohmori, 2002).

The PilT protein is a member of the AAA family of motor ATPases, which are generally known to mediate the unidirectional disassembly of macromolecular complexes (Vale, 2000). Disassembly of the pilus fiber by PilT is predicted to occur in a manner similar to

that of the F_1-ATPase (Kaiser, 2000). In this scenario PilT would break the protein-protein interaction between pilin monomers in an ATP-dependent manner, and dissolve the pilus into a pool of pilin monomers in the membrane, from which they could be recycled (Herdendorf *et al.*, 2002; Merz *et al.*, 2000; Skerker and Berg, 2001). Based on the velocity of pilus retraction and the physical measurements of the pilus fiber it is hypothesized that this process is rapid, with PilT disassembling ~1500 pilin monomers per second (Merz *et al.*, 2000).

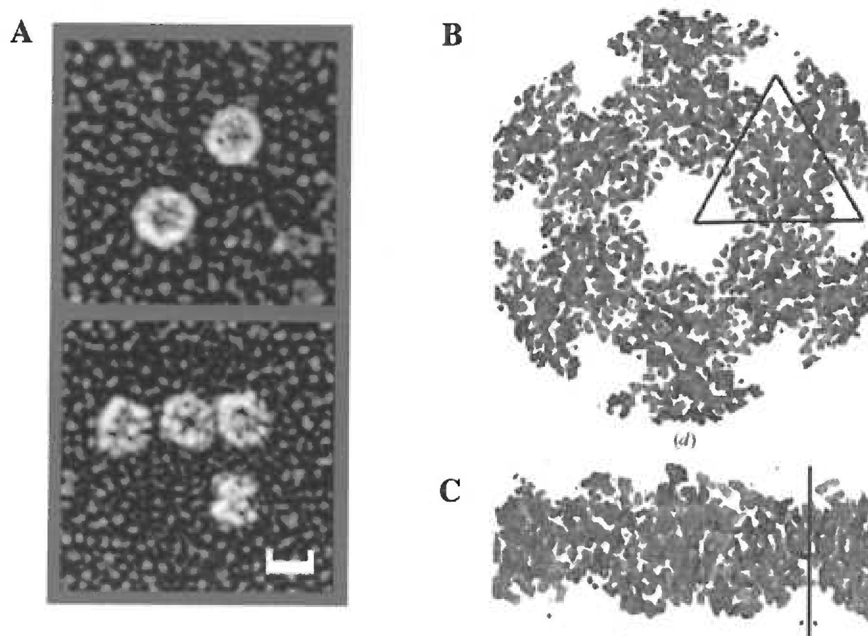

Figure 1-4 – Ultrastructure of the PilT ATPase (Forest *et al.*, 2004)

(A) *N. gonorrhoeae* PilT hexamers as visualized by freeze-etch microscopy.
(B) & (C) *A. aeolicus* PilT electron density map with a single PilT monomer shown within the drawn triangle. (B) represents an aerial view of the hexamer, while (C) shows a side-on view.

Twitching Motility and Pilus Retraction

The process of Tfp extension, adhesion and retraction allow bacteria to crawl along solid surfaces, a process known as twitching motility (Henrichsen, 1972). One model as to

how this might work is shown in Figure 1-5 (Merz, 2002). Pilus extension mediated by PilF elongates the pilus, allowing it to reach out in front of the bacterium (Figure 1-5A). The tip of the pilus then adheres to the surface of the substrate (Figure 1-5B). Retraction of the pilus subsequently drives forward movement, as the bacterium is pulled toward the anchored tip (Figure 1-5C). Live-cell microscopy has revealed these movements to be short and jerky, thus the bacteria are said to be "twitching".

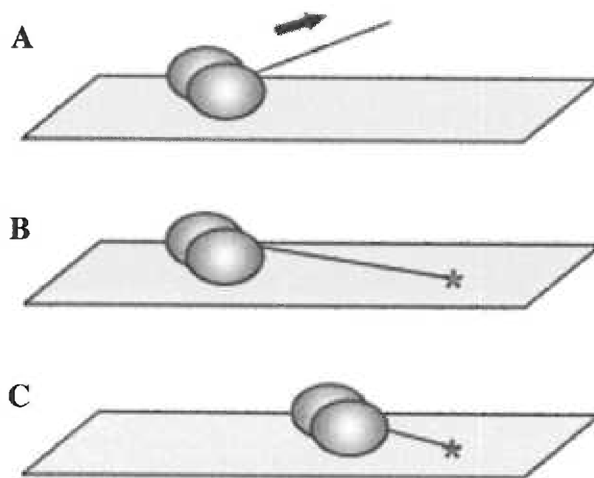

Figure 1-5 – The grappling hook model of twitching motility

(A) The pilus fiber extends via PilF mediated assembly of pilin subunits.

(B) The fiber binds to a substrate or another cell

(C) The fiber retracts via PilT mediated disassembly of pilin subunits. This pulls the bacterium toward the point of fiber attachment

The relationship between twitching motility and pilus retraction was originally demonstrated using phage studies in *P. aeruginosa* (Bradley, 1972a, b). It was observed that phage susceptible bacteria had numerous phage attached to the bacterial cell body, and that many of these phages were specifically concentrated at the base of the pili. In contrast, a nonsusceptible mutant had phage uniformly covering its pili, but very few phage on the cell body itself. It was also observed that these nonsusceptible mutants did not undergo twitching motility. Together these data led to the hypothesis that the pili "retracted" into the cell body, and that the mutant was not capable of retraction (Bradley,

1972a, b, 1974). Later the lesion responsible for the nonsusceptible phenotype was mapped to the *pilT* locus (Whitchurch *et al*., 1991).

Following these studies, a *pilT* homologue was identified in *N. gonorrhoeae* (Brossay *et al.*, 1994). Gonococcal *pilT* mutants were found to be hyperpiliated, and adhered to substrates better than wild-type. In addition, *pilT* mutants were not motile (Wolfgang *et al.*, 1998a). Finally, this mutant was not competent for DNA uptake, a process that is known to be dependent on pilus expression. These and other data suggested that the Tfp of *N. gonorrhoeae* was retractile, that retraction required *pilT*, and that retraction was responsible for motility and DNA uptake.

Recently, three elegant studies have provided conclusive evidence that Tfp physically retract. First, experiments using *M. xanthus* demonstrated that the bacteria move by: (1) attaching to a surface in an endwise fashion, (2) laying down parallel to the surface, and (3) moving over the surface from the initial position of contact by flipping end over end. (Sun *et al.*, 2000). Importantly, while *M. xanthus pilT* mutants retained the ability to undergo the initial tethering step, they were unable to lie down or move from the tethered position, implicating PilT in this process. Using a different approach, pilus retraction was directly observed in *P. aeruginosa* by labeling the pili with a fluorescent dye and visualizing retraction in real-time using total internal reflection microscopy (Skerker and Berg, 2001). Finally, laser tweezers experiments were employed to examine pilus retraction in *N. gonorrhoeae* (Merz *et al.*, 2000). As with *M. xanthus*, the *pilT* mutant was unable to retract its pili. In this experiment, not only was pilus retraction directly

captured by video microscopy, but the force of retraction was determined as well. This force varied between 20-100pN per retraction event, making PilT the strongest molecular motor known to date (Maier *et al.*, 2002; Merz *et al.*, 2000).

Type IV Pilus Mediated Signaling

Tfp play key signaling roles during infection of the host epithelium, as they are the first part of the bacteria to contact the host cell. At least three specific receptors for Tfp have been identified: Membrane Cofactor Protein (MCP or CD46) (Kallstrom *et al.*, 1997), I-domain containing integrins (Edwards and Apicella, 2005), and complement receptor 3 (CR3) (Edwards *et al.*, 2001; Edwards *et al.*, 2002). Through these receptors, and other specific or non-specific interactions with the host cell membrane, the pilus initiates signaling cascades that are necessary for the infection process. While some of these signals are dependent solely on pilus binding, others are stimulated specifically by retractile pili.

Signaling through Pilus Receptors

Of all three pilus receptors known to date, CD46 has been the most studied with regard to *N. gonorrhoeae*-induced signaling (see Figure 1-6). Interactions between the gonococcal pilus and CD46 results in activation of Src kinase c-Yes within 5 minutes of infection (Lee *et al.*, 2002). Activated c-Yes is subsequently recruited to the site of bacterial attachment, and is thought to mediate the phosphorylation of the CD46 cytoplasmic tail. The importance of these signaling events to infection is emphasized by the observation that gonococcal adherence to cells pre-treated with a Src-kinase inhibitor is decreased by

~50%. Pilus binding to CD46 has also been demonstrated to induce calcium signaling within infected cells (Kallstrom *et al.*, 1998). This retraction independent calcium flux requires intracellular calcium stores, and occurs ~10 minutes following bacterial attachment. Importantly, if the intracellular calcium stores are depleted, then pilus-mediated attachment is attenuated (Kallstrom *et al.*, 1998). Retraction of the gonococcal Tfp during bacterial colonization induces an extended calcium flux, however it has yet to be determined if this is mediated through CD46 (Ayala *et al.*, 2005). Other retraction dependent CD46 signals include CD46 recruitment to cortical plaques (Edwards *et al.*, 2000; Gill, 2004 and Weyand and So, submitted), and CD46 shedding from *N. gonorrhoeae* infected cells (Gill *et al.*, 2003). The requirements for these retraction-induced effects with regard to gonococcal infection have yet to be determined.

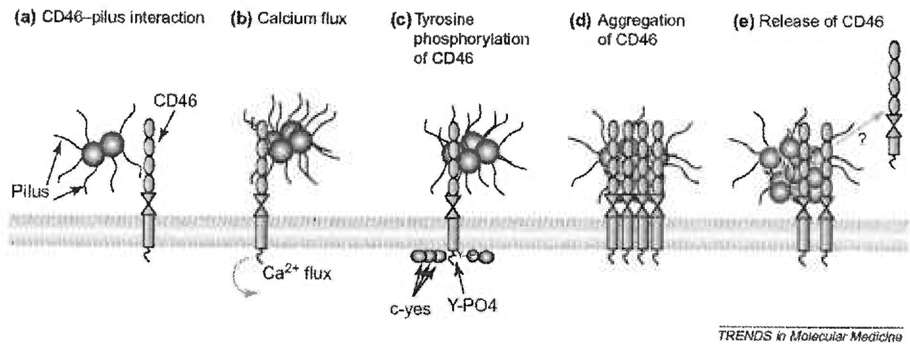

Figure 1-6 – CD46-dependent host cell responses to *N. gonorrhoeae* adherence (Gill and Atkinson, 2004)

Tfp adhesion to CR3 was only recently discovered, therefore the signaling pathways stimulated by this interaction have not been elucidated in detail. Moreover, CR3 is only expressed on cervical epithelial cells, so assumptions with regards to it function in general gonococcal infection cannot be made. Nonetheless, it has been shown that during

infections of primary cervical epithelial cells, pilus interaction with CR3 induces membrane ruffling and bacterial internalization via phagocytosis. As CR3-mediated phagocytosis occurs independently of an inflammatory response (Caron and Hall, 1998), this may provide yet another explanation as to why gonococcal infections in women are so often asymptomatic.

Pilus binding to I-domain containing (IDC) integrins is an even more recent discovery. These membrane proteins were shown to be the initial docking site for piliated bacteria during infection of urethral epithelial cells. While the signaling events downstream of the pilus-integrin association are unknown, it is interesting to note that integrins can oligomerize with both CD46 (Rezcallah *et al.*, 2005) and CR3 (Edwards *et al.*, 2002), Thus, these receptors may cooperate to induce the signaling pathways described above. Moreover, integrins are well known for their role in sensing mechanical force, and thus may play a role in transducing pilus-retraction mediated membrane tension into host cell signals. Overall, Tfp binding may stimulate a number of signaling cascades depending on which receptors, or combination of receptors they bind, and the cell type that is being infected. The influence of mechanical stress, the subject of this thesis, lends yet another variable to Tfp-mediated signaling.

Other Pilus Stimulated Cellular Effects

Most of the pilus-dependent signaling events discovered to date have been analyzed independently of specific pilus-host cell receptor interactions. One of the earliest described effects was that of induced protein clustering underneath the site of bacterial

attachment. These structures of recruited proteins, termed cortical plaques, are enriched in cytoskeletal components, transmembrane glycoproteins, and nonreceptor kinases and their anchors (Merz and So, 1997; Merz *et al.*, 1999). Transmembrane proteins include ICAM1 and signaling receptors EGFR, CD44 CD46 and CD66. Kinases/anchors include cYes/YAP (Lee *et al.*, 2002, Lee and So, unpublished results), PI-3 kinase (Lee *et al.*, 2005) and PKA/AKAP250 (Lee, Weyand and So, unpublished). Cytoskeleton components include cortical actin and ezrin, which tethers its cargoes to the actin cytoskeleton. Plaque formation begins within minutes of bacterial adhesion, and appears strikingly pronounced within 3-4 hours. Importantly, fulminate plaque formation requires both Tfp and a functional PilT, suggesting that recruitment is induced by retraction-mediated membrane tension (Merz *et al.*, 1999). The known functions of this diverse group of recruited proteins suggests that these plaques act as a signaling center during bacterial infection.

Pilus mediated adherence to the host epithelium has also been shown to induce gene expression changes. Plant *et al.* used a limited microarray containing 375 genes representing cell surface proteins and inflammatory factors to determine gene expression changes induced by piliated or non-piliated gonococci after 2 or 6 hours of infection (Plant *et al.*, 2004). Piliated bacteria induced changes in 10 genes: E-Cadherin, P-Cadherin, VEGF, EphA2, EphA4, β_1-integrin, IL-2Rα, DR-6, IGF binding protein 7, and ErbB1. It was suggested that these changes may be crucial for pilus mediated attachment and the initiation of the invasion process. A more recent study found that meningococcal Tfp induces expression of type-IIA secreted phospholipase A(2) (sPLA(2)-IIA) in

alveolar macrophages via an NF-KB dependent process (Touqui *et al.*, 2005). This gene product is known to normally be induced by LPS from other bacteria and has potent microbiocidal activity (Alaoui-El-Azher *et al.*, 2002; Arbibe *et al.*, 1997; Attalah *et al.*, 2003; Murakami *et al.*, 1997; Touqui and Alaoui-El-Azher, 2001; Weinrauch *et al.*, 1996). The consequence of sPLA(2)-IIA gene upregulation during Neisseria infection has yet to be investigated.

The most recently identified Tfp-mediated signaling event is that of phosphoinositide-3 kinase/Akt pathway activation (Lee *et al.*, 2005). As with cortical plaque formation, the activation of PI-3 kinase/Akt is dependent on a functional PilT. Importantly, this pathway was shown to induce PI(3,4,5)P3 recruitment beneath gonococcal microcolonies, and subsequently translocate the lipid to the outer surface of the host cell plasma membrane. Once on the cell surface, PIP3 appears to be "sensed" by the bacteria and functions to accelerate bacterial aggregation and increase *pilT* expression. These effects have been hypothesized to increase the amount of force induced on the host cell, by increasing the number of bacteria within a microcolony, and by increasing the rate of retraction due to higher numbers of PilT motors.

Evidence of Retraction-Mediated Mechanical Stress

Many of the above mentioned signaling cascades are activated and/or enhanced by pilus retraction, and thus provide indirect evidence for pilus-induced membrane tension. Cortical plaques closely resemble focal adhesions that are formed specifically by mechanical forces applied to the plasma membrane during cell-cell and cell-substrate

attachment (Choquet *et al.*, 1997; Sheetz *et al.*, 1998; Wang *et al.*, 1993). These adhesive plaques, like retraction-induced cortical plaques, are enriched in cytoskeletal proteins, transmembrane glycoproteins, and non-receptor kinases. Mechanical stress is also a known stimulator of PI-3 kinase activation (Miao *et al.*, 2002; Raucher *et al.*, 2000), calcium transients (Glogauer *et al.*, 1995; Wu *et al.*, 1999), gene expression changes (Feng *et al.*, 1999; McCormick *et al.*, 2003; Ohki *et al.*, 2002; Wasserman *et al.*, 2002), and microvillus elongation (Raucher and Sheetz, 2000; Shao *et al.*, 1998). All of these pathways have been demonstrated to be induced in a PilT-dependent manner. It is therefore tempting to speculate that these responses are in fact due to pilus retraction-mediated mechanical force. Direct evidence for such force induction by Tfp, however, has yet to be demonstrated.

Mechanical Forces

Forces Generated by a Bacterial Microcolony

Given the range of forces induced by a single Tfp retraction event, we can begin to estimate the forces generated by a single bacterium, and by a microcolony during the early stages of infection. IMPORTANTLY, these calculations are based on assumptions, and are used only as a crude way to estimate the range of forces that might be induced by a microcolony, thus allowing us to compare these hypothetical values to physiological forces induced within the human body. Until we have a better understanding of how pilus retraction occurs within a microcolony, it is impossible to verify the validity of our assumptions. For a detailed explanation of how these values were derived, please see Appendix 3 of this thesis.

We estimate (conservatively) that an average gonococcus contains approximately 10 pili. If we assume that each pilus is in contact with the host cell, all pili retract in unison, and that each pilus pulls with a force of ~100pN, the force induced by a single bacterium can reach 10^3pN. The diameter of a single gonococcus is ~1μm^2. Assuming that this is the area in contact with the host cell, then the above force is equivalent to 0.1N/cm^2.

From this, we can estimate the force induced by a small microcolony of ~100 gonococci. In each microcolony, roughly 1/3 of the bacteria are predicted to be in contact with its substrate, thus in a microcolony of 100 bacteria, we can assume that ~33 bacteria are contacting the host cell. Again assuming that each pilus is capable of inducing 100pN of force per retraction event, the microcolony as a whole could place forces of 3.3×10^4pN on the host cell membrane at any given time. Given that an average microcolony of this size is roughly 10μm in diameter, this is equivalent to a force 0.12N/cm^2.

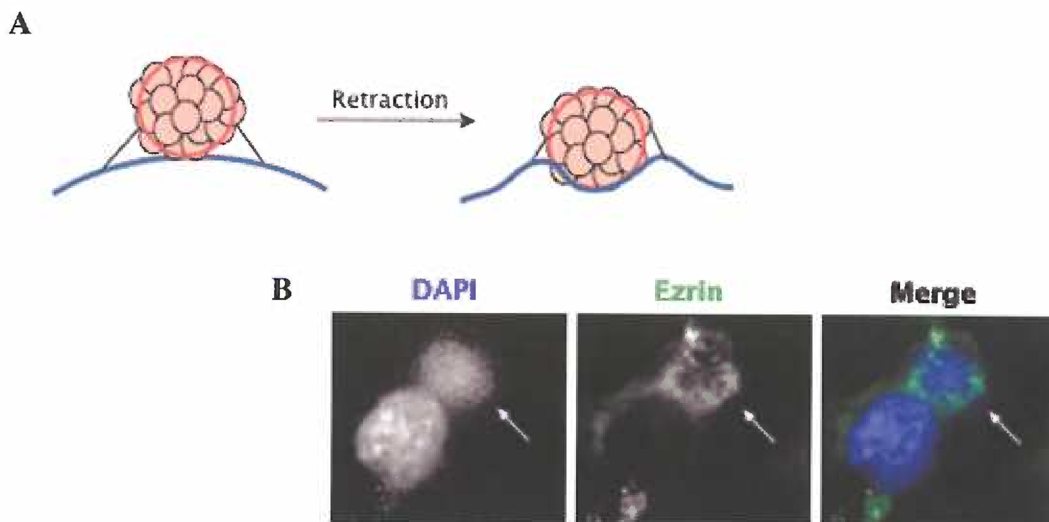

Figure 1-7 – Model of radial membrane tension (adapted from Lee, 2003)

(A) A gonococcal microcolony with extended pili contains an anchor point from which retraction generates substantial amounts of membrane tension, radially extended from the sphere of the microcolony.
(B) Example of radial protein recruitment around a microcolony at 3 hr post infection

This model can further be refined by taking into account the proposed nature of force induction with respect to the structure of the microcolony. If we assume the microcolony to be roughly spherical in shape, the tangent point of the "sphere" would be where the microcolony contacts the host cell. This area would serve as an anchor point, allowing pilus retraction from bacteria at the edge of the microcolony to generate membrane tension (see Figure 1-7A). Thus pilus retraction should generate tension at a radial circumference from the center of the microcolony (Lee, 2003). Indeed, evidence for such tension can be seen by the recruitment of ezrin to the circumference of a microcolony (Figure 1-7B). Assuming that each gonococcus is 1μm in diameter, and that the average diameter of a microcolony is 6μm across the base, the circumference of the microcolony is 19μm, or the equivalent to ~19 gonococci. As each gonococcus can pull with a force of 10^3pN, the force around the circumference of the microcolony can reach 1.9×10^4pN. Given the area around the circumference of the microcolony in which this force is applied, this is equivalent to 0.218N/cm^2. Interestingly, while the total force applied to the cell is the same as above (using an estimate of ~33 bacteria as being in contact with the host cell membrane), the force per area is approximately twice as high. Thus, if retraction from a microcolony can be focused at the circumference of the microcolony, the relative force applied to the cell is significantly higher.

Physiological Forces Experienced by the Body

The forces generated by a microcolony are within the range of physiological forces that various tissues in the body endure. For example, 10^4-10^5pN is the amount of force that is applied to integrin complexes in the periodontal ligament by a human bite (Glogauer and

Ferrier, 1998). Similarly, during a contraction of the human heart, the wall tension in the left ventricle reaches 3×10^5 dynes/cm^2 (Douglas *et al.*, 1987) or 3 N/cm^2 (1×10^5 dynes ~ 1 N). Table 1-1 shows a list of the forces required to mediate the mechanical stress induced signaling events within a cell, and how these forces compare with the calculated force of pilus retraction from a microcolony. Importantly, almost all of these signaling events can be triggered by the range of forces induced by retraction of a single pilus.

Physiological Event	**Force of Event**	**Reference(s)**
Integrin strain from human bite	10^4-10^5pN	(Glogauer and Ferrier, 1998)
Left ventrical wall tension of heart	3N/cm^2	(Douglas *et al.*, 1987)
Extraction of integral membrane prot.	20pN	(Evans *et al.*, 1991)
Microvillus elongation	30pN	(Shao *et al.*, 1998)
Calcium transients	2.6×10^{-4}N/cm^2	(Glogauer *et al.*, 1995; Wu *et al.*, 1999)
Focal adhesion plaques	3000pN	(Sheetz *et al.*, 1998; Wang *et al.*, 1993)
Single pilus retraction	20 - 100pN	(Maier *et al.*, 2002; Merz *et al.*, 2000)
Retraction from single gonococcus	1000pN or 0.1N/cm^2	(Howie and So, unpublished data)
Retraction from 6μm microcolony	3.3×10^4pN or 0.12N/cm^2	(Howie and So, calculated, this thesis)
Radial force from 6μm microcolony	1.9×10^4pN or 0.22N/cm^2	(Howie and So, calculated, this thesis)

Table 1-1 – Relative forces of physiological events

Thesis Overview

At the time that I began research for this thesis, retraction of the *N. gonorrhoeae* Tfp had just been demonstrated, and the associated forces quantitated (Merz *et al.*, 2000). Moreover, evidence of gonococcal pilus retraction-mediated mechanical stress had been accumulating. Cytoskeleton rearrangements in the form of cortical plaques (Merz *et al.*,

1999), calcium signaling (Ayala *et al.*, 2005), and PI-3 kinase activation (Lee *et al.*, 2005) had all been shown to be dependent on PilT.

As shown in Table 1-1, all three of the above events are known to be induced by membrane stress in the range of 100-3000pN. For purposes of comparison, a single retraction event can pull with a force of 100pN, and a microcolony is estimated to generate a force between 1.9×10^4 and 3.3×10^4 pN. With this amount of force placed on the host cell membrane during infection, we hypothesized that the cell response would be similar to those identified by other studies using artificial force application. One such response that had not yet been examined during Neisseria infection was the effect of pilus retraction on host cell gene expression.

Manuscript I describes the results of a microarray experiment that was designed to identify retraction responsive genes within the host cell. Surprisingly, pilus retraction, *per se*, did not regulate a unique set of genes. Rather, retraction *enhanced* the expression of a small subset of infection-regulated genes. The majority of these genes were known to be activated specifically by mechanical stress via MAPK activation. Indeed, I found that all three MAPK cascades (ERK, JNK and P38) are activated by *N. gonorrhoeae* infection and *enhanced* by pilus retraction. These genes are therefore termed gonococcus induced and retraction-enhanced, or GIRE. Importantly, I was able to recapitulate these results by employing a magnet and magnetic beads to artificially induce an upward pulling force. As many of the GIRE genes are known to function in anti-apoptotic signaling, I also tested the hypothesis that retraction-mediated mechanical force would

protect cells from apoptosis. I found that gonococcal infection protected host cells from staurosporine (STS) mediated apoptosis, and that this protection was enhanced by Tfp retraction. Moreover, artificially applied mechanical force was able to replicate this anti-apoptotic effect.

Manuscript II continues on this observation of GIRE cytoprotection, by examining the mechanisms involved. As activated ERK has long been documented as a pro-survival signaling kinase, I proposed that cytoprotection was mediated via this signaling intermediate. Indeed, inhibition of ERK prior to infection with either wild-type or *pilT N. gonorrhoeae* attenuated the ability of the bacteria to protect host cells from STS-induced apoptosis. Pro-survival signaling events downstream of ERK activation were then examined to identify the mechanism of ERK-mediated cytoprotection. Infection-mediated ERK activation was found to induce the down-regulation and/or modification of two BH3-only proteins, Bim and Bad. To examine if decreased levels of Bim and Bad were sufficient to protect cell from cytoprotection, siRNA was used to artificially lower the levels of each protein prior to induction of apoptosis by STS. Decreasing the levels of either protein attenuated apoptosis signaling. Moreover, I showed that this down-regulation of Bim and Bad blocks apoptosis by preventing the release of cytochrome c from the mitochondria into the cytosol.

The final chapter presents a discussion of the results from manuscripts I and II, as well as unpublished data presented in the appendices to this thesis. Potential mechanosensors for pilus retraction are discussed, such as the G-protein coupled receptor, LHR. In addition,

the idea of a "pushing force" (in relationship to the pulling force induced by pilus retraction) is introduced. Finally, the nature of pilus retraction from the microcolony structure is examined. Together these data and ideas should pave the way for others to investigate pilus-retraction mediated signaling to the host cell.

Chapter 2: Manuscript 1

The *Neisseria gonorrhoeae* Type IV Pilus Stimulates Mechanosensitive Pathways and Cytoprotection through a *pilT*-Dependent Mechanism

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Summary

The *Neisseria gonorrhoeae* type IV pilus is a retractile appendage that can generate forces near 100 pN. We tested the hypothesis that type IV pilus retraction influences epithelial cell gene expression by exerting tension on the host membrane. Wild-type and retraction-defective bacteria altered the expression of an identical set of epithelial cell genes during attachment. Interestingly, pilus retraction, per se, did not regulate novel gene expression but, rather, enhanced the expression of a subset of the infection-regulated genes. This is accomplished through mitogen-activated protein kinase activation and at least one other undefined stress-activated pathway. These results can be reproduced by applying artificial force on the epithelial membrane, using a magnet and magnetic beads. Importantly, this retraction-mediated signaling increases the ability of the cell to withstand apoptotic signals triggered by infection. We conclude that pilus retraction stimulates mechanosensitive pathways that enhance the expression of stress-responsive genes and activate cytoprotective signaling. A model for the role of pilus retraction in influencing host cell survival is presented.

Introduction

Many pathogenic and nonpathogenic bacteria produce type IV pili (Tfp), among them, *Neisseria gonorrhoeae*, *N. meningitidis, Pseudomonas aeruginosa, Legionella pneumophila,* enteropathogenic and enterohemorrhagic *Escherichia coli,* and *Vibrio cholerae* (Mattick, 2002). Tfp are fimbriate organelles that play a crucial role in the interaction of the bacterium with its environment, as evidenced by their requirement for motility (Wall *et al.*, 1999), biofilm formation (Bechet and Blondeau, 2003; O'Toole and Kolter, 1998), and horizontal gene transfer (Dubnau, 1999; Karaolis *et al.*, 1999; Yoshida *et al.*, 1999). These appendages also promote bacterial attachment to host cells and contribute to virulence (Bieber *et al.*, 1998; Comolli *et al.*, 1999; Merz *et al.*, 1999; Pujol *et al.*, 1999; Zolfaghar *et al.*, 2003).

Recent evidence has shown that the gonococcal Tfp can physically retract—a process that underlies twitching motility (Merz *et al.*, 2000) (i.e., the ability of the bacterium to move on solid surfaces (Henrichsen, 1983). It is now generally believed that twitching motility occurs via extension, substrate tethering, and retraction of the pilus filament. Two inner membrane/cytoplasmic ATPases, PilF and PilT, take part in these activities. PilF mediates pilus assembly, as *pilF* mutants produce pilin subunits but are not piliated (Freitag *et al.*, 1995). PilT is involved in pilus disassembly, as *pilT* mutants are piliated but cannot retract their pili (Merz *et al.*, 2000; Whitchurch *et al.*, 1991). Neither mutant is motile.

Pilus retraction allows gonococci to form organized microbial communities on the cell surface and on synthetic substrates (Lee *et al.*, 2005), via both specific and nonspecific interactions. During attachment to host cells, microcolonies stimulate the formation of cortical plaques—structures in the cell cortex containing high concentrations of transmembrane receptors, nonreceptor tyrosine kinases and their anchors, and components of the cortical cytoskeleton (Merz *et al.*, 1999; Merz and So, 2000). Though *pilT* mutants adhere normally to both synthetic surfaces and epithelial cells, they form disordered microcolonies, fail to induce cortical plaques, and are less invasive than their wild-type (wt) parent strain (Merz and So, 2000).

Retraction of a single gonococcal pilus can exert forces up to 80–100 pN on its substrate (Maier *et al.*, 2002; Merz *et al.*, 2000). Forces of lesser magnitude can elongate the membrane into microvillus-like structures (Raucher and Sheetz, 2000; Shao *et al.*, 1998), promote cytoskeleton rearrangements and protein clustering (Choquet *et al.*, 1997; Sheetz *et al.*, 1998), induce calcium fluxes (Glogauer *et al.*, 1995; Wu *et al.*, 1999), and alter gene expression (Feng *et al.*, 1999; McCormick *et al.*, 2003; Ohki *et al.*, 2002; Wasserman *et al.*, 2002). Pilus retraction has therefore been speculated to induce host cell signaling by exerting mechanical tension on the membrane (Merz and So, 2000). Indirect support for a mechanical signaling hypothesis comes from observations that *pilT* mutants, unlike wt piliated strains, can neither trigger cortical plaque formation (Merz *et al.*, 1999) nor activate PI-3 kinase (Lee *et al.*, 2005), a member of a mechanical stress–activated pathway. Moreover, a *pilT* mutant induces an attenuated calcium flux in epithelial cells, as compared to infection with wt gonococci (Ayala *et al.*, 2005). Here we provide further

evidence that pilus retraction acts as a mechanical stimulus by activating mechanical stress–signaling pathways that alter epithelial cell gene expression and generate a cytoprotective environment within the host cell.

Results

Pilus Retraction Enhances the Expression of Cell Stress/Survival Genes

We used microarrays to examine the transcriptional profiles of T84 human colorectal epithelial cells infected with retraction-proficient (N400) or retraction-deficient (N400*pilT*) gonococci for 3 h. Infection with N400 or N400*pilT* induced transcriptional changes in the same genes. Contrary to expectations, no genes responded uniquely to infection with either strain. Instead, infection with *pilT* affected the level of expression of a small subset of infection-responsive genes. To segregate the genes responding to pilus retraction, a wt to *pilT* fold-change expression ratio (W/P) was calculated for each infection-regulated gene. This method identified, out of approximately 300 infection-regulated genes, 69 probe sets (representing 52 genes) whose expression appeared to be enhanced by pilus retraction (Figure 2-1).

To confirm the microarray results, real-time quantitative RT-PCR was initially performed on two infection-regulated genes, *DUSP5* and *ADM*. According to our microarray data, *DUSP5* expression was enhanced by pilus retraction (W/P = 1.63), and *ADM* expression was not (W/P ≈ 1.0). RT-PCR results corroborated the microarray analysis, as *DUSP5* transcript levels were significantly higher in N400-infected cells than N400*pilT*-infected cells, whereas *ADM* transcript levels were similar in both sets of cells (Figure 2-2A). Ten additional genes predicted to respond to retraction and five additional genes predicted to be not affected by retraction were similarly analyzed by real-time quantitative RT-PCR

(Figure 2-2B). In every case, the presumptive positives yielded W/P ratios of 1.5 or more, whereas the presumptive negatives yielded W/P ratios of approximately 1.0.

Gene	Affy ID	GenBank	WT	*pilT*	W/P	p value
NR4A1	279_at	L13740	26.93	12.25	2.23	2.10E-06
NR4A1	280_g_at	L13740	23.43	13.10	1.81	2.03E-05
FOS	2094_s_at	K00650	22.53	11.80	1.88	5.09E-04
IL-8	1369_s_at	M28130	19.88	12.55	1.59	1.08E-05
DUSP1	1005_at	X68277	19.74	6.50	3.04	2.06E-06
IL-8	35372_r_at	M17017	19.16	12.75	1.52	1.59E-02
IL-6	38299_at	X04430	18.56	8.29	2.45	3.05E-07
DUSP5	529_at	U15932	16.58	10.25	1.63	7.28E-07
NR4A2	547_s_at	S77154	15.10	8.00	1.89	7.33E-02
EGR2	37863_at	J04076	13.32	8.60	1.63	3.65E-01
DTR	38037_at	M60278	12.77	7.63	1.69	4.16E-02
GRO1	408_at	X54489	9.39	5.78	1.63	1.74E-04
GRO2	37187_at	M36820	9.01	5.60	1.71	1.35E-05
TNFalpha	1852_at	X02910	8.33	3.32	2.56	1.94E-04
SOCS3	40968_at	AB004904	8.29	5.49	1.53	2.23E-01
JUNB	2049_s_at	M29039	8.29	5.66	1.46	1.62E-03
Id1	36619_r_at	S78825	8.18	4.98	1.63	1.08E-02
TCF8	33439_at	D15050	7.77	5.10	1.53	6.39E-03
GRO3	34022_at	M36821	7.73	5.18	1.53	9.01E-04
H2BFR	35562_at	AI076718	7.17	3.27	4.16	2.78E-02
H2AFO	32609_at	AI885852	6.64	2.63	2.55	3.07E-03
BLIMP1	31779_s_at	AF084199	6.15	4.25	1.59	9.40E-02
ATF3	287_at	L19871	5.88	3.47	1.68	1.06E-03
KRT16	601_s_at	M28439	5.44	2.61	1.89	2.07E-03
CSF2	1400_at	M13207	5.12	2.97	1.69	2.93E-03
NR4A2	37623_at	X75918	5.03	2.93	1.73	8.57E-02
CYR61	38772_at	Y11307	4.98	2.35	2.15	6.92E-07
H2AFO	286_at	L19779	4.79	2.17	2.22	1.10E-03
CSF2	1401_g_at	M13207	4.57	2.52	1.80	2.99E-03
DUSP2	1292_at	L11329	4.51	3.07	1.53	1.96E-03
EGR1	789_at	X52541	4.46	2.57	1.74	2.22E-03
H2AFA	35127_at	AI039144	4.38	1.86	2.38	2.40E-02
OVOL1	34047_at	AF016045	4.20	2.30	1.81	1.08E-03
uPA	37310_at	X02419	4.09	2.65	1.53	3.01E-03
GADD45B	39822_s_at	AF078077	3.89	1.96	2.01	3.97E-02

Gene	Affy ID	GenBank	WT	*pilT*	W/P	p value
GADD34	37028_at	U83981	3.66	2.56	1.47	6.29E-03
RHOB	1826_at	M12174	3.10	1.94	1.58	8.58E-03
	37538_at	AL049354	3.08	1.28	2.17	6.16E-02
HSP70-2	31692_at	M59830	3.00	1.59	2.02	1.92E-01
H1F2	37018_at	AI189287	2.86	1.81	1.64	6.68E-01
Id1	36618_g_at	X77956	2.83	1.81	1.58	1.17E-03
KIP2	38673_s_at	D64137	2.52	1.55	1.62	3.07E-03
HBP17	38489_at	M60047	2.47	1.71	1.48	2.32E-04
RHO6	37785_at	U69563	2.46	1.70	1.47	1.08E-03
MIP1A	36103_at	D90144	2.38	1.52	1.57	3.60E-03
HSPF1	752_s_at	D85429	2.32	1.54	1.59	1.24E-02
MEKK1	33009_at	AF042838	2.18	0.95	2.30	3.13E-02
KIP2	1787_at	U22398	2.04	1.30	1.57	4.31E-02
H3FB	34964_at	N35832	2.01	1.35	1.53	1.21E-01
	38207_at	AW006742	1.94	1.32	1.47	1.14E-01
E2F-2	37043_at	AL021154	1.63	1.04	1.57	5.06E-04
CYP1A1	1024_at	X02612	-1.75	-1.12	1.57	3.36E-02
FDZF2	38872_at	U95044	-1.94	-1.19	1.63	2.29E-01
	36070_at	AL049389	-1.94	-1.23	1.57	4.56E-03
ZNF253	35573_r_at	AF038951	-1.94	-1.23	1.57	2.28E-02
PLXNC1	32193_at	AF030339	-1.99	-1.24	1.59	4.50E-01
ALDH1A3	36686_at	U07919	-2.00	-1.28	1.57	1.09E-03
CYP1A1	1025_g_at	X02612	-2.01	-1.28	1.57	1.39E-02
CYP1A1	36767_at	K03191	-2.04	-1.35	1.52	1.60E-02
	40143_at	D50930	-2.23	-1.52	1.47	8.01E-03
SOCS5	32669_at	AB014571	-2.23	-1.24	1.80	5.29E-02
	34283_at	AL050125	-2.57	-1.32	1.94	1.05E-01
	40848_g_at	AB018293	-2.75	-1.81	1.52	5.37E-02
CBX6	39560_at	H10776	-2.86	-1.99	1.47	5.07E-01
SIM2	39608_at	U80456	-2.95	-1.87	1.57	1.05E-01
	41807_at	AL040137	[illegible]	-1.94	1.57	3.61E-02
EIF2C2	38505_at	AL050151	[illegible]	-2.04	1.57	1.82E-02
GRLF1	34724_at	AI670100	[illegible]	-1.63	2.18	9.81E-02
ZNF211	38142_at	U38904	[illegible]	-1.63	2.81	5.06E-02

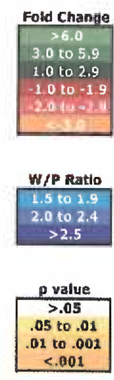


Figure 2-1. Infection-regulated, retraction-enhanced epithelial cell genes

Wt and *pilT* values represent the mean fold-change in the transcript level of each gene in infected cells compared to uninfected cells ($n = 2$). W/P values represent the degree of enhancement of gene expression resulting from pilus retraction and are the result of dividing the wt fold-change value by the *pilT* fold-change value from two independent experiments. The p-value for each gene represents the statistical significance of the difference in its expression level (as determined by Cyber-T analysis) between wt and *pilT*. The color code assigned to each gene represents its degree of response to infection as expressed by its fold-change value, W/P, and p-value.

The identification of genes whose expression is enhanced by pilus retraction raised the question of whether these genes share a common regulatory pathway or perform similar functions. The majority of genes whose expression is enhanced by retraction are involved in the cell stress response and survival (Figure 2-2C). Over half of these can be induced by environmental or other cellular stresses, and a striking number can be induced

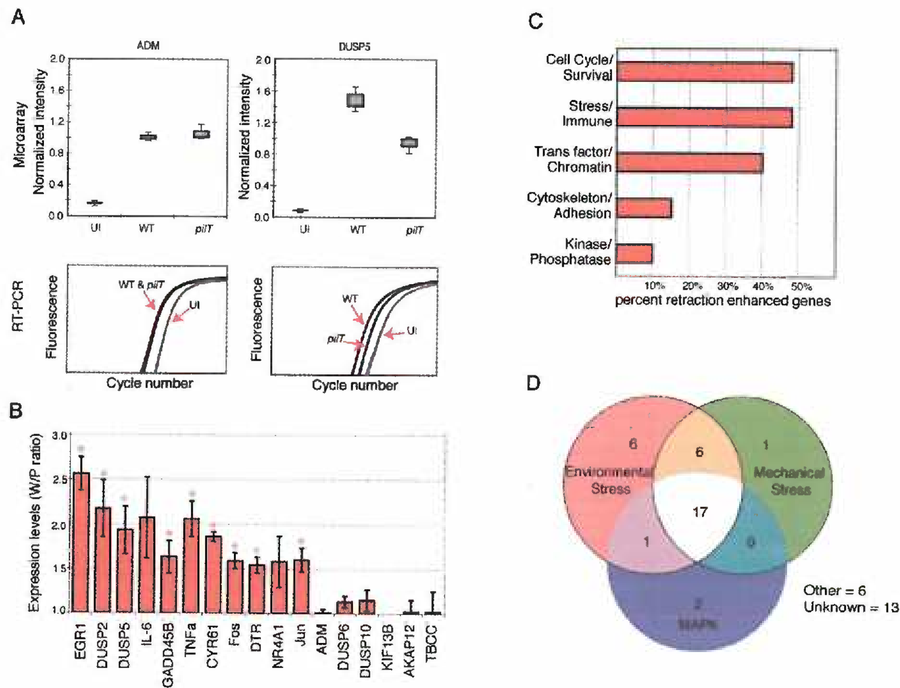


Figure 2-2. Real-time quantitative RT-PCR verification of microarray results and initial characterization of retraction-enhanced genes

(A) Microarray (top panels) and real-time quantitative RT-PCR (bottom panels) expression profiles of ADM and DUSP5 in uninfected cells (UI), N400-infected cells (WT), and N400*pilT*-infected cells *(pilT)*. Microarray data are shown as box-plots ($n = 3$). RT-PCR data are plotted as triplicate samples from one representative experiment.

(B) Real-time quantitative RT-PCR verification of retraction-enhanced expression of selected genes. Data are expressed as average W/P (±SEM, $n = 3$). Genes with a W/P statistically greater than 1.0 ($p < 0.05$) are denoted with an asterisk.

(C) Grouping of retraction-enhanced genes according to function, based on published reports (see Table S1). Some genes have multiple functions and thus appear in more than one group.

(D) Genes in this study that are known to be induced by environmental stress, mechanical stress, or MAPK signaling (see Table S1).

specifically by mechanical stress (Figure 2-2D). Importantly, the majority of the genes from both groups can also be induced by mitogen-activated protein kinases (MAPKs; Figure 2-2D) (For literature citations, see Table S1.) These results indicate that pilus retraction may enhance infection-induced gene expression through the MAPK pathway.

ERK, JNK, and P38 MAPK Are Activated by Infection and Enhanced by Pilus Retraction

The MAPK cascades are well known for their involvement in the stress response, including the response to bacterial infection. Previous studies have shown that JNK is activated in *N. gonorrhoeae*–infected HeLa, Chang, and phagocytic cells (Hauck *et al.*, 1998; Naumann *et al.*, 1998), and MAPK signaling is induced in conjunctival cells by *N. meningitidis* (R. Bonnah and M. So unpublished data). To study the role of MAPK signaling in retraction-enhanced gene expression, we first determined which of these pathways are activated in infected T84 cells.

Compared to resting cells (Figure 2-3A, left panel), the addition of medium alone slightly increased the levels of ERK-p, JNK-p, and P38-p (Figure 2-3A, UI), but levels of each phosphorylated kinase returned to baseline after 90 min. Infection with N400 dramatically increased the levels of all three activated kinases by 60 min post-infection (Figure 2-3A, WT). Densitometric analysis of immunoblots from two independent experiments is shown in Figure 3B. ERK-p levels were elevated throughout the course of infection, with only a slight decrease in phosphorylation visible by 3 h post-infection. In contrast, P38-p and JNK-p levels peaked between 60 and 90 min post-infection and dropped noticeably by 3 h post-infection.

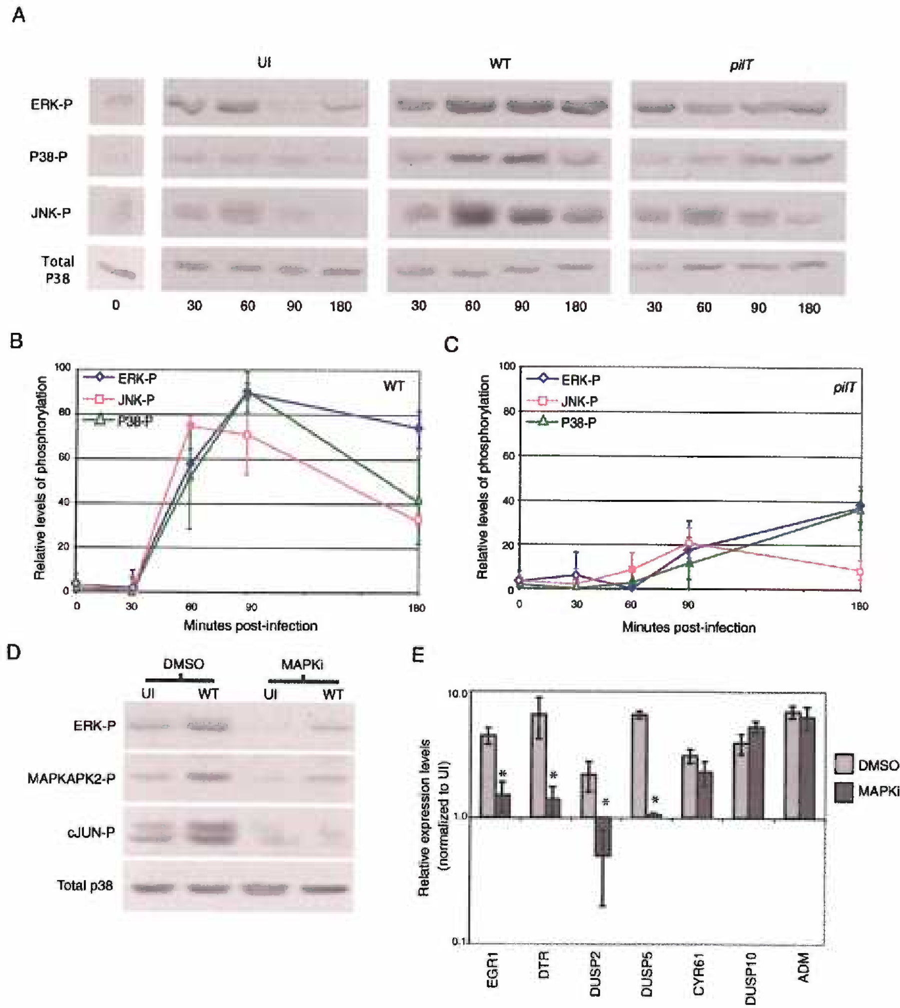


Figure 2-3. Levels of activated MAPK in infected cells and their involvement in retraction-enhanced gene expression

(A) Representative immunoblot showing ERK-p, P38-p, and JNK-p, in uninfected cells before (0 h) and after medium change (UI), or in cells infected with N400 (WT) or N400*pilT (pilT).* Total P38 protein levels in each sample served as the internal control (bottom lanes).

(B and C) ERK-p JNK-p, and P38-p levels over time in cells infected with N400 and N400*pilT,* respectively. Immunoblots from (A) were analyzed by densitometry, and levels of activated kinase from infected cells were normalized to that from uninfected cells (UI). Values represent mean normalized protein levels (±SEM, $n = 2$). Solid markers indicate a significant difference between wt and *pilT*-induced MAPK phosphorylation at that time point ($p < 0.05$); thus, ERK-p is significant at 60, 90, and 180 min; JNK-p is significant at 60 min; and P38-p is significant at 60 and 90 min.

(D) Representative immunoblot showing ERK-p, MAPKAPK2-p, and c-Jun-p in cells preincubated with vehicle (DMSO) or MAPK inhibitors and infected for 90 min with N400 (WT) or left untreated (UI). Total P38 protein levels in each sample served as the internal control (bottom lanes).

(E) Real-time quantitative RT-PCR analysis of the effect of MAPK inhibitors on the expression of retraction-responsive genes. Light bars indicate cells infected with N400 in the presence of vehicle (DMSO); dark bars indicate cells infected with N400 in the presence of MAPK inhibitors. Values represent the fold-change (±SEM, $n = 2$) in transcript levels compared to uninfected, DMSO treated control. A significant difference in expression between the two conditions is denoted by an asterisk ($p < 0.1$).

We next examined MAPK phosphorylation in T84 cells infected with N400*pilT* to determine whether kinase activation was influenced by pilus retraction. Low levels of all three activated MAPKs were detected in N400*pilT*-infected cells only after 90 min of infection (Figure 2-3A, PT). Densitometric analysis of immunoblots from two independent experiments is shown in Figure 2-3C. Although the kinetics of MAPK activation appear to be different in wt- and *pilT*-infected cells, a firm conclusion cannot be drawn from these results, given the delayed onset of activation and the low levels of phosphorylation of each enzyme. Taken together, these results demonstrate that infection by piliated gonococci activates all three MAPK pathways and that pilus retraction enhances this activation.

MAPK Signaling Is a Mediator of Retraction-Dependent Enhancement of Gene Expression

We next determined whether MAPK signaling regulates the expression of retraction-enhanced genes. T84 cells were preincubated with vehicle or MAPK inhibitors SB203588, U0126, and SP600125, and assessed for ERK, P38, and JNK activation by immunoblotting for ERK-p, MAPKAPK2-p, and c-Jun-p, respectively. MAPK inhibitors dramatically reduced the levels of all three activated kinases in both uninfected and N400-infected cells (Figure 2-3D). They also significantly reduced the transcript levels of four of the five retraction-responsive genes in N400-infected cells, as judged by real-time quantitative RT-PCR (Figure 2-3E). In contrast, the inhibitors did not affect the transcript levels of genes with a W/P of approximately 1.0. Interestingly, *cyr61* expression was unaltered by MAPK inhibitors. This gene was shown by microarray (W/P = 2.15) and

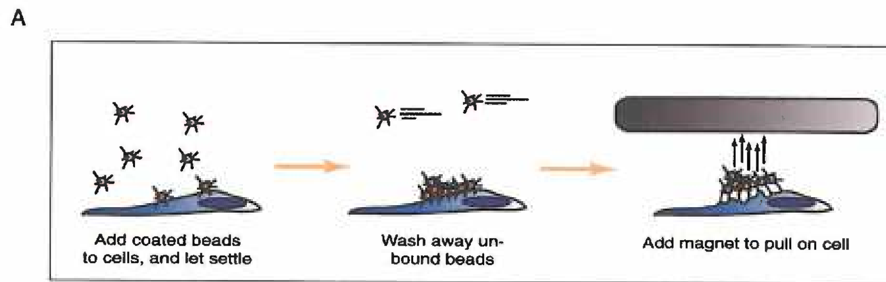
RT-PCR analysis (W/P = 1.86) to respond to retraction. These results implicate MAPK signaling in the regulation of some, but not all, retraction-responsive genes. They indicate that other pathways also influence the response of genes to pilus retraction.

Mechanical Stress Activates MAPK Signaling and Upregulates Retraction-Responsive Genes

A significant number of the retraction-responsive genes are known to be induced specifically by mechanical strain on the cell membrane. Although substantial force is generated by pilus retraction in vitro, this force has not yet been demonstrated to influence host responses to infection. To examine this issue, we determined whether artificial mechanical force on the epithelial cell membrane could mimic retraction-induced MAPK activation and retraction-enhanced gene expression. To generate mechanical stress in a manner similar to that of pilus retraction, a modified magnet-based force assay was used (Glogauer and Ferrier, 1998). Magnetic beads were coated with crude pili preparations (CPPs) from piliated gonococci and added to T84 cells (Figure 2-4A). Within 30 min, small clusters of approximately two to ten beads attached to the cells, with each cell containing two to three clusters of beads (data not shown). Cell monolayers were then placed 10 mm beneath the magnet. At this distance, the magnet generates an upward force of 4 pN per bead (Figure 2-4B), or approximately 20–100 pN per cell.

T84 cells seeded with CPP-coated beads and exposed to the magnet were first examined for the presence of actin recruitment into cortical plaques (see Introduction). The clustering of actin near these beads would indicate that the magnetic force was sufficient

A



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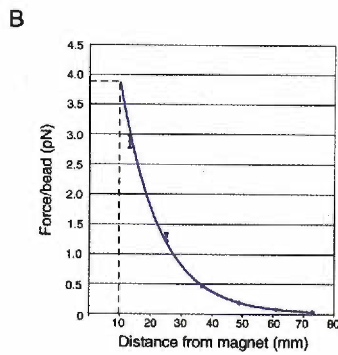

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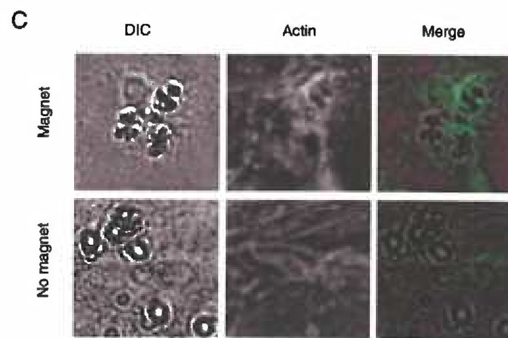

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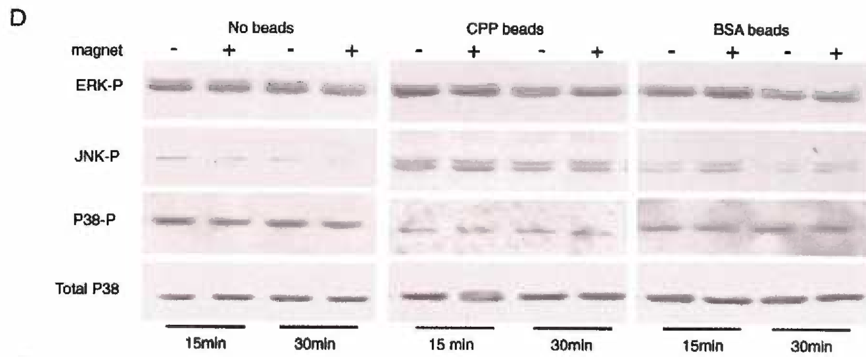

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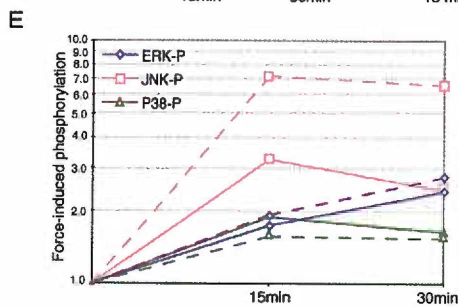

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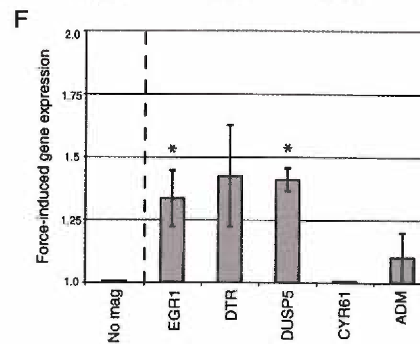

Figure 2-4. Artificial force triggers MAPK phosphorylation and induces the expression of retraction-enhanced genes

(A) Representation of the magnet/magnetic bead assay.

(B) Average force generated on one bead as a function of magnet distance from the culture dish. Data represent the forces calculated from four identical magnets (±SEM). All subsequent assays were performed using a magnet distance of 10 mm, which corresponds to a force of 4 pN per bead (dotted line).

(C) Magnet-induced clustering of actin beneath magnetic beads. CPP-coated beads were seeded onto T84 cells and exposed to the magnet for 1 h (top panels) or left untreated (no magnet, bottom panels). Differential interference contrast images (left panels) reveal the location of the beads; phalloidin staining (middle panels) shows the presence of actin at the same site. Right panels show the two previous images merged.

(D) Representative immunoblot of ERK-p, JNK-p, and P38-p in cells seeded with CPP-coated beads, BSA-coated beads, or no beads, and exposed to the magnet for 15 or 30 min. Total P38 protein levels in each sample served as the internal control (bottom panels).

(E) Quantitation of ERK-p, JNK-p, and P38-p signals by densitometry from the representative immunoblot shown in (D), normalized to the no-bead control. Solid lines indicate signals from cells exposed to membrane-coated beads; dotted lines indicate signals from cells exposed to BSA-coated beads.

(F) Real-time quantitative RT-PCR analysis of the transcript levels of selected genes in cells seeded with CPP beads and exposed to the magnet for 3 h. Data represent the average fold-change (±SEM, n = 2) compared to a no-magnet control. A significant difference in expression on force induction is denoted by an asterisk ($p < 0.1$).

to mimic pilus retraction forces from the bacterial microcolony. In the presence of magnetic force, actin concentrated in the cell cortex around membrane-coated beads (Figure 2-4C, top panel). In contrast, actin did not cluster with the beads in the absence of the magnet (Figure 2-4C, bottom panel). Thus, the force generated by this magnet system was sufficient to recruit actin to the site of the attached beads.

We next determined whether magnetic forces applied to CPP-coated beads were sufficient to activate MAPK and alter gene expression. The levels of all three phosphokinases were slightly reduced when the magnetic field was applied to cells

incubated with medium alone (Figure 2-4D, no beads). Levels of each phosphorylated kinase from bead-treated samples (Figure 2-4D, CPP) were normalized to those from the no-bead samples to account for the effect of the magnet alone on MAPK phosphorylation. Following normalization, increased levels of all three phosphokinases are evident within the short time course (Figure 2-4E, solid lines).

In parallel experiments, cells were seeded with Bovine Serum Albumin (BSA)-coated beads and exposed to the magnet. Under these conditions, less force was applied to the cells, as fewer bead clusters attached to the cells, and each cluster contained only two to three beads on average (data not shown). Again, levels of each phosphorylated kinase from bead-treated samples (Figure 2-4D, BSA beads) were normalized to those from the no-bead samples to account for the effect of the magnet alone on MAPK phosphorylation. Despite lower forces, BSA-coated beads also activated ERK, JNK, and P38 (Figure 2-4E, dashed lines). Interestingly, force-induced activation of both JNK and ERK was higher in cells treated with BSA-coated beads. This can most likely be attributed to the fact that BSA-coated beads, unlike CPP beads, induce no MAPK activation in the absence of force (data not shown). Thus when force-induced MAPK activation is calculated, the CPP-coated beads are normalized to a higher level of "background" activation than are the BSA-coated beads. The observation that force induction via both CPP- and BSA-coated beads can induce these signals strongly indicates that activation of MAPK cascades is, in part, a response to stress forces on the membrane rather than to force mediated through specific adhesin–receptor contacts between the bacterium and the host.

To examine the effect of mechanical stress on gene expression changes, cells seeded with CPP-coated beads were exposed to magnetic force for 3 h, and gene expression levels were analyzed by real-time quantitative RT-PCR. Transcript levels were expressed as the ratio of signals from magnet-stimulated cells to those from cells not subjected to magnetic force. All three "enhanced" genes tested, *EGR1, DTR,* and *DUSP5,* were upregulated in cells exposed to magnetic force (Figure 2-4F). In contrast, neither *ADM* (W/P $\approx$ 1.0) nor *cyr61* (which did not respond to MAPK inhibitors; see Figure 2-3E) was affected by the magnet.

In this and the previous experiment, magnet-induced changes were of lower magnitude than those induced by infection. The most plausible explanation for this difference is that pilus retraction from a microcolony likely generates greater force than a magnet acting on a small cluster of beads. In our magnet assay, an average force of 20–100 pN was placed on each cell. During an infection, each *pilus* can induce this amount of force. Thus, if there are 10–100 bacteria per microcolony, and each bacterium expressed 10 pili (a conservative estimate), pilus retraction from a single microcolony could place forces of 10^4–10^5 pN on the cell. Nonetheless, our method of artificial force application did indeed activate all three MAPK cascades and increased the expression level of each gene examined by approximately 1.5-fold. (Note that a minimum 1.5-fold change in expression level was found to accurately identify retraction-responsive genes in the microarray experiment.) Together, these results demonstrate that retraction-enhanced MAPK activation and gene expression changes can be replicated by artificial force.

Pilus Retraction Mediates Host Cell Cytoprotection

Many of the retraction-responsive genes are known to protect cells from apoptosis and from a variety of cellular stresses. Moreover, prolonged ERK activation accompanied by transient JNK and P38 activation (as observed in a wt infection; see Figure 2-3A and 2-3B) is hypothesized to mediate cytoprotection (Davis, 2000; Lin, 2003; Roulston *et al.*, 1998; Wada and Penninger, 2004). We therefore investigated whether pilus retraction was involved in determining cell fate by assaying infected cells for cleaved poly(ADP-ribose) polymerase (PARP) and cleaved caspase 8. PARP is a 116-kDa nuclear protein that mediates DNA repair in response to cell stress and is required to maintain cell viability (Oliver *et al.*, 1998; Satoh and Lindahl, 1992). During programmed cell death, the protein is cleaved by caspase 3 or caspase 7, a terminal step in the caspase cascade (Nicholson *et al.*, 1995; Tewari *et al.*, 1995). Caspase 8, however, is an initiator caspase that is upstream of caspase 3, caspase 7, and PARP, and represents an earlier event in the apoptosis cascade. Thus, increased levels of cleaved PARP or caspase 8 indicate that a cell is undergoing apoptosis.

Cells infected with N400 for 6 h contained lower levels of both cleaved PARP and cleaved caspase 8 than did uninfected cells (Figure 2-5A). In contrast, N400*pilT*-infected cells had higher levels of cleaved PARP and cleaved caspase 8 than did both uninfected and wt-infected cells. These results indicate that piliated gonococci that cannot retract pili induce low levels of programmed cell death in a culture. In contrast, gonococci capable of retracting their pili lower the tendency for cells to enter the apoptosis pathway.

We next determined whether this cytoprotective effect of pilus retraction was sufficient to protect cells from staurosporine (STS)-induced apoptosis. STS is a cell-permeant protein kinase inhibitor that induces apoptosis at micromolar concentrations (Couldwell *et al.*, 1994; Yue *et al.*, 1998). Infection of urethral epithelium with *N. gonorrhoeae* was recently reported to protect these cells from STS-induced apoptosis (Binnicker *et al.*, 2003). Both N400 and N400*pilT* infection protected T84 cells from STS-induced

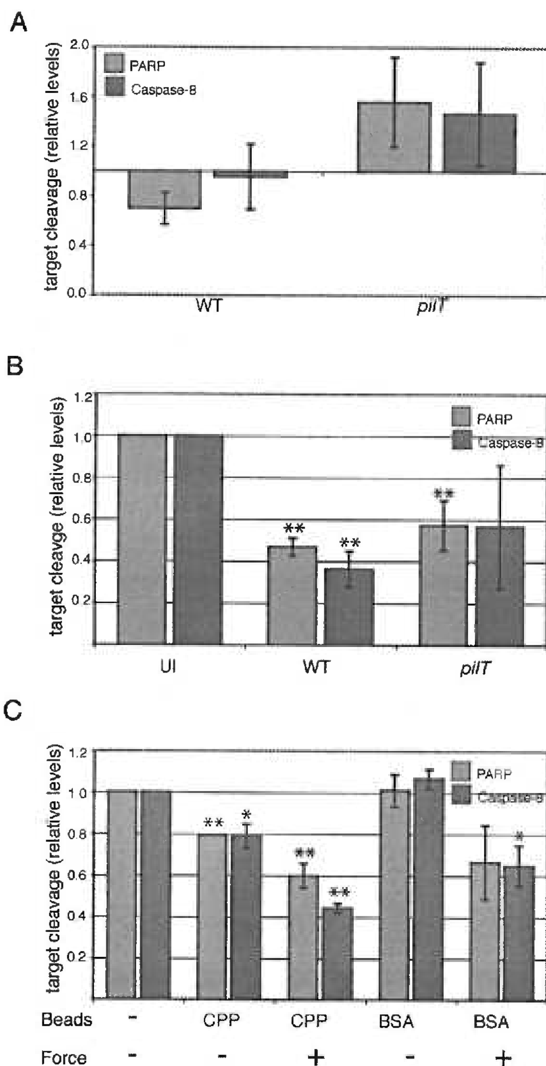

Figure 2-5. Pilus retraction during bacterial attachment promotes host cell cytoprotection

(A) Levels of cleaved PARP and cleaved caspase 8 in T84 cells infected for 6 h with N400 (WT) or N400*pilT (pilT),* normalized to cleaved PARP or cleaved caspase 8 levels in uninfected cells.

(B) Levels of cleaved PARP and cleaved caspase 8 in T84 cells infected with N400 (WT) or N400*pilT (pilT)* or left uninfected (UI) for 4 h, then incubated with STS (1 μM) for an additional 4 h to induce apoptosis. A significant difference from uninfected cells is denoted by two asterisks ($p < 0.05$).

(C) Cleaved PARP and cleaved caspase 8 levels in cells exposed to magnetic force. T84 cells were seeded with CPP- or BSA-coated beads and exposed to the magnet for 2 h or were left unexposed, then incubated with STS (1 μM) for an additional 4 h away from the magnet. The cleaved PARP and cleaved caspase 8 level in cells without beads and not exposed to magnetic force is arbitrarily assigned a value of 1.0, and all other treatments are expressed relative to this value. For all experiments, cleaved protein levels were quantified by densitometry of immunoblot signals. Values represent the mean levels of cleaved target (±SEM) from two independent experiments. A significant difference from untreated cells is denoted by two asterisks ($p <$ 0.05) or by a single asterisk ($p < 0.1$).

apoptosis, as compared to uninfected cells (Figure 2-5B). However, cleaved PARP and cleaved caspase 8 levels in N400*pilT*-infected cells were higher than in wt-infected cells, indicating that pilus retraction enhances protection from STS-induced apoptosis.

Finally, we examined whether this retraction-enhanced cytoprotection is specifically mediated by mechanical force. In the absence of force, CPP-coated beads provided moderate protection from STS-induced apoptosis, demonstrated by lower cleaved PARP and cleaved caspase 8 levels than the no-bead cell control (Figure 2-5C). This result is similar to that seen in *pilT*-infected cells and suggests that components in the bacterial membrane are sufficient to protect against STS-induced apoptosis. Cells seeded with CPP-coated beads and exposed to the magnetic field had still lower cleaved PARP and cleaved caspase 8 levels, consistent with data from wt-infected cells (Figure 2-5B). BSA-coated beads did not protect against STS-induced apoptosis in the absence of magnetic force. However, when force was applied to these cells, the level of cleaved PARP and cleaved caspase 8 was reduced nearly to the value observed for membrane-coated beads in the presence of the magnet. Together, these data indicate that nonspecific membrane tension is capable of protecting the host cell against apoptosis.

Discussion

Retraction of the *N. gonorrhoeae* Tfp during bacterial attachment elicits host cell signaling cascades essential for the establishment of intimate attachment and promotion of bacterial invasion (Merz and So, 2000). We tested the hypothesis that Tfp retraction induces changes in epithelial cell gene expression during bacterial attachment. Pilus retraction, per se, did not regulate a unique set of genes. Rather, retraction enhanced the expression of a small subset of infection-regulated genes (see Figure 2-1), many of which are known to respond specifically to mechanical stress and to be induced by the MAPK cascade. We confirmed that wt bacteria activated MAPKs ERK, JNK, and P38 at a higher level than the *pilT* mutant. Moreover, MAPK inhibitors lowered the expression level of all but one retraction-responsive gene selected for further examination (see Figure 2-3). These results strongly indicate that MAPK signaling plays a major role in the enhancement of gene expression by pilus retraction.

Importantly, artificial force placed on the cell membrane using magnets and magnetic beads can replicate the gene expression changes and MAPK activation observed using wt bacteria, indicating that pilus retraction may induce these events via mechanical force. Although the total force produced by pilus retraction within a bacterial microcolony is not known, we estimate that it is on the order of 10^4–10^5 pN, based on 100 pN per retraction event, approximately 10 pili per bacteria, and roughly 10–100 bacteria per microcolony. In comparison, this amount of force is equivalent to that applied to integrin

complexes in the periodontal ligament by a human bite (Glogauer and Ferrier, 1998). Retraction forces from a microcolony could therefore be physiologically relevant.

We cannot exclude the possibility that pilus retraction enhances these signaling events by mechanisms independent of membrane tension (i.e., through secondary receptor engagement or via an inherent difference in the pilus structure/composition between wt and *pilT* bacteria). Our data with CPP-coated beads strongly argue against these possibilities, however. The CPP preps used for bead coating were from wt cultures, and thus were identical. In addition, the magnet pulled the beads upward. This should pull the bead farther from the cell surface, making secondary receptor engagement less likely. The possibility remains, however, that pilus differences or secondary receptor engagement may act in concert with membrane tension to generate the higher levels of MAPK activation and gene expression changes seen with infection. Further research is needed to examine this possibility. Nonetheless, we are confident that force plays at least some role in the signaling events identified through this work.

We have begun to assess the biological functions of enhanced gene expression and MAPK activation during gonococcal infection. ERK, JNK, and P38 play a role in determining cell survival during stress and entry into the apoptosis pathway (Wada and Penninger, 2004). Moreover, nearly half of the identified retraction-enhanced genes are known to be involved in cell cycle/survival signaling. We show that cells infected with wt bacteria have lower levels of cleaved PARP and cleaved caspase 8 than do uninfected and *pilT*-infected cells. Pilus retraction is therefore predicted to enhance the ability of the

cell to withstand apoptosis-inducing signals generated by infection. Indeed, cells infected with wt bacteria withstood STS-induced apoptosis better than uninfected cells and cells infected with *pilT*.

The effect of *N. gonorrhoeae* infection on cell fate has been a long-standing controversy. The neisserial porin has been reported to protect cells from apoptosis (Massari *et al.*, 2003) as well as to induce programmed cell death (Muller *et al.*, 1999). These conflicting observations are likely a result of differences in experimental systems and bacterial strains. We believe that our results may clarify the issue of *N. gonorrhoeae* and programmed cell death, through the identification of another bacterial factor (i.e., pilus retraction) involved in such a response.

A number of factors influence the ability of the cell to withstand apoptosis, including the signaling cascades that are activated and the degree and duration of the activation of these cascades (Wada and Penninger, 2004). They also include the virulence genes expressed by the infecting bacteria. The bacterial strains used for previous studies on *N. gonorrhoeae* and apoptosis differed in their piliation state and their ability to invade the host cell. Our results indicate that piliated bacteria, in the absence of pilus retraction, slightly increase the tendency of the infected cell to undergo apoptosis (see Figure 2-5A). However, these bacteria are still able to moderately protect infected cells from STS-induced apoptosis, indicating that a certain level of cytoprotection is provided by other bacterial factors. In contrast, bacteria that can retract their pili, and thus presumably

induce mechanical stress on the host-cell membrane, strongly mediate pro-survival signaling.

The influence of mechanical stress on apoptosis has been studied in some detail. Importantly, such studies indicate that different stress patterns result in different cellular outcomes. Extended, repetitive mechanical force increases the expression of genes encoding cytoprotective heat shock proteins and lowers the number of apoptotic cells in a culture (Barkhausen *et al.*, 2003). Suppression of apoptosis requires permanent membrane tension or rhythmic, pulsatile forces (Graf *et al.*, 2003), which are thought to allow the cell to adapt to new environmental conditions. Retraction events in *N. gonorrhoeae* generate strong, pulsatile forces every 1–20 s (Merz *et al.*, 2000). The nature of the pilus retraction force may therefore be the key to counteracting infection-induced apoptosis. Our data strongly indicate that pilus retraction from a microcolony is capable of stimulating mechanoprotective signals.

In light of the results presented here and elsewhere, we propose a model to explain how pilus retraction by *N. gonorrhoeae* influences survival signaling in the infected cell (Figure 2-6). Initial contact between the bacterium and the epithelial cell activates MAP kinases and alters gene expression at a low level. The cell senses “stress” from the infection, the degree of which varies depending on the metabolic state of the cell and the constellation of virulence factors expressed by the infecting strain. As a result of this stress, the cell is poised to enter the apoptosis pathway. In the absence of pilus retraction and membrane tension, the low levels of activated MAP kinases may or may not be

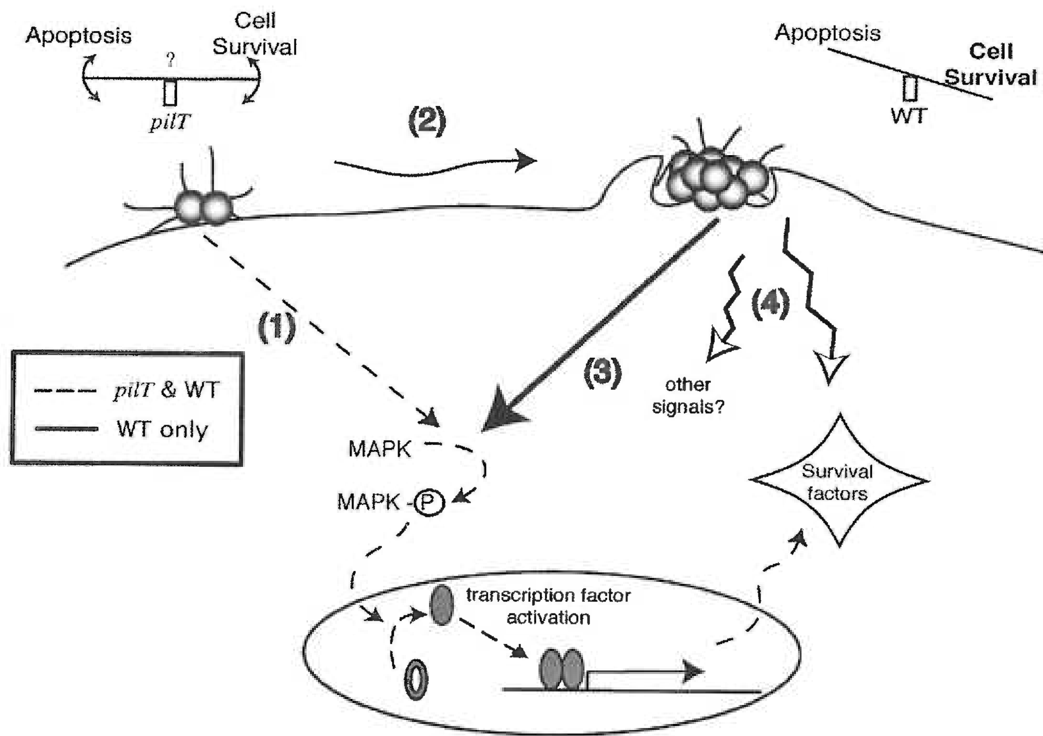

Figure 2-6. Model of the role of pilus retraction in promoting a cytoprotective environment during gonococcal infection of an epithelial cell

(1) Initial contact between the bacterium and host cell activates low levels of MAPK, and transcription of infection-induced genes. This level of signaling may or may not be able to protect the cell from apoptosis; thus, the host cell "teeters" on the edge of life and death.

(2) As the infection proceeds, microcolonies of gonococci are formed, and more pili are locally available to retract.

(3) Pilus retraction amplifies MAPK activation, which in turn enhances the transcription of mechanical stress–induced genes.

(4) Pilus retraction may also stimulate other pathways that mediate gene expression and survival signaling. Overall signaling events tip the balance in favor of cell survival.

enough to counteract this stress. As the infection proceeds, microcolonies are formed. Pilus retraction from microcolonies is hypothesized to exert stress forces on the membrane, amplifying the levels of activated MAPK, enhancing the transcription of infection-induced genes, and possibly activating other as-yet-unidentified pathways. The end result is the enhanced stimulation of pro-survival pathways and an overriding of pro-

apoptotic stress signals. In other words, the fate of the infected cell is decided by the type of signaling networks induced by infection and the extent of activation of these networks. Pilus retraction tips the balance in favor of cell survival.

We have used a tissue culture system to study the interplay between pilus retraction, host cell signaling, and gene expression during the attachment phase of *N. gonorrhoeae* infection. How these interactions may affect the disease in vivo remains to be clarified. Our results make teleologic sense when the bacterial life cycle and gonococcal disease are taken into consideration. *N. gonorrhoeae* does not survive on fomites and has no intermediate host. Transmission depends on person-to-person spread. Simple mucosal gonorrhea infections can be mild, and inflammatory responses begin days after exposure (Morse, 1996). Moreover, a significant number of infected individuals carry gonococci without overt symptoms of disease (Morse, 1996; Turner *et al.*, 2002). Indeed, the ability of the bacterium to survive as a species requires a relatively healthy host. Our model for pilus retraction is consistent with these considerations.

Materials and Methods

Reagents.

Antibodies to PARP, caspase 8, c-Jun, phospho-c-Jun (Ser63), P44/42 MAPK, phospho-p44/42 MAPK (Thr202/Tyr204), phospho-MAPKAPK2 (Thr334), p38 MAPK, phospho-p38 MAPK (Thr108/Tyr182), SAPK/JNK, and phospho-SAPK/JNK (Thr183/Tyr185) were purchased from Cell Signaling Technology (Beverly, Massachusetts, United States). MAPK inhibitors SB203588, U0126, and SP600125 were purchased from Calbiochem (San Diego, California, United States) and used at a final concentration of 10 μM unless otherwise stated. STS was purchased from Cell Signaling Technology and used at a final concentration of 1 μM to induce apoptosis. Neodymium iron boron (NdFeB) magnets (Eneflux Armtek Magnetics, Bethpage, New York, United States) measured 2 in. in diameter by 1 in. thick and were grade 30 (MGOe).

Cell lines, bacterial strains, and infections.

T84 human colonic epidermoid cells (American Type Culture Collection, Manassas, Virginia, United States) were maintained in DMEM-F-12 plus 5% heat-inactivated, filter-sterilized fetal bovine serum at 37 °C and 5% CO_2. For all experiments, cells were seeded into 35-mm dishes and allowed to become confluent before infection. *N. gonorrhoeae* strains N400 and N400*pilT* (Wolfgang *et al.*, 1998b) were used for all infections and were maintained on GCB agar plus Kellogg's supplements at 37 °C and 5% CO_2. Piliation and Opa phenotypes were monitored by colony morphology. Only piliated, Opa$^-$

bacteria were used. For infection experiments, bacteria were resuspended in GCB liquid medium and added to the epithelial cells at a multiplicity of infection of 50.

RNA isolation and microarray analysis.

T84 cells were infected with N400 or N400*pilT* or treated with GCB medium alone for 3 h. For RNA isolation, labeling, and microarray hybridization procedures, see Protocol S1. Comparative analysis was performed using MAS 5.0 algorithms to determine fold-change values between uninfected and infected samples from the same experiment, with uninfected samples representing the baseline. Statistical analysis was performed on natural-log transformed data using Cyber-T (http://visitor.ics.uci.edu/genex/cybert/). Subsequent data analysis was performed using Excel (Microsoft, Redmond, Washington, United States) and GeneSpring version 4.0 (Silicon Genetics, Redwood City, California, United States). Genes with a "presence call" *p*-value of less then 0.1 across all chips were eliminated from analysis, as were genes that were given a "no change" call across all samples. A gene was identified as differentially regulated if the fold-change was greater than ±1.5 in at least two out of three experiments. "Enhanced" genes were identified by calculating the ratio of the fold-change for the wt-infected cells to the fold-change for the *pilT*-infected cells (W/P). Gene expression was considered to be enhanced by pilus retraction if the W/P, averaged from at least two out of three individual experiments, was greater than 1.5, and the individual W/P from each experiment was greater than1.25.

Real-time RT-PCR analysis.

One microgram of total RNA (as isolated above) was reverse-transcribed to generate cDNA, using the iScript cDNA synthesis kit (Bio-Rad, Hercules, California, United States). As a control, parallel samples were run in which reverse transcriptase was omitted from the reaction mixture. Quantitative real-time PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, California, United States). Amplification was carried out using TaqMan master mix (Applied Biosystems), and pre-designed TaqMan probes (Assays on Demand, Applied Biosystems) according to the manufacturer's instructions. Assay numbers are given in Table 2-1. Reactions were performed in triplicate in a 20-μl volume, with the following cycle parameters: 95 °C/10 min enzyme activation, 95 °C/15 s, 60 °C/1 min for 40 cycles. Data analysis was performed using the comparative Ct method (Applied Biosystems) to determine relative expression levels.

Gene	RefSeq ID	Assay Number	Description
GAPDH	NM_002046	Hs99999905_m1	Glyceraldehyde-3-phosphate dehydrogenase
EGR1	NM_001964	Hs00152928_m1	Early-growth response 1
DUSP2	NM_004418	Hs00358879_m1	Dual-specificity phosphatase 2
DUSP5	NM_004419	Hs00244839_m1	Dual-specificity phosphatase 5
IL-6	NM_000600	Hs00174131_m1	Interleukin 6
GADD45B	NM_015675	Hs00169587_m1	Growth arrest and DNA-damage-inducible, beta
TNFα	NM_000594	Hs00174128_m1	Tumor necrosis factor
CYR61	NM_001554	Hs00155479_m1	Cysteine-rich, angiogenic inducer, 61
Fos	NM_005252	Hs00170630_m1	C-fos
DTR	NM_001945	Hs00181813_m1	Diphtheria toxin receptor
NR4A1	NM_173158	Hs00544986_m1	Nuclear receptor subfamily 4, group A, member 1
Jun	NM_002228	Hs00277190_s1	C-Jun
ADM	NM_001124	Hs00181605_m1	Adrenomedullin
DUSP6	NM_001946	Hs00169257_m1	Dual-specificity phosphatase 6
DUSP10	NM_144728	Hs00200527_m1	Dual-specificity phosphatase 10
KIF13B	NM_015254	Hs00209573_m1	Kinesin family member 13B
AKAP12	NM_005100	Hs00374507_m1	A kinase (PRKA) anchor protein (gravin) 12
TBCC	NM_003192	Hs00268437_s1	Tubulin-specific chaperone c

DOI: 10.1371/journal.pbio.0030100.t001

Table 2-1. Assays on demand (Taqman probes and primers) used for real-time quantitative RT-PCR in this study

Immunoblotting.

T84 cells were infected with N400 or N400*pilT* or treated with GCB medium alone for specified times. Following infection, cells were lysed with 150 μl of 1X SDS lysis buffer (62.5 mM Tris-HCl [pH 6.8], 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromophenol blue), scraped into Eppendorf tubes, vortexed for 15 s, and immediately stored at -20 °C. For PARP and caspase 8 assays, samples were incubated with 150 μl of cell lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP40, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml Leupeptin) for 20 min on ice, followed by a 15-s sonication. Samples were boiled for 5 min at 100 °C, then separated by SDS 8% polyacrylamide gels and transferred onto nitrocellulose sheets. Membranes were probed with the specified antibodies following the manufacturer's protocol.

CPPs and bead coating.

N. gonorrhoeae CPPs were generated from piliated, Opa– gonococci. Bacteria were scraped from overnight cultures (grown on plates) into HBSS and vortexed for 2 min, followed by centrifugation at 14,000*g* for 5 min. Supernatants were removed, quantitated by spectrophotometric analysis, and stored at -80 °C until use. Pili preparations were assayed for the presence of pili via indirect immunofluorescence microscopy and immunoblot, using anti-pilin antibody (data not shown). Bio-Mag Plus carboxy-modified paramagnetic microspheres (Bangs Laboratories, Fishers, Indiana, United States), were activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (EDAC), and incubated with piliated *N. gonorrhoeae* CPPs or BSA as per the manufacturer's

instructions. Bead coating was confirmed by immunoblotting using antibodies to BSA (ICN Biomedicals, Irvine, California, United States) and pilin (antibody SM1; data not shown).

Immunofluorescence microscopy.

T84 cells were grown on coverslips to 50% confluency and incubated with either BSA-coated or CMP-coated magnetic beads for 15 min. Unbound beads were washed off and the magnet placed at a distance of 10 mm from the cell surface for 1 h. The medium was then aspirated, and the cells fixed for 15 min at room temperature in 4% paraformaldehyde. Cells were blocked and permeabilized in isotonic PBS containing BSA (3%, w/v) and saponin (0.02% w/v) for 1 h at room temperature, followed by staining with Alexa-Fluor 594 phalloidin (Molecular Probes, Eugene, Oregon, United States) at 1:1,000 for 30 min. Samples were rinsed extensively in PBS before mounting in Fluoromount-G (Fisher Scientific, Hampton, New Hampshire, United States). Images were obtained with a Deltavision Restoration Microscope (Applied Precision, Issaquah, Washington, United States) fitted with a Nikon (Tokyo, Japan) 60_ oil-immersion objective and processed at a Silicon Graphics (Mountain View, California, United States) workstation with accompanying API software. The images were subsequently exported to Adobe Photoshop (version 7.0) and Adobe Illustrator (version 11.0) (Adobe Systems, San Jose, California, United States) for manuscript preparation.

Calculation of magnetic force.

To quantify the amount of force that the magnet exerts per magnetic bead, the change-in-mass method [31] was used. Briefly, the mass of a known number of dry beads (0.12 g) was measured on an electronic balance in the presence and absence of the magnet. Given the mean bead diameter of 1.5 μm and the bead density of 2.5×10^3 kg/m^3 (Bangs Laboratories), the number of beads in this sample was calculated to be 1.2×10^{10}. The change in mass of the beads in the presence of the magnet was entered into the equation: force = Δmass x acceleration (with acceleration being equal to gravity, or 9.81 m/s^2) to give a value for the force. Change-in-mass measurements were taken at varying distances from the magnet to determine force as a function of distance (see Figure 2-4B).

Magnetic force experiments.

T84 cells were grown to confluency in 35-mm culture dishes. Before assay, the cells were incubated with prewarmed, serum-free medium for 2 h. Cells were then incubated for 30 min with medium alone, or with CPP- or BSA-coated beads diluted in the same medium. Cells were then washed with fresh, serum-free medium to remove unbound beads. Magnets were placed at a distance of 10 mm from the bottom of the tissue culture dish, and the dishes were incubated for the specified time at 37 °C, 5% CO_2. The samples were then processed for RNA isolation or SDS-PAGE, as described above. Control samples were treated in parallel but were not exposed to the magnet.

Statistics.

Statistical analysis was performed using standard *t*-test analysis with SPSS version 11.0 (SPSS, Chicago, Illinois, United States) unless otherwise stated

Protocol S1: BBC MIAME Checklist version 1.0

Affymetrix Experiments

EXPERIMENT DESIGN

Authors / Submitters

Magdalene So

Heather L. Howie

Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, Oregon.

Type of experiment: for example, is it a comparison of normal vs. diseased tissue, a time course, or is it designed to study the effects of a gene knock-out?

Compared the gene expression profiles of uninfected T84 human epithelial cells to cells infected with wild-type *Neisseria gonorrhoeae*, or an isogenic mutant (*pilT*) that is unable to undergo pilus retraction, to test the hypothesis that pilus retraction alters the host cell transcriptome.

Experimental factors: the parameters or conditions tested, such as time, dose, or genetic variation.

3 experimental conditions:

Uninfected

Wild-type infected (3hr infection with strain N400)

pilT-infected (3hr infection with strain N400*pilT*)

The number of hybridizations performed in the experiment.

9 total hybridizations

The type of reference used for the hybridizations, if any.

No references used

Hybridization design: if applicable, a description of the comparisons made in each hybridization, whether to a standard reference sample, or between experimental samples. An accompanying diagram or table may be useful.

Comparisons between uninfected and wild-type infected; comparisons between uninfected and *pilT* infected: comparisons between wild-type infected and *pilT* infected

Quality control steps taken.

Isolated total RNA was analyzed on an Agilent Bioanalyzer 2100 to verify that starting material was of good quality. Standard Affymetrix control steps were then taken. Labeled RNA was hybridized to a Test Array containing probe sets for all Affymetrix Expression Array controls. Background noise, Avg Diff, and ratio of Avg Diff values for probe sets representing the 5' and 3' ends of actin and GAPDH transcripts were used to determine target quality. Targets that did not meet the empirically

determined cut-off values within the project were re-made. Quality tested samples were then hybridized to the HG_U95Av2 array.

Number of replicates (Biological or Technical)

3 biological replicates for each condition tested

URL of any supplemental websites or database accession numbers.

Data will be hosted on the Oregon Health & Science University Biostatistics and Bioinformatics Shared Resource web page (www.ohsu.edu/cbcibm/bbsr) following publication.

SAMPLES USED, EXTRACT PREPARATION AND LABELING

- **The origin of the biological sample (for instance, name of the organism, the provider of the sample) and its characteristics: for example, gender, age, developmental stage, strain, or disease state.**

  Organism: Homo Sapien

  Cell line: T84 (derived from human colorectal cancer)

  Passage: 4-9

  Bacterial strains: N400 and N400*pilT*

  Bacterial strain origin: Michael Koomey

  Passage: 4-6

Manipulation of biological samples and protocols used: for example, growth conditions, treatments, separation techniques.

Fresh DMEM-F12 media (+ 5% FBS) was added to confluent T84 cells seeded onto wells of a 6-well plate. N400 or N400*pilT* suspended in GCB media were added to the cell monolayers at an MOI (multiplicity of infection) of 50. Uninfected cells were treated with an equivalent volume of GCB media. Plates were incubated at 37°C/5% CO_2 for 3 hours. Following incubation, media was aspirated and replaced with buffer RLT (+ beta-mercaptoethanol). Cells were scraped off of plates, and transferred to qiashredder columns (Qiagen Inc.) to homogenize each sample, followed by storage at -80°C.

Protocol for preparing the hybridization extract: for example, the RNA or DNA extraction and purification protocol.

Total RNA was isolated using the Qiagen RNeasy kit (Qiagen Inc.).

Labeling protocol(s).

Standard Affymetrix Protocols (Affymetrix GeneChip Expression Analysis Technical Manual, rev.3. 2001)

External controls (spikes).

Standard Affymetrix external spikes added to hybridization mixture: BioB, BioC, BioD and CreX at 1.5pm, 5.0pm, 25pm and 100pm respectively.

HYBRIDIZATION PROCEDURES AND PARAMETERS

The protocol and conditions used during hybridization, blocking and washing.

Standard Affymetrix Protocols (Affymetrix GeneChip Expression Analysis Technical Manual, rev.3. 2001)

MEASUREMENT DATA AND SPECIFICATIONS

Quantitations based on the images

Original Affymetrix .dat proprietory output files

Type of scanning hardware and software used: this information is appropriate for a materials and methods section.

Software – Affymetrix Microarray Suite 5.0

Scanning Hardware – HP GeneArray Scanner

Type of image analysis software used: specifications should be stated in the materials and methods.

Affymetrix Microarray Suite 5.0 was used for image analysis and feature extraction

A description of the measurements produced by the image-analysis software and a description of which measurements were used in the analysis.

Probe level measurements produced by Affymetrix Microarray Suite 5.0. Analysis performed at level of raw signal. (See Affymetrix Microarray Suite 5.0 reference on web site for description).

The complete output of the image analysis *before* data selection and transformation (spot quantitation matrices).

Original Affymetrix output files

Data selection and transformation procedures.

Genes displaying a "detection p-value" less than 0.1 in 8 or more arrays (out of a total of 9 arrays) were removed from the analysis. Regulated genes were identified based on the following criteria:

1) A 1.5 fold difference in expression in wild-type and/or *pilT* infected cells as compared to uninfected cells in at least 2 out of 3 replicates.
2) An average W/P ratio (wild-type fold-change divided by *pilT* fold-change) of ≥ 1.5 or ≤ .67 from 2 out of 3 replicates
3) Both values used to calculate average W/P ratios must be ≥1.25 or ≤ 0.8

A gene was considered for further analysis only if condition (1) was met. A gene was considered to be regulated by pilus retraction if both conditions (2) and (3) were met.

For statistical analysis, data were ln transformed, and analyzed using CyberT (**http://visitor.ics.uci.edu/genex/cybert**) to generate p-values from regularized t-tests using a Bayesian estimate of the variance among gene measurements within an experiment. These p-values are used only as supporting data for the genes identified by the three criteria listed above.

Final gene expression data table(s) used by the authors to make their conclusions *after* data selection and transformation (gene expression data matrices).

See worksheet Final.xls for results

ARRAY DESIGN

- **Platform Type**

  Synthesized Oligonucleotide Array

- **Surface and Coating Specifications**

  Glass

- **Availability of the Array (name/make)**

  Affymetrix Human Genome HG_U95Av2

- **For each feature (spot) on the array, its location on the array and the ID of its respective reporter (molecule present on each spot) should be given.**

  See www.affymetrix.com

  - **For each reporter, its type (e.g., cDNA or oligonucleotide) should be given, along with information that characterizes the reporter molecule unambiguously, in the form of appropriate database reference(s) and sequence (if available).**

  See www.affymetrix.com

Table S1

Gene	Affy ID	GenBank	Function	Induction	References (in addition to Locus Link descriptions)
NR4A1	279_at	L13740	TC,CS,SI	MS,ES,MAPK	(Bandoh *et al.*, 1997; Honkaniemi *et al.*, 2000; Li *et al.*, 2000; Pekarsky *et al.*, 2001; Song *et al.*, 2002; Suzuki *et al.*, 2003)
NR4A1	280_g_at	L13740	TC,CS,SI	MS,ES,MAPK	(Bandoh *et al.*, 1997; Honkaniemi *et al.*, 2000; Li *et al.*, 2000; Pekarsky *et al.*, 2001; Song *et al.*, 2002; Suzuki *et al.*, 2003)
Fos	2094_s_at	K00650	TC,CS	MS,ES,MAPK	(Li *et al.*, 2002)
IL-8	1369_s_at	M28130	SI	MS,ES,MAPK	(Akhtar *et al.*, 2003; Chen *et al.*, 2003; Hoffmann *et al.*, 2002; Liang *et al.*, 2002a; Liang *et al.*, 2002b; Oudin and Pugin, 2002; Yamamoto *et al.*, 2002)
DUSP1	1005_at	X68277	KP,CS,SI	MS,ES,MAPK	(Denkert *et al.*, 2002; Li *et al.*, 1999; Theodosiou and Ashworth, 2002)
IL-8	35372_r_at	M17017	SI	MS,ES,MAPK	(Akhtar *et al.*, 2003; Chen *et al.*, 2003; Hoffmann *et al.*, 2002; Liang *et al.*, 2002a; Liang *et al.*, 2002b; Oudin and Pugin, 2002; Yamamoto *et al.*, 2002)
IL-6	38299_at	X04430	SI	MS,ES,MAPK	(Kishikawa *et al.*, 2002; Kobayashi *et al.*, 2003; Mann, 2003; Meduri, 2002; Naka *et al.*, 2002)
DUSP5	529_at	U15932	KP	MS,ES,MAPK	(Ueda *et al.*, 2003)
NR4A2	547_s_at	S77154	TC	MS,ES,MAPK	(Bandoh *et al.*, 1997; Chai and Tarnawski, 2002; Honkaniemi *et al.*, 2000; Maira *et al.*, 1999; Park *et al.*, 2003)
EGR2	37863_at	J04076	TC,CS	CC,MAPK	(Chavrier *et al.*, 1988; Hirano *et al.*, 2002; Unoki and Nakamura, 2003; Zeng *et al.*, 2003)
DTR	38037_at	M60278	CS,SI	MS,ES,MAPK	(Ingram *et al.*, 2003; Kuhn *et al.*, 2002; Park *et al.*, 1999; Tschumperlin *et al.*, 2002; Wallasch *et al.*, 2002; Xia *et al.*, 2003)
Gro1	408_at	X54489	CS,SI	MS,ES	(Dhawan and Richmond, 2002; Dunican *et al.*, 2000; Hagiwara *et al.*, 1998; Thorpe *et al.*, 2001; Wang *et al.*, 2003)
Gro2	37187_at	M36820	CS,SI	MS,ES	(Hess *et al.*, 2001; Joshi-Barve *et al.*, 1993; Quinn *et al.*, 2002; Tarzami *et al.*, 2002)
TNFa	1852_at	X02910	CS,SI	MS,ES,MAPK	(Gupta, 2002; Hehlgans and Mannel, 2002; Lamb *et al.*, 2003; Long *et al.*, 2001; Palmieri *et al.*, 2002)
SOCS3	40968_at	AB004904	SI	MS,ES,MAPK	(Bode *et al.*, 2001; Krebs and Hilton, 2001; Mysorekar *et al.*, 2002; Yasukawa *et al.*, 2001)
JunB	2049_s_at	M29039	TC,CS	MS,ES,MAPK	(Balmanno and Cook, 1999; Frazier-Jessen *et al.*, 2002; Granet *et al.*, 2002; Shaulian and Karin, 2002)
Id1	36619_r_at	S78825	TC,CS	U	(Kang *et al.*, 2003; Miyazono and Miyazawa, 2002; Sikder *et al.*, 2003)
TCF8	33439_at	D15050	TC	U	(Grooteclaes and Frisch, 2000; Postigo *et al.*, 2003)
Gro3	34022_at	M36821	CA,SI	MS,ES	(Curnock *et al.*, 2002; Hagiwara *et al.*, 1998; Haskill *et al.*, 1990; Yang *et al.*, 1997)
H2BFR	35562_at	AI076718	TC	CC	(LaBella *et al.*, 1988)
H2AF0	32609_at	AI885852	TC	CC	(Augusto *et al.*, 2003; Oswald *et al.*, 1996)
BLIMP1	31779_s_at	AF084199	SI	ES	(Gyory *et al.*, 2003; Schebesta *et al.*, 2002)
ATF3	287_at	L19871	TC,CS,SI	MS,ES,MAPK	(Hai *et al.*, 1999; Kawauchi *et al.*, 2002; Kool *et al.*, 2003; Liang *et al.*, 1996; Nakagomi *et al.*, 2003; Sepulveda *et al.*, 2002)
KRT16	601_s_at	M28439	CS,CA	MS,CC	(Jiang *et al.*, 1993; McClowry *et al.*, 2002; Paramio *et al.*, 1999)
CSF2	1400_at	M13207	CS,SI	MS,ES,MAPK	(Kosaki *et al.*, 1998; Quentmeier *et al.*, 2003; Reibman *et al.*, 2002)
NR4A2	37623_at	X75918	TC	MS,ES,MAPK	(Bandoh *et al.*, 1997; Chai and Tarnawski, 2002; Honkaniemi *et al.*, 2000; Maira *et al.*, 1999; Park *et al.*, 2003)
CYR61	38772_at	Y11307	CS,CA,SI	MS,ES,MAPK	(Brigstock, 2003; Han *et al.*, 2003; Kunz *et al.*, 2003; Lau and Lam, 1999; Leu *et al.*, 2002; Tamura *et al.*, 2001)
H2AF0	286_at	L19779	TC	CC	(Augusto *et al.*, 2003; Oswald *et al.*, 1996)
CSF2	1401_g_at	M13207	CS,SI	MS,ES,MAPK	(Kosaki *et al.*, 1998; Quentmeier *et al.*, 2003; Reibman *et al.*, 2002)
DUSP2	1292_at	L11329	KP,CS	MS,ES,MAPK	(Rohan *et al.*, 1993; Yin *et al.*, 2003)
EGR1	789_at	X52541	TC	MS,ES,MAPK	(O'Donovan *et al.*, 1999; Schwachtgen *et al.*, 1998; Silverman *et al.*, 1999; Stula *et al.*, 2000)
H2AFA	35127_at	AI039144	TC	CC	(Bosch and Suau, 1995; Manniron i *et al.*, 1994)
OVOL1	34047_at	AF016045	TC	U	
uPA	37310_at	X02419	CS,CA,SI	MS,ES,MAPK	(Aguirre Ghiso *et al.*, 1999; Blasi, 1999; Cirillo *et al.*, 1999; Essig *et al.*, 2001; Irigoyen *et al.*, 1997; Miura *et al.*, 2000; Redmond *et al.*, 1999; Stefansson and Lawrence, 2003; Tarui *et al.*, 2003; Wang *et al.*, 1995; Watanabe *et al.*, 2003)
Gadd45B	39822_s_at	AF078077	CS,SI	ES	(De Smaele *et al.*, 2001; Jin *et al.*, 2002; Kyriakis, 2001; Liebermann and Hoffman, 2002, 2003)
Gadd34	37028_at	U83981	CS,SI	ES,MAPK	(Hung *et al.*, 2003; Liebermann and Hoffman, 2002; Oh-Hashi *et al.*, 2001; Sarkar *et al.*, 2002)
RhoB	1826_at	M12174	CS,CA,SI	ES	(Fritz and Kaina, 2001a, b; Gampel *et al.*, 1999; Jahner and Hunter, 1991; Prendergast, 2001; Ridley, 2001; Zalcman *et al.*, 1995)
	37538_at	AL049354	U	U	
HSP70-2	31692_at	M59830	CS,SI	MS,ES	(Gabai *et al.*, 2002a; Gabai and Sherman, 2002; Galvin *et al.*, 2002; Liu *et al.*, 2003; Nollen and Morimoto, 2002; Park *et al.*, 2001; Pirkkala *et al.*, 2001; Volloch *et al.*, 2000)
H1F2	37018_at	AI189287	TC	CC	(Marzluff *et al.*, 2002)
Id1	36618_g_at	X77956	TC,CS	U	(Kang *et al.*, 2003; Miyazono and Miyazawa, 2002; Sikder *et al.*, 2003)
KIP2	38673_s_at	D64137	KP,CS,SI	MS,ES	(Billotte *et al.*, 2001; Chang *et al.*, 2003b; Hsu *et al.*, 2002; Petermann *et al.*, 2002)

HBP17	38489_at	M60047	CS	MAPK	(Chen *et al.*, 2001; Harris *et al.*, 2000; Tassi *et al.*, 2001)
Rho6	37785_at	U69563	CA	U	(Chardin, 1999; Katoh *et al.*, 2002; Nobes *et al.*, 1998; Oinuma *et al.*, 2003; Wennerberg *et al.*, 2003)
MIP1A	36103_at	D90144	SI	ES	(Li *et al.*, 2003; Shi *et al.*, 1996; Takahashi *et al.*, 2002)
HSPF1	752_s_at	D85429	SI	MS,ES	(Ohtsuka and Hata, 2000; Sironen *et al.*, 2002)
MEKK1	33009_at	AF042838	KP,CS	U	(Schlesinger *et al.*, 1998)
KIP2	1787_at	U22398	KP,CS,SI	MS,ES	(Billotte *et al.*, 2001; Chang *et al.*, 2003b; Hsu *et al.*, 2002; Petermann *et al.*, 2002)
H3FB	34964_at	N35832	TC	CC	(Frank *et al.*, 2003)
	38207_at	AW006742	U	U	
E2F2	37043_at	AL021154	TC,CS	CC	(de Bruin *et al.*, 2003; Leone *et al.*, 2001; Zhu *et al.*, 2003)
CYP1A1	1024_at	X02612	SI	ES	(Barouki and Morel, 2001; Morgan *et al.*, 1998)
FDZF2	38872_at	U95044	TC	U	
	36070_at	AL049389	U	U	
ZNF253	35573_r_at	AF038951	TC	U	(Han *et al.*, 1999)
PLXNC1	32193_at	AF030339	CA	U	(Comeau *et al.*, 1998; Pasterkamp *et al.*, 2003)
ALDH1A3	36686_at	U07919	SI	U	(Okamura *et al.*, 1999; Pappas *et al.*, 2001)
CYP1A1	1025_g_at	X02612	SI	ES	(Barouki and Morel, 2001; Morgan *et al.*, 1998)
CYP1A1	36767_at	K03191	SI	ES	(Barouki and Morel, 2001; Morgan *et al.*, 1998)
	40143_at	D50930	U	U	
SOCS5	32669_at	AB014571	SI	ES	(Yasukawa *et al.*, 2000)
	34283_at	AL050125	U	U	
	40848_g_at	AB018293	U	U	
CBX6	39560_at	H10776	TC	U	(Jones *et al.*, 2001)
SIM2	39608_at	U80456	TC	U	
	41807_at	AL040137	U	U	
	38505_at	AL050151	U	U	
GRLF1	34724_at	AI670100	CA	U	(Brouns *et al.*, 2000; Zangerl *et al.*, 2002)
ZNF211	38142_at	U38904	TC,CS	U	(Becker *et al.*, 1997)

Functions: TC (transcription factor), KP (kinase/phosphatase), CS (cell cycle/survival), CA (cytoskeleton/adhesion), SI (stress/immune response) and U (unknown)

Induction mechanisms: MS (mechanical stress), ES (environmental stress), CC (cell cycle), MAPK (mitogen activated protein kinase) and U (unknown)

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Chapter 3: Manuscript 2

ERK Activation by *Neisseria gonorrhoeae* modulates Host Cell Pro-Apoptotic Proteins Bad and Bim

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Summary

Infection with *Neisseria gonorrhoeae* protects the host cell from apoptosis via cytoprotective signaling that is enhanced by type IV pilus retraction. We tested the hypothesis that this process is mediated in part by gonococcal-induced and retraction-enhanced activation of extracellular signal-regulated kinase (ERK). Inhibition of ERK prior to infection with either wild-type or *pilT* bacteria attenuated the ability of the bacteria to protect host cells from STS-induced apoptosis. ERK mediated the down-regulation of two BH3-only proteins, Bad and Bim, through a proteasomal dependent mechanism. Bad was additionally modified by ERK-dependent phosphorylation on Ser112, a well-characterized survival signal. These events prevent mitochondrial cytochrome c release and subsequent apoptotic signaling. Using siRNA, we show that decreased levels of Bad or Bim are sufficient to protect T84 cells from STS-induced apoptosis. Thus, *N. gonorrhoeae* stimulation of pro-survival signaling is mediated, at least in part, by ERK dependent down-regulation and modification of Bad and Bim.

Introduction

Neisseria gonorrhoeae is the causative agent of gonorrhea, the second most reported sexually transmitted disease in the United States (CDC, 2003). The bacterium most often initiates infection at mucosal surfaces of the urogenital tract, and can lead to serious complications if left untreated. While gonorrhea is typically regarded as a disease with clear symptoms in men, it has long been recognized that infection in women is often asymptomatic. When clinical symptoms do appear in either sex, they usually occur days or even weeks following exposure (Morse, 1996). Moreover, approximately 5.3% of the population between the ages of 18 and 35 are persistent carriers of *N. gonorrhoeae*, with no overt symptoms of disease (Turner *et al.*, 2002). These observations imply that symptomatic disease may be accidental, and that the infecting organisms intend minimal harm to the human host.

Consistent with this view, we and others have demonstrated that, indeed, *in vitro* infection of epithelial cells with *N. gonorrhoeae* does not induce cell damage (Beck and Meyer, 2000; Binnicker *et al.*, 2003, 2004; Howie *et al.*, 2005). On the contrary, cells infected with wild-type, piliated *N. gonorrhoeae* are better able to withstand apoptotic stimuli than non-infected cells. Importantly, this cytoprotection was partially attenuated when epithelial cells were infected with a *pilT* mutant (Howie *et al.*, 2005), which expresses non-retractable type IV pili (Tfp) (Merz *et al.*, 1999; Merz *et al.*, 2000). Exerting an artificial upward pulling force on uninfected cells can replicate the host cell responses to wild-type infection, strongly suggesting that mechanical stress at least

partially mediates this process. As *pilT* mutants cannot induce mechanical stress on the host cell membrane, they are unable to stimulate the full level of cytoprotection seen in wild-type infected cells. Thus, we term this cytoprotective signaling gonococcal-induced and retraction-enhanced (GIRE).

While the specific mechanism(s) underlying GIRE cytoprotection have not yet been identified, we have shown that Tfp retraction enhances the activation of at least two potential survival-signaling pathways: the phosphoinositide-3 (PI-3) kinase/Akt pathway (Lee *et al.*, 2005) and the extracellular-signal regulated kinase (ERK) pathway (Howie *et al.*, 2005). Both pathways are known to be induced by mechanotransduction in a variety of cell types (Hughes-Fulford, 2004; Liu *et al.*, 1999). However, to date, only ERK activation has been shown to be stimulated by piliated *N. gonorrhoeae* and specifically enhanced by the application of mechanical force (Howie *et al.*, 2005). Moreover, the kinetics of ERK activation during infection, i.e., rapid and sustained phosphorylation, strongly suggest that the kinase plays a role in cell survival (He *et al.*, 2004; Petlickovski *et al.*, 2005; Tong *et al.*, 2002).

We tested the hypothesis that ERK activation plays a role in the ability of piliated *N. gonorroheae* to induce a cytoprotective state in the host. Inhibition of ERK signaling prior to infection attenuated the ability of both wild-type and *pilT* to mediate cytoprotection in T84 human colorectal epithelial cells. The downstream targets of *N. gonorrhoeae*-induced ERK activation were two BH3-only proteins, Bad and Bim. These proteins were both down-regulated by activated ERK, through a process that involves

proteasomal activity. Consistent with a blockage of apoptosis signaling at the level of BH3-only signaling, infection with either wild-type or *pilT* prevents the release of cytochrome c from the mitochondria. Decreasing the levels of either of these pro-apoptotic proteins, via small-inhibitory RNA (siRNA), is sufficient to protect T84 cells from STS-induced apoptosis. These results reveal a pathway by which *N. gonorrhoeae* infection and pilus retraction work together to prevent apoptosis signaling in the host epithelium.

Results

ERK Activation During Infection is Cytoprotective

ERK activation is a well-documented pro-survival signal. We therefore tested the hypothesis that ERK activation mediates GIRE cytoprotection. T84 human colorectal epithelial cells were pre-incubated with ERK inhibitor (U0126) prior to infection with either wild-type *N. gonorrhoeae* (N400) or a pilus retraction-deficient mutant (N400*pilT*). After a 4-hour infection, cells were treated with Staurosporine (STS) for an additional 4hr to induce apoptosis.

Immunoblots were performed on cell lysates to determine the relative induction of apoptosis by assaying the levels of cleaved PARP and cleaved caspase-8 induced by each treatment (Figure 3-1). PARP and caspase-8 are cleaved during apoptosis signaling, thus, a higher level of cleaved protein is directly correlated with increased apoptosis in the culture. To establish baseline levels of cleaved PARP and caspase-8, we first compared levels of each protein from uninfected cells to cultures infected with either N400 or N400*pilT*. As expected, infection with either strain resulted in reduced levels of both cleavage products. N400-infected cells had slightly lower levels of cleaved PARP and caspase-8 than did N400*pilT*-infected cells. Although the difference between levels of cleaved protein in wild-type- versus *pilT*-infected cells is only moderate, the difference has been repeatedly observed, and is consistent with previous results (Howie *et al.*, 2005).

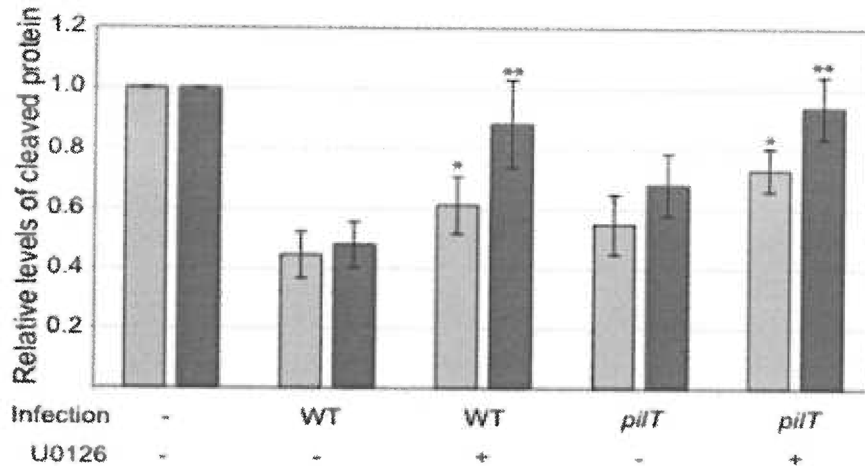

Figure 3-1. Effect of ERK inhibition on infection-mediated cytoprotection.

Levels of cleaved PARP and cleaved caspase 8 in T84 cells pre-treated with U0126, then infected with N400 (WT) or N400*pilT* (*pilT*) or left uninfected for 4hr, followed by incubation with STS (1mM) for an additional 4hr to induce apoptosis. Cleaved protein levels were quantified by densitometry of immunoblot signals. Light grey bars represent cleaved PARP levels and dark grey bars represent cleaved caspase 8 levels. Values represent the mean levels of cleaved target (+/- SEM) from 4 independent experiments. A significant difference from the corresponding non-U0126 treated sample is denoted by two asterisks ($p < 0.05$) or by a single asterisk ($p < 0.1$).

Cultures pre-incubated with ERK inhibitor U0126 prior to infection with either strain had increased levels of cleaved PARP and caspase 8, compared to levels of cleaved protein identified in the baseline control samples. These results indicate a higher level of apoptosis within the ERK-inhibited culture. Thus, ERK activation at least partially mediates GIRE cytoprotection. Interestingly, the differences in cleavage between wild-type- and *pilT*-infected cells remains evident for PARP cleavage levels, but is completely abolished for the levels cleaved caspase-8. Moreover, ERK inhibition almost completely prevents GIRE reduction of caspase-8 cleavage, while only attenuating GIRE reduction of PARP cleavage. Together, these results suggest that ERK activation by itself can block the caspase-8 pathway following STS treatment. Conversely, other GIRE signaling pathways are likely involved in blocking caspase-8-independent apoptotic signaling.

Bad is Modified by Infection and Pilus Retraction

Activated ERK mediates cytoprotection through a number of mechanisms, including induction of gene expression and phosphorylation of pro- or anti-apoptotic proteins (Ballif and Blenis, 2001). One of the best-studied substrates of activated ERK is the pro-apoptotic protein Bcl-2 antagonist of cell death (Bad). Bad functions as a cellular sensor for survival signals, and is phosphorylated at a basal level as long as such signals are present. In the absence of survival signals, Bad becomes dephosphorylated (Datta *et al.*, 1997; Datta *et al.*, 2000; Datta *et al.*, 2002; del Peso *et al.*, 1997; Lizcano *et al.*, 2000; Virdee *et al.*, 2000; Zha *et al.*, 1996), traffics to the mitochondria, and antagonizes the function of pro-survival Bcl-2-like proteins (Cheng *et al.*, 2001; Wei *et al.*, 2001). Thus, the relative phosphorylation state of Bad functions as a cellular "teeter-totter" which ultimately controls the induction or repression of apoptotic signaling.

ERK is one of at least three signaling pathways that converge to phosphorylate Bad. ERK and PKA induce phosphorylation of Bad at Ser112 and/or Ser155, and PI3-kinase mediates phosphorylation at Ser136 (Bonni *et al.*, 1999; Harada *et al.*, 2001; Schurmann *et al.*, 2000; Tan *et al.*, 1999). We examined Bad phosphorylation at each of these sites in cells infected with N400 and N400*pilT* (Figure 3-2). In uninfected cells there was little to no change in Bad phosphorylation over a 6hr period. Conversely, N400 infection induced the phosphorylation of Bad at Ser112. This phosphorylation increased over time, reaching maximum levels by 4-6 hr post-infection. Phospho-Ser112 levels were only increased slightly in cells infected with N400*pilT*, indicating that pilus retraction plays a role in this signaling event. No phosphorylation was detected at the other ERK-dependent

site (Ser155) during infection with either strain. Importantly, N400 and N400*pilT*-induced phosphorylation at Ser112 could be partially blocked by pre-incubating cells with an ERK inhibitor prior to infection (Figure 3-4A and C). These data indicate that GIRE ERK activation at least partially mediates phosphorylation of Bad, but only at Ser112. The involvement of PKA in the phosphorylation of Ser112 cannot be inferred from this data. Finally, no phosphorylation was detected at Ser136 during infection with either N400 or N400*pilT*, indicating that PI3-K is not involved in Bad phosphorylation.

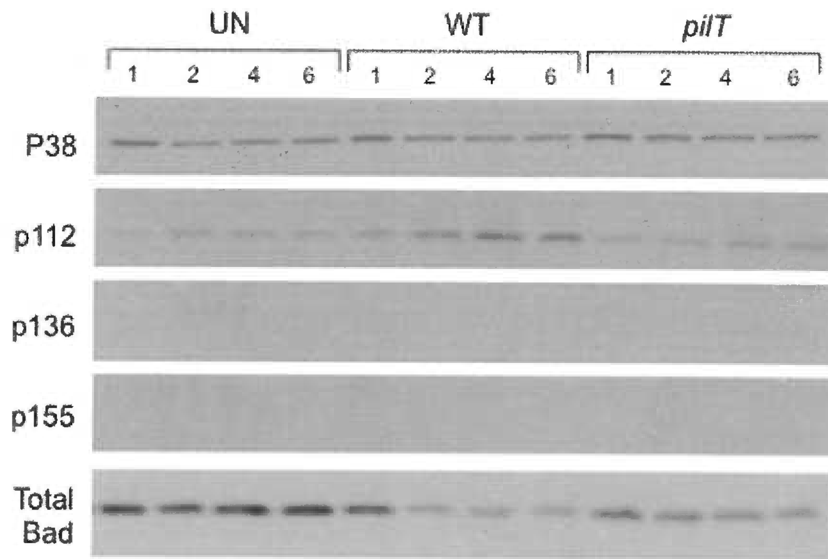

Figure 3-2. Phosphorylated Bad and total Bad levels in infected T84 cells.

Representative immunoblot showing levels of total Bad and three species of phosphorylated Bad (p112, p136 and p155) in uninfected T84 cells (UN) and T84 cells infected with N400 (WT) or N400*pilT* (*pilT*) for 1, 2, 4 and 6hr. Total P38 protein levels in each sample served as the internal control.

In addition to enhancing Bad phosphorylation at Ser112, infection with N400 and N400*pilT* led to decreased levels of total Bad protein (Figure 3-2). Maximal protein down-regulation was detected by 2 hr post infection, and levels of total Bad remained low throughout the 6hr timecourse of infection (longer time points were not tested). As

with Ser112 phosphorylation, Bad down-regulation was at least partially dependent on a functional *pilT*, as N400 induced a greater reduction in total Bad levels than did N400*pilT*.

Down-Regulation of Other BH3-Only Proteins During Infection

Bad is a member of a group of proteins termed "BH3-only" proteins, which act as sensors for a variety of apoptotic signals and, when stimulated, converge on a central cell-death signaling pathway (Festjens *et al.*, 2004). Activity of many of these proteins is tightly controlled at the transcriptional or post-transcriptional level in order to prevent aberrant cell death (Puthalakath and Strasser, 2002). Recent studies have demonstrated that *Chlamydia* infection leads to a broad degradation of host cell BH3-only proteins, including Bad (Dong *et al.*, 2005; Fischer *et al.*, 2004; Ying *et al.*, 2005). As this is the only published example of induced Bad down-regulation, we hypothesized that *N. gonorrhoeae* infection might induce a broad down-regulation of BH3-only proteins as well.

Lysates from T84 cells infected for 6hr with N400 or N400*pilT* were analyzed by immunoblotting for a number of pro- and anti-apoptotic Bcl-2 family proteins, including those in the BH3-only family. Infected and uninfected cells had identical levels of Bid, Bmf and Bok (Figure 3-3A and B), as well as Bcl2, Bax and Bak (data not shown). In contrast, total levels of the BH3-only protein Bim were lower in cells infected with either N400 or N400*pilT*. Quantitative analysis of immunoblots by densitometry demonstrated that levels of both Bad and Bim were significantly lower in infected cells than in

uninfected cells (Figure 3-3B). Moreover, pilus retraction was at least partially responsible for this down-regulation, as total levels of both Bad and Bim were significantly lower in N400 infected cells than in N400*pilT* infected cells. These results indicate that, unlike Chlamydia, *N. gonorrhoeae* specifically targets Bad and Bim for down-regulation.

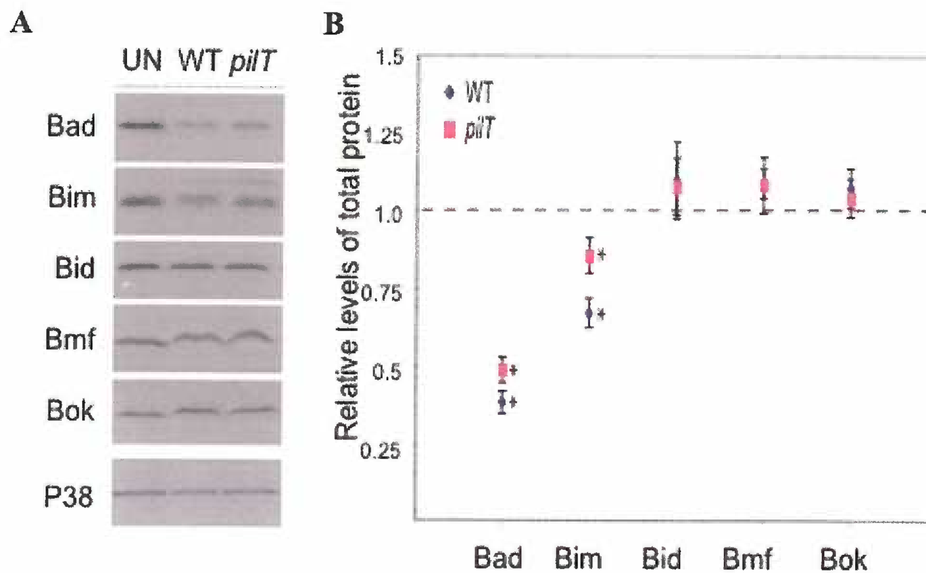

Figure 3-3. Levels of BH3-only proteins in infected and uninfected cells.

(A) Representative immunoblot showing levels of selected BH3-only proteins (Bad, Bim, Bid, Bmf and Bok) in uninfected T84 cells (UN) or T84 cells infected with N400 (WT) or N400*pilT* (*pilT*) for 6hr. Total P38 protein levels in each sample served as the internal control.

(B) Relative levels of the BH3-only proteins shown in (A). Each signal was quantitated by densitometry, normalized to its internal P38 signal, and expressed relative to the normalized value from uninfected cells (represented as dotted line). Values are mean normalized protein levels averaged from at least 3 independent experiments (+/- SEM). Asterisk indicates differences between infected and uninfected signals with a p value <0.05.

Down-Regulation of Bad and Bim is Mediated by ERK and Proteasomal Activity.

We next determined the mechanism behind GIRE down-regulation of Bad and Bim. As Bim has been shown to be regulated at the level of transcription (Dijkers *et al.*, 2000; Harris and Johnson, 2001; Putcha *et al.*, 2001; Shinjyo *et al.*, 2001; Whitfield *et al.*,

2001), we first determined whether down-regulation was at the level of mRNA. T84 cells were infected with N400 and N400*pilT* for 2 or 6 hr, at which time mRNA was isolated and used as template for real-time RT-PCR. Bad and Bim transcript levels were nearly identical in uninfected cells and cells infection with N400 or N400*pilT* (Table 3-1). Thus the infection-induced decrease in Bad and Bim does not occur at the level of mRNA.

		WT	*pilT*
Bad	2hpi	-1.05± .071	-1.1± .000
	6hpi	-1.15± .071	-1.3± .000
Bim	2hpi	1.15± .071	1.0± .141
	6hpi	-1.10± .141	1.0± .000

Table 3-1. Bim and Bad mRNA levels following infection.

Levels of Bim and Bad mRNA following infection with N400 (WT) and N400*pilT* (*pilT*). Values indicate mean mRNA levels +/- SEM from at least 2 independent experiments.

Next, we examined the involvement of ERK activation in the down-regulation of Bad and Bim protein levels. Both proteins are targets of ERK, and Bim degradation has been shown to be mediated by this pathway (Harada *et al.*, 2004; Ley *et al.*, 2003; Ley *et al.*, 2004; Luciano *et al.*, 2003; Marani *et al.*, 2004). T84 cells were pre-incubated with ERK inhibitor U0126 for 1hr, followed by a 6hr infection with N400 or N400*pilT*. Total cell lysates were then assayed by immunoblot to determine the relative levels of Bad and Bim (Figure 3-4A). When ERK activation was blocked by pre-treatment with inhibitor, down-regulation of both Bad and Bim was prevented, with total levels of each protein returning to that seen in uninfected cultures (Figure 3-4A, D and E). Thus both Bad and Bim are down-regulated in an ERK-dependent manner.

As ERK-mediated degradation of Bim is known to proceed via the proteasomal pathway (Ley *et al.*, 2003; Ley *et al.*, 2004; Luciano *et al.*, 2003), we next determined if this

pathway was involved in *N. gonorrhoeae*-mediated down-regulation of Bad or Bim. T84 cells were pre-incubated with proteasome inhibitor MG132 for 1hr, followed by a 6hr infection with N400 or N400*pilT*. Total cell lysates were again assayed by immunoblot to determine the relative levels of Bad and Bim (Figure 3-4B). MG132 attenuated the ability of both strains to down-regulate Bim. This is consistent with the well-documented mechanism of Bim degradation by the proteasomal pathway (Figure 3-4D). Interestingly, MG132 significantly *enhanced* the ability of infection to down-regulate Bad (Figure 3-4E).

Taken together, these results suggest that Bad and Bim are targeted for down-regulation via activated ERK, and that proteasomal activity plays a role in this process. While proteasomal activity seems to be directly involved in Bim down-regulation, it appears to be indirectly involved in the down-regulation of Bad. To our knowledge this indirect involvement of the proteasome in Bad protein down-regulation has not been demonstrated previously. The precise mechanism(s) by which ERK and the proteasome cooperate to induce down-regulation of Bad thus remain to be elucidated.

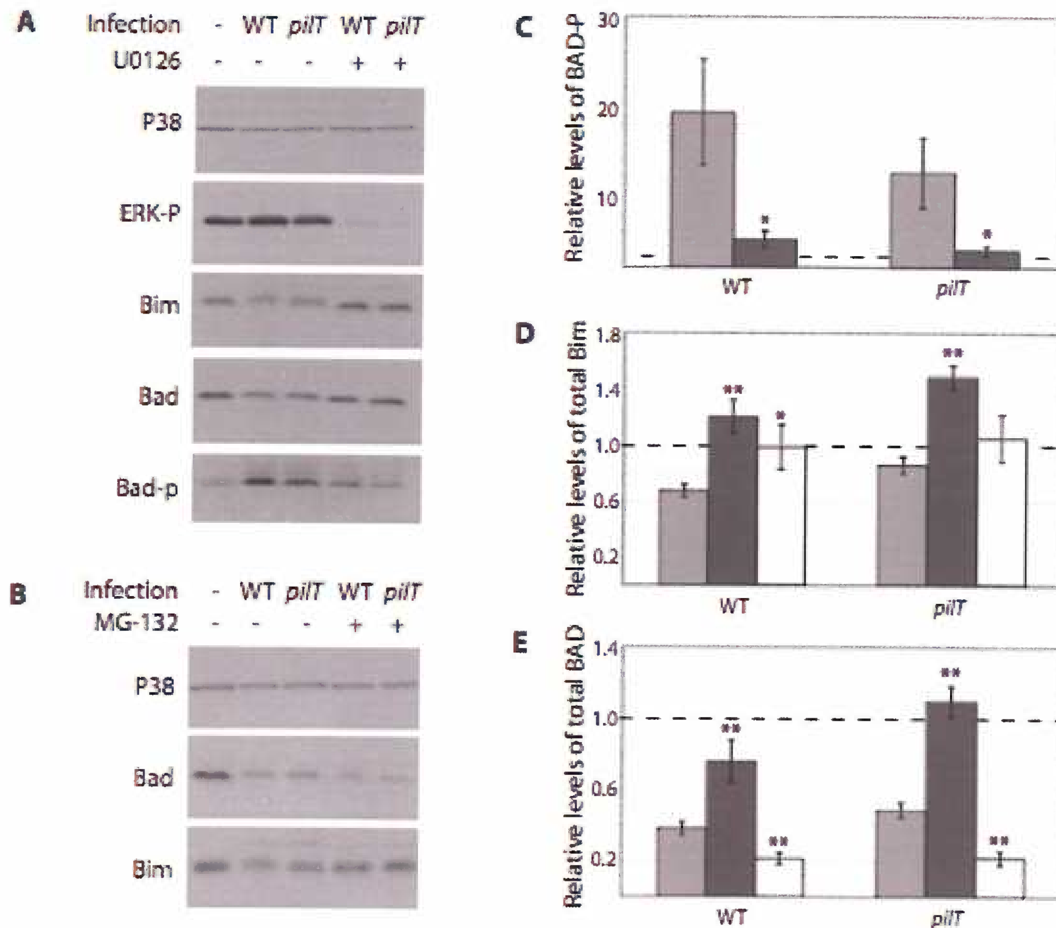

Figure 3-4. Inhibition of Bad and Bim degradation and Bad phosphorylation by ERK and proteasome inhibitors.

(A) Representative immunoblot showing levels of total Bad, total Bim, and phospho-Bad Ser112 (Bad-p) in uninfected T84 cells (UN), and T84 cells infected with N400 (WT) or N400*pilT* (*pilT*) for 6hr in the presence or absence of ERK inhibitor U0126. Total P38 protein levels in the same sample served as the internal control.

(B) Representative immunoblot showing total Bad and Bim levels in uninfected T84 cells (UN), and T84 cells infected with N400 (WT) or N400*pilT* (*pilT*) for 6hr in the presence or absence of proteasome inhibitor MG-132. Total P38 protein levels in the same sample served as the internal control.

(C, D and E) Levels of Bad-p and total Bim and Bad in immunoblots shown in (A) and (B). Each signal was quantitated by densitometry, normalized to its internal P38 signal, and expressed relative to the normalized value from uninfected cells (represented as dotted line). Light grey bars represent values from infected cells without inhibitor. Dark grey bars: values from infected cells treated with ERK inhibitor U0126. White bars: values from infected cells treated with proteasome inhibitor MG132. Values are mean normalized protein levels averaged from at least 3 independent experiments (+/- SEM). A single asterisk above a bar indicates differences between untreated and inhibitor-treated cells with a p value <0.05, while a double asterisk indicates a difference with $p < 0.01$.

Neisseria Infection Prevents Cytochrome c Release from Mitochondria.

Each BH3-only protein identified to date functions as a sensor for some specific apoptotic stimulus. BH3-only protein stimulation subsequently leads to Bax and Bak oligomerization, mitochondrial pore formation, and the release of cytochrome c into the cytosol. Because infection with *N. gonorrhoeae* results in decreased levels of two of these proteins, we hypothesized that the infection-induced cytoprotection occurs at this "sensing" stage. Such a block would prevent depolarization of the mitochondrial membrane and subsequent cytochrome c release. *N. gonorrhoeae* infection has been shown to prevent mitochondrial cytochrome c release in urethral epithelial cells (Binnicker *et al.*, 2003). The mechanism responsible for this process has not been investigated. Moreover, cytochrome c release in *N. gonorrhoeae*-infected T84 cells has not previously been analyzed. Therefore, to examine these issues, we used two assays to determine the location of cytochrome c in *N. gonorrhoeae*-infected, STS-treated cells.

We first determined cytochrome c localization qualitatively by indirect immuno-fluorescence microscopy on infected and uninfected T84 cells (Figure 3-5). In uninfected cells without STS, nuclei appeared intact, and the cytochrome c signals were entirely compartmentalized (Figure 3-5A). Similar staining is observed in *N. gonorrhoeae* infected, non-STS treated cells, suggesting that infection itself does not induce cytochrome c release (Figure 3-5C). When uninfected cultures were treated with STS for 4hr, the nuclei of many cells became fragmented, and the cytochrome c signal in these cells appeared dispersed or absent altogether (Figure 3-5B). This classical apoptotic

staining was almost completely absent in STS-treated cells that were pre-infected with wild-type *N. gonorrhoeae* (Figure 3-5D).

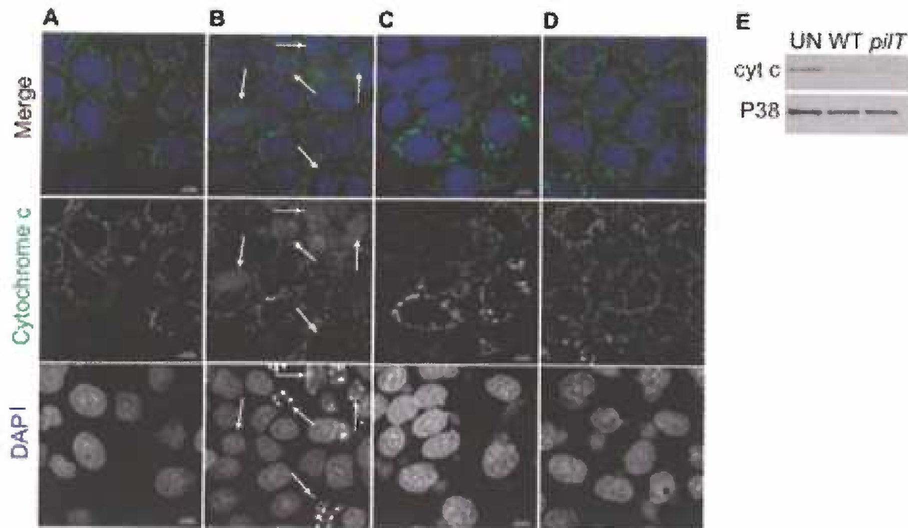

Figure 3-5. *N. gonorrhoeae* inhibition of cytochrome c release from the mitochondria.

Indirect immunofluorescence microscopy showing location of cytochrome c in uninfected T84 cells without STS treatment (A) and after STS treatment (B); and in infected T84 cells without STS treatment (C) and after STS treatment (D). Arrows indicate cells in which cytochrome c release and nuclear fragmentation has occured. (E) Representative immunoblot showing cytochrome c levels in the cytosol of uninfected T84 cells (UN), or T84 cells infected with N400 (WT) or N400*pilT* (*pilT*) for 4hr, followed by STS treatment. Total P38 protein levels in each sample served as the internal control. All cultures were incubated with medium or infected with bacteria for 4hr, followed, where noted, by incubation with 1mM STS for an additional 4hr.

Next, we used immunoblot analysis to globally analyze cytochrome c location in a cell culture during infection and STS treatment. T84 cells were either left uninfected or infected with N400 or N400*pilT* for 4 hrs, followed by treatment with STS to induce apoptosis. Cells were then harvested, separated into cytosolic and mitochondrial fractions and the cytosolic fractions then probed for the presence of cytochrome c. Compared to the uninfected, STS-treated control cells, cells infected with either N400 or N400*pilT* showed little if any release of cytochrome c upon STS treatment (Figure 3-5E). Together,

these data demonstrate that Neisseria infection prevents apoptosis signaling at or prior to cytochrome c release from the mitochondria.

Decreased Levels of Bad or Bim are Sufficient for Cytoprotection.

Because Bad and Bim play such an early role in sensing apoptosis, we hypothesized that decreased levels of either of these BH3-only proteins was sufficient to protect T84 cells against STS-induced apoptosis. Bad and Bim siRNAs have been demonstrated to be cytoprotective for a number of cell types and apoptotic stimuli (Abrams *et al.*, 2004; Chae *et al.*, 2005; Han *et al.*, 2005; Harada *et al.*, 2004; Kang *et al.*, 2004; Kuribara *et al.*, 2004; Li *et al.*, 2005; Marani *et al.*, 2004; Sunters *et al.*, 2003; Tong *et al.*, 2005; Yin *et al.*, 2005), however, cytoprotection specific to STS and for T84 cells has not yet been demonstrated. T84 cells were transfected with siRNAs directed against Bad or Bim, then treated with STS to induce apoptosis. To monitor siRNA efficiency, cell lysates were immunoblotted for total Bad and Bim levels 72hr post-transfection. Cells treated with the highest concentration of siRNA used (100uM), had noticeably decreased levels of both Bad and Bim (Figure 3-6A). Bim levels were decreased roughly 90%, while Bad levels were decreased approximately 70%. Importantly, siRNA targeted against Bad had no effect on total Bim levels, and vice versa.

T84 cells transfected with either Bad or Bim siRNA were then analyzed for their ability to survive STS treatment. 72 hr post-transfection, cultures were treated with STS for 4 hr, and then analyzed by immunoblotting for cleaved PARP. Compared to untransfected cells, cells transfected with either Bad or Bim siRNA contained lower levels of cleaved

PARP (Figure 3-6B). Thus reducing Bad or Bim levels attenuated apoptosis signaling. Additionally, both Bad and Bim siRNA appeared to protect cells from apoptosis in a concentration dependent manner. Linear regression analysis demonstrated that the degradation of either Bad or Bim is directly correlated to the relative levels of cleaved PARP in the culture (Figure 3-6C; $R^2 = 0.90$). Thus, lower levels of total Bad or Bim *by themselves* results in higher levels of cytoprotection.

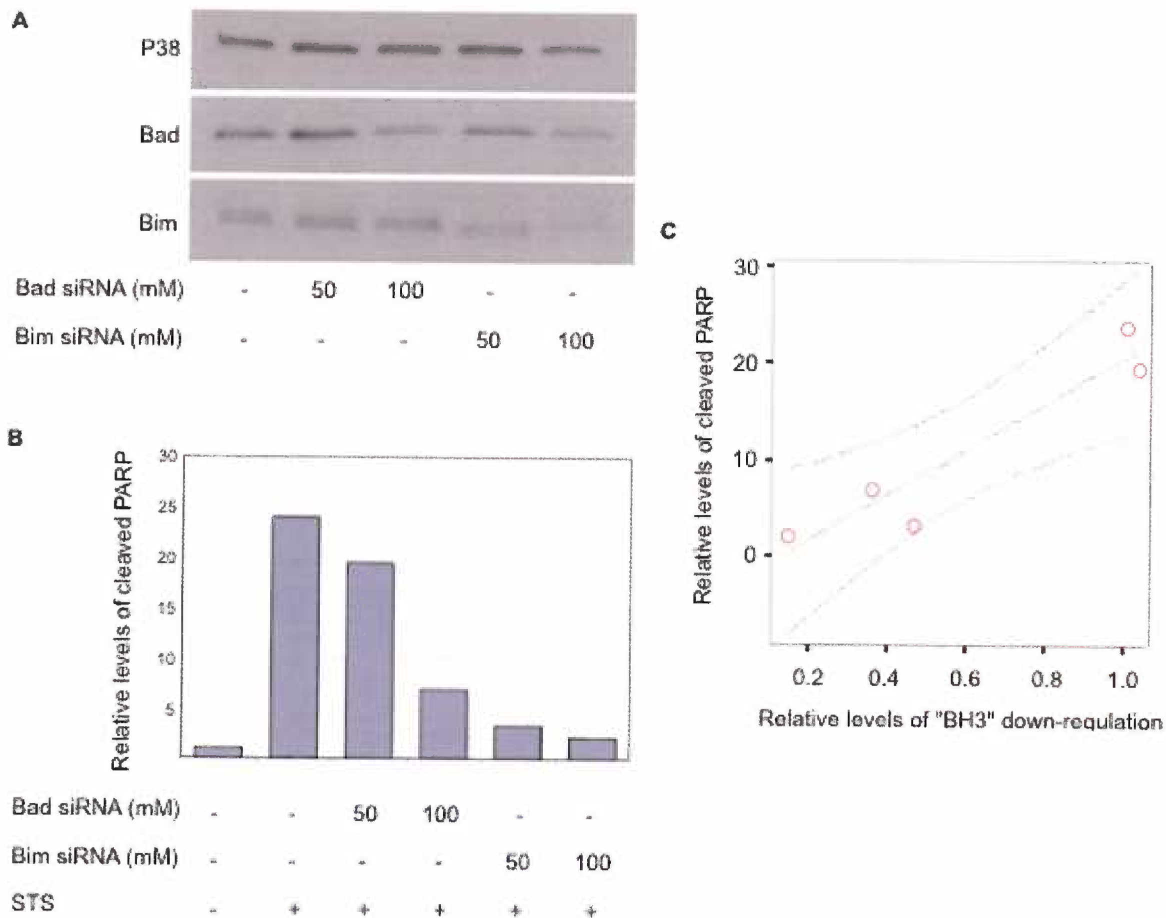

Figure 3-6. siRNA reduction of Bad and Bim levels and cytoprotection against STS-induced apoptosis.

(A) Representative immunoblot showing total Bad and Bim protein levels in uninfected T84 cells 72hr after transfection with 50 or 100 mM siRNA. Total P38 protein levels in each sample served as the internal control.

(B) Levels of cleaved PARP in uninfected T84 cells transfected with 50 or 100 mM siRNA for 72hr, followed by incubation with 1 mM STS for 4 hr to induce apoptosis. Cleaved PARP levels were determined by immunoblot and the signals were quantified by densitometry. Each signal was normalized to its internal P38 signal. Values represent cleaved PARP levels from one representative experiment.

(C) Linear regression analysis of cleaved PARP and relative levels of Bim and Bad downregulation, with a 95% mean prediction interval (R-square = 0.90).

Discussion

Infection with piliated *N. gonorrhoeae* induces a cytoprotective state within the host cell (Binnicker *et al.*, 2003; Howie *et al.*, 2005); a process that is amplified by the application of mechanical force on the host cell membrane by pilus retraction (Howie *et al.*, 2005). Retraction force also enhances infection-induced activation of a key survival kinase, ERK. We tested the hypothesis that gonococcal-induced, retraction-enhanced (GIRE) ERK activation is involved in host cell cytoprotection. Here we have demonstrated that inhibition of ERK signaling attenuates the ability of *N. gonorrhoeae* to protect host cells from STS-induced apoptosis (Figure 3-1). However, ERK inhibition does not completely abolish cytoprotection. This may be explained by either (a) incomplete inhibition of ERK following U0126 treatment, or (b) the involvement of ERK-independent GIRE cytoprotective pathways. Nevertheless, our results show that ERK is a key signaling molecule through which infection mediates cytoprotection.

In accordance with its role as a pro-survival signaling intermediate, ERK activation is known to influence the level and activity of numerous proteins involved either in promoting survival and/or inhibiting apoptosis. Here, we wished to determine the effects of ERK-dependent survival signaling by focusing on signaling pathways known to be rapidly modified by activated ERK. One of these pathways leads to the phosphorylation of the pro-apoptotic protein Bad at Ser112, and Ser155, a step that inactivates its apoptotic activity. We found Bad to be phosphorylated on Ser112 during infection in a

pilus retraction and ERK activation dependent manner. Surprisingly, we also found total Bad protein levels to be down-regulated during infection (Figure 3-2).

Induced degradation of total Bad protein levels was recently identified as a mechanism by which *Chlamydia* promotes cytoprotection in infected host cells (Dong *et al.*, 2005; Fischer *et al.*, 2004; Ying *et al.*, 2005). As Bad was previously thought to be primarily controlled at the level of phosphorylation, this down-regulation represented a novel mechanism for the regulation of this protein. *Chlamydia*-induced Bad degradation occurs very slowly (taking approximately 24 hours) and is mediated by the BH3 domain of the protein. Moreover, Chlamydia infection leads to the down-regulation of all host cell BH3-only proteins, not just Bad. In contrast, *Neisseria* infection specifically targets Bad and one other BH3-only protein, Bim for down-regulation (Figure 3-3). This down-regulation occurs rapidly: minimal protein levels are apparent by 2 hr post-infection, and reduced levels are maintained throughout a 6hr timecourse. Neisseria-induced down-regulation of both Bad and Bim is mediated by ERK activation, and is dependent on proteasomal activity (albeit via different mechanisms) (Figure 3-4). Importantly, while proteasomal involvement in Bim regulation has been well characterized, it is an entirely new component of Bad regulation. To our knowledge this mechanism of Bad regulation represents a completely novel means of apoptosis regulation via ERK signaling.

Bim degradation protects cells from apoptosis induction by UV, paclitaxel gluccocorticoids and other stimuli (Abrams *et al.*, 2004; Chae *et al.*, 2005; Han *et al.*, 2005; Harada *et al.*, 2004; Kuribara *et al.*, 2004; Li *et al.*, 2005; Liu *et al.*, 2005; Marani

et al., 2004; Sunters *et al.*, 2003; Tong *et al.*, 2005). Down-regulating Bad levels has a similar anti-apoptotic effect (Kang *et al.*, 2004; Sheng *et al.*, 2005; Yin *et al.*, 2005). Here, we have demonstrated that down-regulation of Bad or Bim through siRNA protects T84 cells against STS-induced apoptosis. What might be the advantage for *Neisseria* to down-regulate both Bim and Bad as a cytoprotective strategy during infection? First, different BH3-only proteins act as sentinels for different apoptotic stimuli. Bad is sensitive to growth factors and changes in basal activity of ERK, PI3-K or PKA. Bim is sensitive to cytokine withdrawal, calcium flux, and microtubule-destabilizing agents (Reviewed in Festjens *et al.*, 2004). Down-regulation of both Bad and Bim may therefore protect against a larger set of apoptotic stimuli than either could alone. Secondly, Bim and Bad belong to two distinct functional classes of BH3-only proteins (Letai *et al.*, 2002). Bim is classified as an "activator" BH3-only protein, which is capable of directly binding and activating Bax and Bak, leading to mitochondrial pore formation and cytochrome c release. Conversely, Bad is classified as a "sensitizer" BH3-only protein: it does not directly stimulate apoptosis, rather it sensitizes the cell to apoptotic stimuli by neutralizing BCL-2 like anti-apoptotic proteins. By down-regulating both Bad and Bim, two distinct mechanisms of apoptotic signaling are attenuated. Indeed, infection with *N. gonorrhoeae* almost completely blocks STS-induced release of cytochrome c from the mitochondria, which is indicative of impairment of mitochondrial membrane depolarization, a process mediated, in part, by BH3-only proteins (Figure 5 and Binnicker *et al.*, 2003).

The question remains as to what other pro-survival signaling pathways may be induced by infection with *N. gonorrhoeae* and/or enhanced by retraction of the type IV pilus. Certainly ERK activation is known to be involved in other cytoprotective signaling events, including the regulation of the inhibitor of apoptosis proteins (IAPs) and gene expression changes (Chang *et al.*, 2003a; Hu *et al.*, 2004). Other mechano-sensitive survival pathways also are likely to play a role in retraction-mediated survival. Of note is the PI3-kinase pathway, which has been shown to be activated in a pilus retraction-dependent manner. This pathway (a) controls the regulation of the Forkhead family (FKHR, FKHRL1 and AFX), IK-B kinase, mdm-2, CREB, IAPs and YAP via phosphorylation; (b) regulates Flice-inhibitory protein (FLIP) gene expression, which in turn prevents death receptor signaling via caspase 8; and (c) mediates the metabolic regulation of cell survival through inhibition of glycogen synthase kinase 3 (GSK) (Reviewed in Song *et al.*, 2005). Finally, *Neisseria* infection may regulate apoptosis via mechanisms completely independent of pilus retraction. These pathways have yet to be elucidated, but will likely be dependent on the virulence factors expressed on the infecting strain. Given the seemingly infinite adaptability of Neisseria as a species, it is likely that all of these pathways may play a role at some time during the infection process.

In summary, we have identified at least one mechanism by which the type IV pilus of *N. gonorrhoeae* induces a cytoprotective state in the host cell. Moreover, we have identified a novel mechanism of ERK-mediated cytoprotective signaling, i.e., the down-regulation of total Bad protein levels. The enzymatic steps responsible for such down-regulation

remain to be identified, however, we have demonstrated that both ERK activation and the proteasome are involved in this process. Future studies will elucidate not only this mechanism of down-regulation, but also other cytoprotective pathways activated by *Neisseria* infection and/or pilus retraction.

Materials and Methods

Reagents

Antibodies to PARP, cleaved-PARP, caspase-8, phospho-p44/42 MAPK (Thr202/Tyr204), p38 MAP kinase, BAD, phospho-BAD (Ser112), Bim, Bid, Bmf and Bok were purchased from Cell Signaling Technologies. Antibodies to cytochrome c were purchased from Molecular Probes and Calbiochem. SiRNA specific for Bim and Bad were purchased from Cell Signaling Technologies. Cytosol/Mitochondria fractionation kit was purchased from Caliochem. MEK inhibitor U0126 and proteasome inhibitor MG-132 were purchased from Calbiochem and used at a final concentration of 10μM unless otherwise stated. Staurosporine was purchased from Cell Signaling Technologies and used at a final concentration of 1μM.

Cell lines, bacterial strains and infections

T84 human colonic epidermoid cells (American Type Culture Collection) were maintained in DMEM-F-12 plus 5% heat-inactivated, filter-sterilized fetal bovine serum (FBS) at 37°C and 5% CO_2. For all experiments, cells were seeded into tissue culture dishes and allowed to become confluent prior to infection. GC strains N400, and N400*pilT* (Wolfgang *et al.*, 1998b) were used for all infections, and maintained on GCB agar plus Kellogg's supplements at 37°C and 5% CO_2. Piliation and Opa phenotypes were monitored by colony morphology. Only piliated, Opa^- bacteria were used. For infection experiments, bacteria were resuspended in GCB liquid medium and added to the epithelial cells at an MOI (multiplicity of infection) of 50.

Immunoblotting

T84 cells were infected with N400, N400*pilT* or treated with GCB media alone for specified times. Following infection, cells were lysed with 150μl 1X SDS lysis buffer (62.5mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50mM DTT, 0.1% w/v bromophenol blue), scraped into eppendorf tubes, vortexed for 15 seconds and immediately stored at -20°C. For PARP and caspase-8 assays, samples were incubated with 150μl cell lysis buffer (20mM Tris, pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 0.5% NP40, 2.5mM sodium pyrophosphate, 1mM β-Glycerolphosphate, 1mM Na_3VO_4, 1μg/ml Leupeptin) for 20 minutes on ice, followed by a 15-second sonication. For cytochrome c assays, the cells were processed as per the manufacturers instructions (Calbiochem). Samples were boiled for 5 minutes at 100°C, then separated by SDS-polyacrylamide gels and transferred onto nitrocellulose sheets. Membranes were probed with the specified antibodies following the manufacturer's protocol.

RNA isolation

Following infections, culture media was aspirated and replaced with buffer RLT (+ beta-mercaptoethanol). Cells were scraped off of plates, and transferred to qiashredder columns (Qiagen, Inc) to homogenize each sample. Samples were then stored at -80°C until further processing. After all samples had been frozen down, total RNA was isolated using the Qiagen RNeasy kit (Qiagen, Inc).

Real time RT-PCR analysis

1μg of total RNA (as isolated above) was reverse-transcribed to generate cDNA, using the iScript cDNA synthesis kit (BioRad). As a control, parallel samples were run in which reverse transcriptase was omitted from the reaction mixture. Quantitative real-time PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Amplification was carried out using Taqman master mix, and pre-designed TaqMan probes (GAPDH, Hs99999905_m1; Bim, Hs00197982_m1; Bad, Hs00188930_m1) according to the manufacturer's instructions (Assays on Demand, Applied Biosystems). Reactions were performed in triplicate in a 20μl volume, with the following cycle parameters: 95°C/10min enzyme activation, 95°C/15 seconds, 60°C/1min for 40 cycles. Data analysis was performed using the comparative Ct method (Applied Biosystems) to determine relative expression levels.

Immunofluoresence microscopy

T84 cells were grown on coverslips to 50% confluency and were infected with N400, N400*pilT* or treated with GCB media alone for 4hr. Samples were either fixed and analyzed at this time (see below) or were further treated by the addition of STS (1μM) for 4 hr. The media was then aspirated, and the cells fixed for 15 minutes at room temperature in 4% paraformaldehyde. Cells were blocked and permeabalized in isotonic PBS containing BSA (3%, w/v) and saponin (0.02% w/v) for 1 hour at room temperature, followed by staining with anti-cytochrome c antibody, fluoresceine-conjugated anti-mouse secondary antibody, and DAPI. Samples were rinsed extensively in PBS before mounting in Fluoromount-G (Fisher Scientific). Images were obtained with a Deltavision

Restoration Microscope (Applied Precision Instruments, Inc) fitted with a Nikon 60x oil immersion objective, and processed at a Silicon Graphics workstation with accompanying API software. The images were subsequently exported to Adobe Photoshop (version 7.0) and Adobe Illustrator (version 11.0) for manuscript preparation.

Gene Silencing using siRNA

Small-interfering RNAs were introduced into T84 cells using nucleofection, according to the manufacturer's instructions (Amaxa). Briefly, cells were grown to confluency in tissue culture flasks, then trypsinized and counted using a hemocytometer. For each nucleofection, $1x10^6$ cells were aliquoted into eppendorf tubes, and centrifuged at 1,500 rpm for 2min. Residual media was aspirated and cells resuspended in 100μl of nucleofector reagent R. The appropriate amount of siRNA was then added, and the cell mixture transferred to an electroporation cuvette. Cells were nucleofected using program T-16, then transferred to 1ml DMEM-F12 + 5% FBS and seeded into 1 well of a 12-well plate. Cells were assayed 24 or 72hr post-nucleofection.

Statistics

Statistical analysis was performed using standard t-test analysis with SPSS version 11.0 unless otherwise stated.

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Chapter 4

Discussion and Conclusions

Type IV Pilus Retraction Triggers Host-Cell Mechanotransduction

The data presented in this thesis demonstrate that retraction of the *Neisseria gonorrhoeae* type IV pilus stimulates mechano-sensitive signaling events within the infected host cell. One consequence of this signaling is the establishment of a cytoprotective environment. Importantly, all of the identified signaling events appear to be activated to a certain level by the initial stages of the infection process, and further enhanced by pilus retraction at later stages of infection. Thus pilus retraction acts as an amplifier of signals necessary for gonococcal infection.

As discussed in manuscript I, gonococcal infection induces the up- or down-regulation of ~300 epithelial cell genes at 3 hr post infection. Approximately 50 of these genes show enhanced up- or down-regulation in response to pilus retraction. The majority of these genes are known from other studies to be activated specifically by mechanical stress via MAPK activation. As with the retraction-enhanced gene expression changes, MAPK cascades were activated by *N. gonorrhoeae* infection and enhanced by pilus retraction. Moreover, these results could be recapitulated by employing a magnet and magnetic beads to artificially induce an upward pulling force on the epithelial cell. These findings suggest that the host cell senses pilus retraction as a mechanical force, and responds to this force by activating mechanically-sensitive signaling pathways.

Many of the identified gonococcal-induced and retraction-enhanced (GIRE) genes are known to function in cell survival signaling. Therefore, I tested the hypothesis that retraction-mediated mechanical force would protect cells from apoptotic events. Indeed,

staurosporine (STS) induced apoptosis was attenuated by gonococcal infection, with enhanced protection observed in the presence of pilus retraction or artificial force application. Once again this demonstrates an initial level of signaling mediated by gonococcal infection, and an enhancement by pilus retraction.

In manuscript II I show that inhibition of ERK prior to gonococcal infection attenuated the ability of the bacteria to protect host-cells from STS-induced apoptosis. GIRE ERK activation was found to induce the down-regulation and/or modification of two BH3-only proteins, Bim and Bad. To examine if decreased levels of Bim and Bad were sufficient to protect cell from cytoprotection, siRNA was used to artificially lower the levels of each protein prior to induction of apoptosis by STS. Indeed, by decreasing the levels of either protein, apoptosis was attenuated. Moreover, I show that this down-regulation of Bim and Bad blocks apoptosis at the level of mitochondria depolarization, by preventing the release of cytochrome c into the cytosol. The data presented in this manuscript suggest that one function of pilus retraction mediated mechanotransduction is to protect the host cell against apoptotic stimuli. Such cytoprotection may keep the host cell alive long enough for gonococcal replication and transcytosis.

In this chapter, I further examine the effects of pilus retraction on the host cell, by integrating the data presented in this thesis with recent findings from other studies of mechanical signaling and/or Neisseria-host cell interactions. Specifically, I focus on the mechanisms by which the host cell may sense pilus retraction, and other signaling events during gonococcal infection that may be attributable to pilus retraction mediated

mechanical force, such as calcium signaling and AKT/PI-3 kinase activation. This is followed by a discussion on additional anti-apoptotic pathways hypothesized to by activated by pilus retraction. Finally, I speculate on the nature of pilus retraction within a microcolony structure, as well as on the types of forces that such a structure may transduce to the host cell surface. Together these ideas provide important insights into the mechanistics behind pilus retraction mediated signaling.

Host Cell Sensing of Mechanical Signals

Most cells in the body can respond to mechanical stress by activating clearly defined signaling pathways. Such a response dictates that a cell possesses a means to sense this stress and translate the mechanical signal into the appropriate biological response. Many forms of mechanical stress can initiate such responses, including gravitational pressure, shear stresses caused by fluid flow, acoustic waves and contractile forces exerted from one cell to another (Janmey and Weitz, 2004). Importantly, the cellular response to such stresses is dependent not only on the type of stress, but also on the geometry and timecourse of such stress (Janmey and Weitz, 2004). A cell may respond differently to constant shear stress than to turbulent shear stress. Likewise, a strong, rapid pulse of mechanical perturbation may cause a different response than a weaker mechanical stress of longer duration. Due to this wide range of possible force mechanisms and responses to such forces, the mechanisms of sensing and transmitting such forces are likewise diverse. Applied forces may be distributed over various cellular components and at multiple loci including the extracellular matrix, the membrane lipid bilayer, integral membrane proteins and the cytoskeleton (Ko and McCulloch, 2000). Importantly, a mechanical

stimulus may activate more than one type of mechanosensor, with each mechanosensor capable of activating multiple downstream events (Apodaca, 2002). This section deals with possible mechanosensors of Tfp retraction, and the mechanisms by which they are thought to induce signaling cascades within the infected cell.

Mechanosensitive Ion Channels

The best-studied mechanosensor candidates are mechanosensitive (MS) ion channels. These channels are composed of specialized molecules in the plasma membrane that can undergo distortion in response to an external or internal mechanical stimulus, which either opens or closes a conductive pathway through the molecule (Sukharev and Corey, 2004). MS ion channels in non-sensory cells can be divided into two main classes – stretch activated or stretch inactivated. Further classifications are based on ion selectivity, and/or sensitivity to calcium, voltage, etc (Morris, 1990). The best studied MS ion channels include membrane K^+ channels (Olesen *et al.*, 1988), non-selective cation channels (MacRobbie, 1998; Nilius *et al.*, 1993; Ono *et al.*, 1997, 1998; Schwarz *et al.*, 1992a; Schwarz *et al.*, 1992b), and voltage gated Na^+ channels (Traub *et al.*, 1999).

MS ion channels can be directly regulated via two main types of gating; a) gating by forces conveyed through lateral tension in the membrane (Figure 4-1A), and b) gating by a mechanical stimulus transduced through elements tethered to the cytoskeleton (Figure 4-1B) (Sukharev and Corey, 2004). In addition, these channels can be indirectly regulated by force conveyed to a mechanically sensitive protein that does not form the channel (Figure 4-1C) (Lin and Corey, 2005). In this case, a second messenger carries the

signal to a ligand-activated channel. Regardless of the mechanism of activation, MS ion channel sensitivity is dependent on subcortical actin in most cases. It is thought that force induced actin restructuring "desensitizes" MS ion channels to subsequent force applications, in order to prevent the unrestricted flow of ions (such as calcium) into the cell (Ko and McCulloch, 2000). Indeed, agents that disrupt cytoskeletal integrity (cytochalasin-B) or decrease cytoskeletal tension (N-ethylmaleimide) *enhance* MS ion channel sensitivity to a mechanical stimulus (Glogauer *et al.*, 1998; Wan *et al.*, 1999).

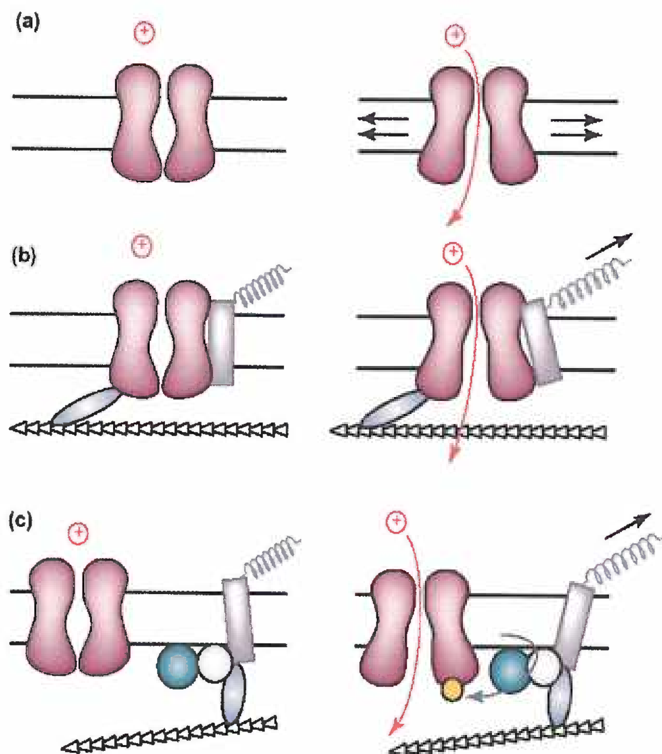

Figure 4-1 - Mechanisms for activation of ion channels by mechanical stimuli. (from Lin and Corey, 2005)

(A) Direct activation by force conveyed through lipid tension.

(B) Direct activation by force conveyed through structural proteins. Linking proteins might be intracellular, or extracellular, or both, and force might be parallel or normal to the membrane.

(C) Indirect activation by force conveyed to a mechanically sensitive protein that does not form the channel. A second messenger carries the signal to a ligand-activated channel.

Evidence for Tfp-retraction dependent activation of at least one MS ion channel, resulting in Ca^{2+} influx, has already been demonstrated (Ayala *et al.*, 2005). Additionally, given that cortical actin is robustly recruited to the site of microcolony formation in a Tfp-retraction dependent manner (Merz *et al.*, 1999), it is tempting to speculate that this restructured actin may play a role in desensitizing MS ion channels during infection.

That the retraction dependent calcium flux appears sustained, but of relatively low intensity, provides additional evidence for this argument (Ayala *et al.*, 2005).

Integrins

A second type of protein that can function as a mechanosensor in eukaryotic cells are integrins. Integrins are a family of transmembrane receptors that mediate cell attachment to extracellular matrix (ECM) proteins vitronectin, fibronectin, laminin and collagen, and are the main structure that physically connects the eukaryotic cytoskeleton to the underlying substratum (Plopper *et al.*, 1995). Integrins are heterodmeric proteins composed of non-covalently associated alpha and beta subunits. To date, 18 alpha subunits and 8 beta subunits have been identified that can heterodimerize with one another to produce over 24 distinct receptors with different binding specificities and affinities (Hynes, 2002; Shyy and Chien, 2002).

In addition to "tethering" functions, integrins also play a key role in regulating signaling pathways within the cell. This function is most often seen as a result of cell movement. Cells exert tractional forces on the substratum to which they are attached, through structures of proteins called focal adhesions (Harris *et al.*, 1980; Pelham and Wang, 1999). These tractional forces are conveyed from the cytoskeleton to the ECM via integrins, and vice versa (Huang and Ingber, 1999). Such tractional forces result in activation of integrins within the focal adhesion, resulting in the phosphorylation of two key adapter proteins, focal adhesion kinase (FAK) and Shc (Parsons and Parsons, 1997; Parsons *et al.*, 2000; Schaller, 2001; Schlaepfer and Hunter, 1998; Wary *et al.*, 1996;

Wary *et al.*, 1998). FAK mediates the phosphorylation of proteins involved in Rap/MAP kinase signaling (Ishida *et al.*, 1996; Schlaepfer and Hunter, 1997), Rho signaling (Ilic *et al.*, 1998), cell motility (Parsons *et al.*, 2000), and apoptosis (Frisch *et al.*, 1996; Hungerford *et al.*, 1996; Ilic *et al.*, 1998; Wen *et al.*, 1997). Shc mediates phosphorylation of proteins involved in the Ras/ERK pathway, thus mediating cell cycle progression (Wary *et al.*, 1998). Importantly, other mechanical stresses are thought to be transduced through the cytoskeleton as well, and thus activate integrins, and integrin mediated signaling in a similar manner.

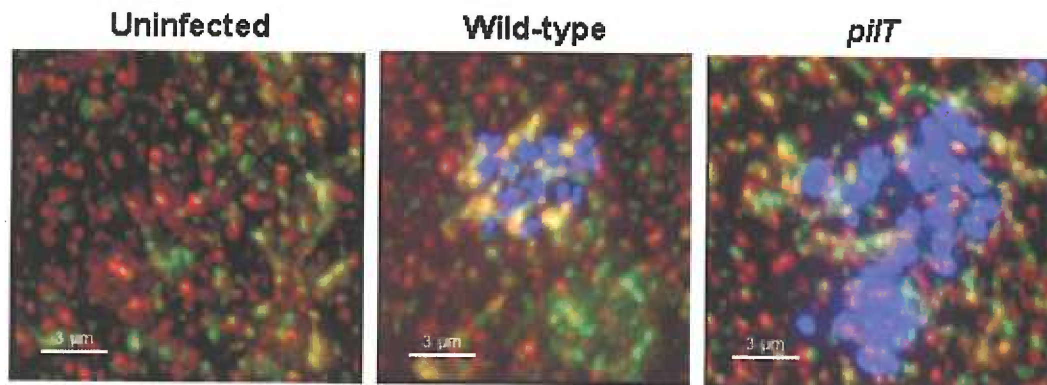

Figure 4-2 – Integrin and CD46 colocalize within cortical plaques

α5β1 integrin (green) and CD46 (red) are both recruited to the cortical plaques beneath bacteria (blue), in a *pilT* dependent mechanism.

As discussed in the introduction to this thesis, *N. gonorrhoeae* Tfp have recently been shown to bind integrins during bacterial attachment to urethral epithelial cells (Edwards and Apicella, 2005). Another pilus Tfp receptor, CR3 is known to oligomerize with integrins in the plasma membrane of primary cervical epithelial cells (Edwards *et al.*, 2002). Studies involving Group A Streptococcus (GAS) infection of epithelial cells have demonstrated that CD46 and α5β1 are coordinately engaged by the bacterium during infection (Rezcallah *et al.*, 2005). Similarly, I have demonstrated that during gonococcal

infection, α5β1 integrins are recruited to the site of microcolony formation, and colocalize with CD46 within the cortical plaques (Howie and So, unpublished data) (see Figure 4-2). Together, these data demonstrate that *N. gonorrhoeae* Tfp interact with integrins in numerous cell types, and suggest that pilus retraction may play a role in this interaction. As Tfp retraction has been shown to induce the signaling pathways known to be activated by integrin-mediated signaling, it is likely that this interaction plays a role in mechanosensing by the host cell.

G-Protein Coupled Receptors and Receptor Tyrosine Kinases

G-protein coupled receptors (GPCRs) are the largest family of cell surface receptors, and are characterized by the presence of seven transmembrane spanning regions. Receptor binding to its cognate ligand triggers a conformational change within the receptor, leading to an association of heterotrimeric G-proteins (composed of an α, β, and γ subunit). Upon this interaction, the G-protein undergoes its own conformational change, resulting in the release of GDP from the α-subunit, and subsequent binding of GTP. The Gα and Gβγ subunits then dissociate from the receptor and each other, and go on to stimulate downstream signaling cascades, resulting in the activation of various second messengers, enzymes and ion channels (Hall and Lefkowitz, 2002; Johnson and Friedman, 1993).

Receptor tyrosine kinases (RTKs) are single transmembrane-spanning receptors, composed of an extracellular ligand binding domain, and an intracellular catalytic domain. Ligand binding to the extracellular domain induces receptor dimerization, which

in turn leads to the activation of the catalytic domain, and subsequent autophosphorylation of specific tyrosine residues in the cytoplasmic portion of the receptor (Lemmon and Schlessinger, 1994). This autophosphorylation stabilizes the active receptor conformation, and creates phospho-tyrosine docking sites for adapter proteins containing SH2 (Src homology 2) and PTB (phosphotyrosine binding) domains. Signaling proteins which are known to bind these sites include RasGAP, PI-3 kinase, PLC, SHP, Shc, Grb2, and Crk (Pawson and Scott, 1997).

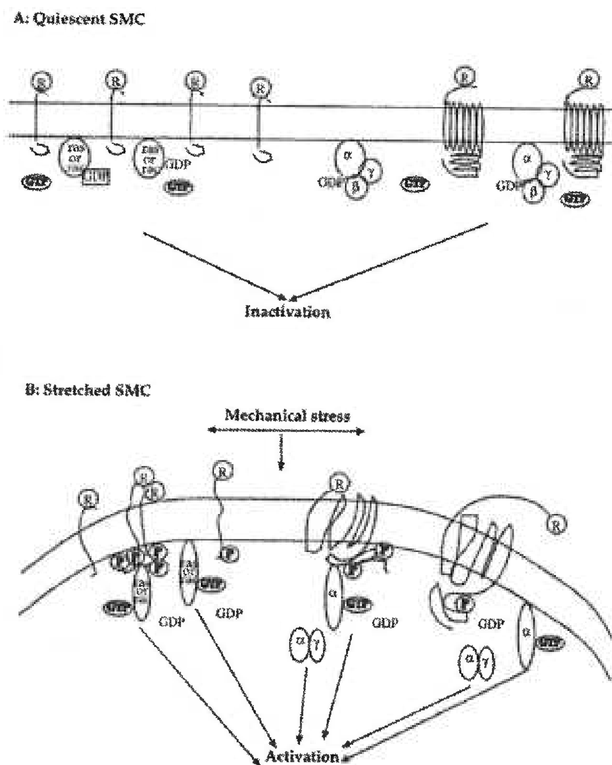

Figure 4-3 – Schematic diagram of proposed receptors and G proteins as mechanosensors. (Li and Xu, 2000)

(A) In quiescent cells, growth factor receptors and GPCRs are monomeric and inactive. Small molecular G proteins ras and rac bind GDP and α, β, and γ subunits from heteromeric G proteins bind to GDP, which are inactive.

(B) In stretched cells, increased elongational and translational mobility in membrane results in exposure of kinase domains of receptor and/or conformation of membrane-bound G proteins, which lead to receptor autophosphorylation or facilititating exchange of GDP for GTP, leading to activation of downstream targets.

Importantly, increasing experimental evidence suggests that both GPCRs and RTKs can be activated by mechanical stress (Figure 4-3). In endothelial cells, activation of G proteins has been reported to be one of the earliest detectable signaling events upon application of shear stress (Gudi *et al.*, 1998a; Gudi *et al.*, 1996; Gudi *et al.*, 1998b; Ishida *et al.*, 1997). Mechanical stress induced MAPK activity can be blocked with a

non-hydrolyzable GDP analog, suggesting the involvement of G proteins in this process (Tseng *et al.*, 1995). Evidence also suggests that stretch induced generation of IP3 and DAG seem to be dependent on G protein mediated stimulation of PLC (Ingber, 1993). Finally, it has been demonstrated that cyclic strain treatment of smooth muscle cells (SMCs) induces rapid phosphorylation of the RTK, PDGF-R (Li and Xu, 2000). These observations have led to the hypothesis that mechanical stress may directly perturb cell the surface or alter receptor conformation, thereby initiating downstream signaling pathways normally induced by activated receptors (Li and Xu, 2000).

Little is known regarding the involvement of GPCRs or RTKs during Neisseria infection. Two RTKs of the epidermal growth factor receptor family, ErbB1 and ErbB2 have been found to be recruited to cortical plaques underneath bacterial microcolonies (Hoffmann *et al.*, 2001; Merz *et al.*, 1999). ErbB2 was further found to be activated during infection, leading to subsequent phosphorylation of Src. Only one GPCR has been identified to play a role in gonococcal infection – Luteinizing Hormone Receptor (LHr). LHr was shown to be a receptor for non-piliated *N. gonorrhoeae* on Hec-1B cervical carcinoma cells (Spence *et al.*, 1997). In a subsequent study, it was shown that LHr was activated by infection, an event which ultimately promoted the trancytosis of the gonococci across the polarized epithelium (Spence *et al.*, 2002). Appendix 1 describes additional experiments that I performed to determine the role of LHr in gonococcal infection. These experiments demonstrate that LHr is clustered underneath the site of microcolony attachment, and provide evidence that a GPCR (possibly LHr) is activated during infection in a Tfp retraction dependent manner. Regardless of the lack of data on these

receptors with regard to gonococcal infection, the fact that they are modulated in a pilus retraction dependent manner suggests that they may be involved in mechanosensation.

Mechanical Stress Induced Signaling

In manuscript I, the ERK, JNK and P38 MAPK cascades were identified as three of the signaling pathways activated by pilus retraction induced mechanotransduction. These pathways were originally analyzed due to their connection to GIRE gene expression. However, not all of the GIRE genes were affected by MAPK activation. For example, CYR61 gene expression was not sensitive to MAPK inhibitors. Thus, additional signaling pathways must be activated by pilus retraction. Indeed, in addition to MAPK activation, numerous signaling events are known to be induced by mechanical stress. The following is a discussion of some of these potential signaling events with respect to gonococcal infection and the role that these pathways may play in the infection process.

Calcium Signaling

Mechanical force can initiate calcium signaling through a number of mechanisms (Figure 4-4). Membrane tension can induce a calcium influx from the external environment through the opening of stretch-activated ion channels or voltage-gated Ca^{2+} channels (as a result of stretch activated channel induced depolarization) (Davis *et al.*, 1992a; Davis *et al.*, 1992b; McCarron *et al.*, 1997). Internal calcium stores can be mobilized by membrane tension induced phospholipase C (PLC) activation. Activated PLC hydrolyses phosphotidylinositol 4,5-bisphosphate (PIP_2) to generate inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Berridge, 1993; Lee *et al.*, 1994). IP3, in turn, mobilizes

intracellular calcium (Berridge, 1993), while DAG, in the presence of calcium, activates protein kinase C (PKC) (Lee *et al.*, 1994). PKC activation can, in turn, modify the activity of a number of targets including cytoskeletal proteins, MAPKs and transcription factors (Gschwendt, 1999; Mochly-Rosen and Gordon, 1998; Yano *et al.*, 1999). Overall, calcium signaling can play a role in cell growth, differentiation, motility, apoptosis and necrosis. The specific responses induced are dependent on the total levels of calcium, the number and duration of calcium transients, and the point of origin of the calcium flux (Montero *et al.*, 2000).

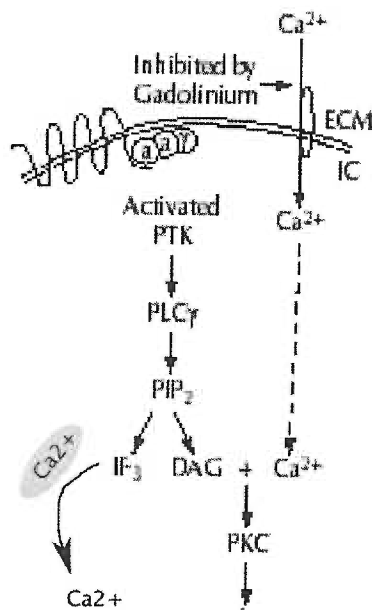

Figure 4-4 – Mechanisms of force induced calcium signaling (modified from Vlahakis and Hubmayr, 2003)

Mechanical force can induce calcium fluxes from both external and internal stores.

DAG, diacylglycerol; ECM, extracellular matrix; IC, intracellular; IP3, inositol 1,4,5-triphosphate; PIP2, phosphoinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; SRE, stretch response elements.

Infection of epithelial cells with *N. gonorrhoeae* induces two distinct calcium fluxes. The earliest calcium flux occurs within 1-2 minutes post-infection and is almost immediately inactivated (Ayala *et al.*, 2005; Muller *et al.*, 1999). This calcium spike is mediated by the bacterial protein porin, a trimeric ion channel associated with both the bacterial outer membrane and pili. Upon contact, porins insert into the host cell

membrane and form active, gated channels that trigger an influx of calcium from extracellular stores (Gotschlich *et al.*, 1987; Muller *et al.*, 1999). A second calcium flux occurs ~3-5 minutes post-infection, and unlike the porin induced calcium flux, has a relatively slow rate of decay. This calcium transient is mediated by the gonococcal type IV pili, and induces calcium release from intracellular stores (Ayala *et al.*, 2001; Ayala *et al.*, 2005; Kallstrom *et al.*, 1998). Importantly, this transient is also dependent on the initial flux of calcium mediated by porin insertion (Ayala *et al.*, 2005). Pilus retraction is involved in both calcium transients, as evidenced by a decrease in the intensity of both the porin- and pilin dependent fluxes during infection with a *pilT* mutant (Ayala *et al.*, 2005). It remains to be tested whether these pilus retraction-mediated effects are due to mechanical force or to additional adhesin/receptor interactions occurring after pilus retraction brings the bacterial and host cell membranes into close apposition. Future studies using artificial force application are needed to differentiate between these possibilities.

Recent studies have begun to elucidate the role of the porin- and pilus-dependent calcium transients in the infection process. The porin-induced transient has been shown to induce endosome exocytosis, while the pilus-mediated calcium flux triggers lysosome exocytosis (Ayala *et al.*, 2001; Ayala *et al.*, 2002). The two exocytic events promote bacterial intracellular survival, presumably by depleting the cell of Lamp1-containing degradative compartments (Hopper *et al.*, 2000; Lin *et al.*, 1997). Additional studies have begun to investigate calcium mediated PKC activation during infection (Ayala and So, unpublished results). Given the role of calcium signaling in PKC and MAPK activation,

it is possible that calcium transients induced during the early stages of infection also promote cytoprotective signaling within the host cell. Overall, calcium signaling is one of the earliest events during gonococcal infection, and ultimately sets the stage for survival of both the bacterium and host.

Phosphoinositide-3 Kinase Signaling

Phosphoinositide-3 (PI-3) kinase, like MAPK, is another central signaling molecule that is involved in numerous cellular processes like cell growth, motility, differentiation, survival and intracellular trafficking (Cantley, 2002; Foster *et al.*, 2003). PI-3 kinases are normally recruited to activated receptor tyrosine kinases (RTKs) at the plasma membrane and associate with the RTK via the PI-3 kinase regulatory subunit to become activated. While RTKs are normally activated by growth factor binding, recent evidence has shown that mechanical stress can mediate RTK activation as well (Li and Xu, 2000). In this manner, membrane tension leads to PI-3 kinase activation (Raucher *et al.*, 2000). It has also been demonstrated that shear stresses can signal through integrins to activate the protein Cbl. Activated Cbl then associates with the regulatory subunit of PI-3 kinase, rendering PI-3 kinase active (Miao *et al.*, 2002). In both cases, activated PI-3 kinase goes on to stimulate downstream signaling pathways.

N. gonorrhoeae infection induces the activation of PI-3 kinase within 30 minutes of infection, with sustained activation detectable for at least 3 hr (Lee *et al.*, 2005). During this time, PI-3 kinase activity is essential for bacterial infectivity, as inhibitors specific to PI-3 kinase can attenuate pilus-mediated invasion by roughly 50-80%. Importantly, PI-3

kinase activation was greatly enhanced by pilus retraction, suggesting that mechanical force is involved in this process. Indeed, when an artificial upward force was placed on host cell membranes using a magnet and magnetic beads, PI-3 kinase activation could be detected (Higashi and So, unpublished results).

Following activation, PI-3 kinase can stimulate the formation of [PI(3,4,5)P3]. During *N. gonorrhoeae* infection [PI(3,4,5)P3] becomes recruited to the site of bacterial attachment, and subsequently "flips" to the outer surface of the plasma membrane, where it is proposed to accelerate microcolony formation, and upregulate the pilus retraction gene *pilT* (Lee et al., 2005). As microcolonies form and enlarge, they generate more membrane tension, and sustain the activation of force-response signaling events. While the exact mechanism of [PI(3,4,5)P3] flipping has not been determined, a recent study identifying *Geobacter sulfurreducens* type IV pili as electrical conductors may provide a clue (Reguera *et al.*, 2005). This study demonstrated that Geobacter type IV pili are capable of transferring electrons from the bacterial cell surface to the substrate to which the bacteria are attached to. If gonococcal Tfp have a similar activity, they may be able to locally depolarize the host cell membrane, a process which is known to induce phospholipid translocation. Whether or not gonococcal or other type IV pili are capable of electrical conductance remains to be elucidated.

Potential Cytoprotective Signaling Pathways Induced by Pilus Retraction

As described in manuscript II, GIRE activation of ERK is involved in establishing a cytoprotective environment within the infected host cell. ERK was found to mediate the

downregulation of two pro-apoptotic BH3 only proteins Bad and Bim, as well as induce the phosphorylation (and subsequent inactivation) of the majority of the remaining Bad in the cytosol. However, ERK is also known to influence apoptotic signaling by additional pathways not investigated in this study. Moreover, the other force induced signaling pathways described above are known to play a role in cytoprotective signaling as well. Thus, while Bad and Bim downregulation are important for GIRE cytoprotection, we hypothesize that a number of other signaling events are also involved in this process. This section provides a brief overview of apoptotic signaling events, as well as the possible sites of pilus-retraction mediated cytoprotection.

Overview of Apoptotic Signaling Pathways

Apoptosis is an evolutionarily conserved mechanism that selectively removes ageing, damaged, or otherwise unwanted cells (Kerr *et al.*, 1972). Developmental processes are also controlled by apoptotic events. Most forms of apoptosis are mediated by caspases (cysteinyl, aspartate-specific proteases), which become activated in a hierarchial order when cells are stimulated by death signals (Algeciras-Schimnich *et al.*, 2002; Bratton *et al.*, 2000; Salvesen and Dixit, 1997; Stegh and Peter, 2001). Apoptotic stimuli activate "initiator caspases" which, in turn, cleave and activate "effector caspases." Effector caspases go on to degrade a number of intracellular proteins, leading to the characteristic biochemical and morphological changes typically associated with apoptosis (Saraste and Pulkki, 2000; Strasser *et al.*, 2000; Van Cruchten and Van Den Broeck, 2002). In mammalian cells, caspase cleavage is initiated by two separate pathways; the "extrinsic pathway" also known as the death receptor pathway, and the "intrinsic pathway," known as the mitochondrial pathway (Figure 4-5) (Abe *et al.*, 2000; Ozoren and El-Deiry, 2003;

Strasser *et al.*, 2000; Thorburn, 2004). These two pathways ultimately converge at the point of effector caspase activation to mediate the final stages of cell death.

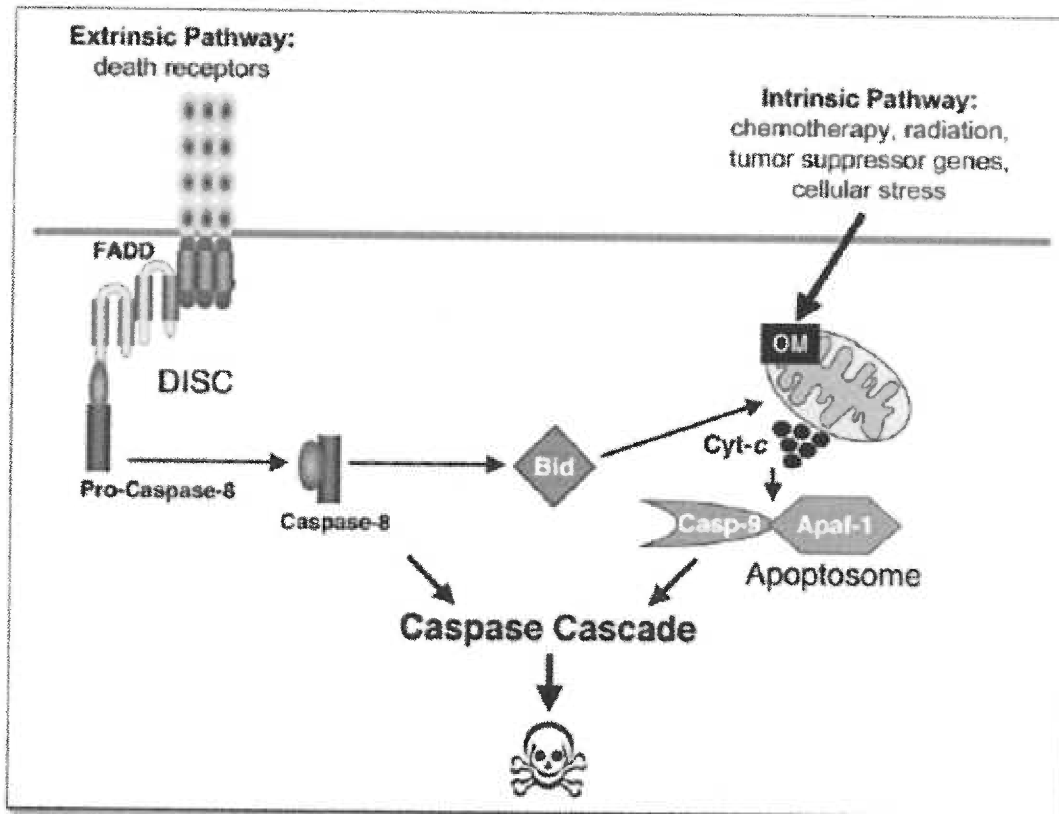

Figure 4-5 – An overview of the extrinsic and intrinsic apoptosis pathways

The extrinsic pathway is dependent on the interaction of certain ligands (i.e. FasL, TNFα and TRAIL) with "death receptors" (CD95/Apo-1, TNF receptors and TRAIL receptors respectively) (Abe *et al.*, 2000; Ashkenazi and Dixit, 1999; Ozoren and El-Deiry, 2003; Peter and Krammer, 2003; Schulze-Osthoff *et al.*, 1998; Thorburn, 2004). Once these ligands bind to their cognate death receptors, the death receptors trimerize and stimulate the recruitment of adapter molecules. Both the death receptors and the recruited adapter molecules contain a globular interaction domain, called the Death Domain (DD) through

which interaction occurs. Recruited adapter molecules also contain another protein-protein interaction domain, called the Death Effector Domain (DED), which interacts with pro-caspase 8/10. Together, this complex of recruited proteins is termed the Death Inducing Signaling Complex, or DISC (Ashkenazi and Dixit, 1998; Boatright *et al.*, 2003). Once formed, the DISC promotes the proximity-induced activation of caspase-8, which in turn cleaves downstream effector caspases to complete the apoptotic process (Salvesen and Dixit, 1999; Yang *et al.*, 1998).

The intrinsic pathway is regulated by mitochondrial membrane integrity (Green and Kroemer, 2004; Kroemer, 2003; Newmeyer and Ferguson-Miller, 2003; Ravagnan *et al.*, 2002; Zamzami and Kroemer, 2001). In this pathway, cellular "stresses" such as UV, heat shock and DNA damage are sensed by a group of cytosolic proteins (termed BH3-only proteins), which, in turn, transduce these stress signals to the mitochondria (Borner, 2003; Bouillet and Strasser, 2002). These signals trigger an increase in the permeability of the mitochondrial outer membrane, allowing the escape of proteins normally trapped in the mitochondrial inner membrane space (Bernardi *et al.*, 1999; Loeffler and Kroemer, 2000). These proteins, including cytochrome c, Omi/Htr2A, Smac/Diablo, AIF and endoG, function to activate apoptosis inducing factor-1 (Apaf-1) and assist in the formation of the apoptosome (Li *et al.*, 2001; Susin *et al.*, 1999; Verhagen *et al.*, 2000; Verhagen *et al.*, 2002). Like the DISC in the extrinsic pathway, the apoptosome functions as a platform for protein recruitment, ultimately facilitating the self-activation of pro-caspase 9, the initiator caspase of the intrinsic pathway (Acehan *et al.*, 2002).

Importantly, the intrinsic and extrinsic pathways are not isolated from one another. Rather, their component signaling molecules engage in a fair level of crosstalk. Extrinsically activated caspase 8 can mediate the cleavage of Bid, a BH3-only protein that functions to induce increased permeabilization of the mitochondrial membrane (Luo *et al.*, 1998). Likewise, intrinsic activation of effector caspase-6 can induce caspase-8 cleavage as a means to amplify apoptosis signaling (Philchenkov, 2004). Thus, activation of one pathway does not exclude the involvement of other pathways in promoting apoptosis.

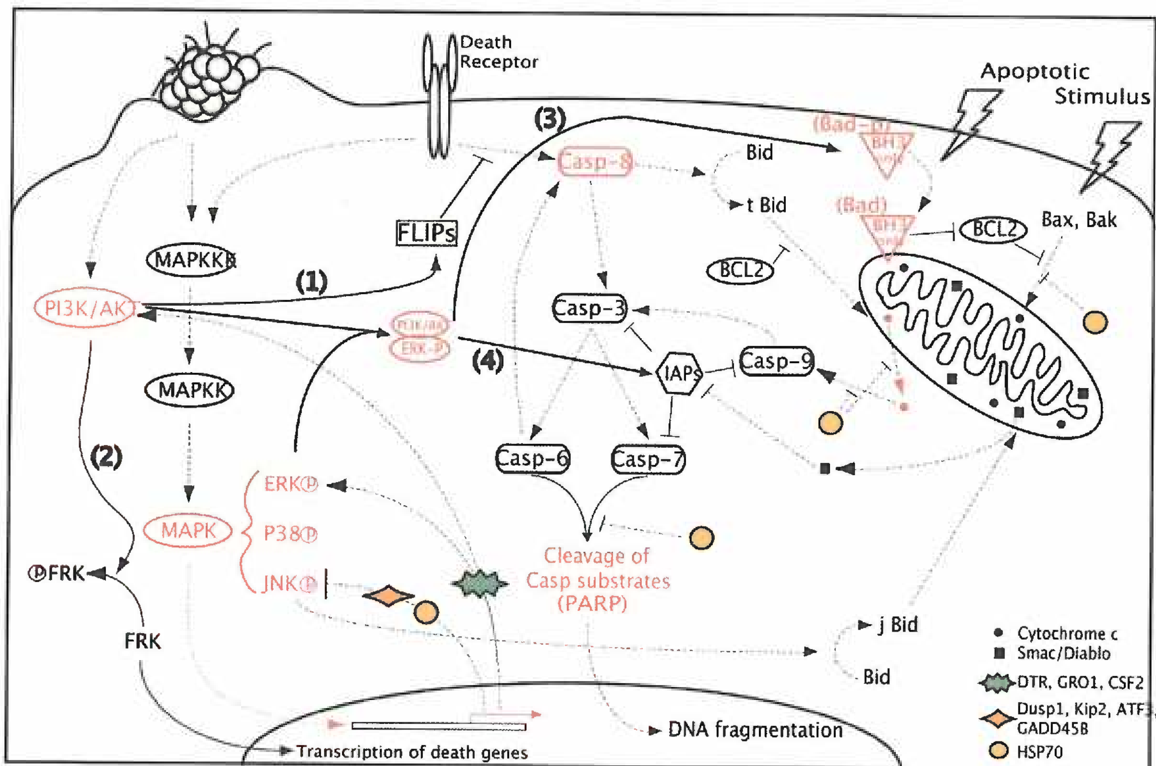

Figure 4-6. Potential cytoprotective signaling pathways induced by pilus retraction

Solid lines: pathways affected by PI-3K/Akt and/or ERK. Dotted lines: all other pathways. Green puff: site of regulation by pilT-enhanced DTR, GRO1 and CSF2. Brown diamond: site of regulation by pilT-enhanced DUSP1, Kip2, ATF3, GADD45B. Orange circle: site of regulation by *pilT*-enhanced HSP70. Red highlights indicate signaling pathways already examined within this thesis or by others.

Regulation of FLICE-Like Inhibitor Protein (Figure 4-6, pathway 1)

FLIP (FLICE-like inhibitory protein) is structurally related to the initiator caspases of the extrinsic apoptosis cascade, caspase-8 and caspase-10 (Goltsev *et al.*, 1997; Han *et al.*, 1997; Hu *et al.*, 1997; Inohara *et al.*, 1997; Irmler *et al.*, 1997; Rasper *et al.*, 1998; Shu *et al.*, 1997; Srinivasula *et al.*, 1997). Unlike caspase-8 or -10 FLIP contains an enzymatically inactive DED, negating its ability to interact with DED containing adapter proteins. However, FLIP can still dimerize with caspases-8 and -10. This dimerization prevents the proximity-induced activation of caspases-8 and -10, thus rendering FLIP a dominant-negative inhibitor of apoptosis.

Activation of the PI-3K/Akt signaling pathway induces the up-regulation of FLIP gene expression (Panka *et al.*, 2001; Skurk *et al.*, 2004; Suhara *et al.*, 2001). Higher levels of FLIP can more thoroughly compete with caspase 8 during dimerization, thus inhibiting downstream death receptor signaling via caspase-8 (Nam *et al.*, 2003; Skurk *et al.*, 2004). *N. gonorroheae* infection and pilus retraction activate the PI-3K/Akt pathway, predicting that infection and/or the mechanical stress of pilus retraction will up-regulate FLIP.

Regulation of Forkhead Proteins (Figure 4-6, pathway 2)

The forkhead family of proteins are a group of ~40 mammalian transcription factors (Burgering and Medema, 2003). The proteins are characterized by a highly conserved winged-helix DNA binding domain, termed the forkhead box, thus the proteins are abbreviated FOX (Forkhead Box) (Kaestner *et al.*, 2000; Weigel and Jackle, 1990). FOX transcription factors play important roles in apoptosis, cell proliferation, differentiation,

tissue-specific gene expression, embryogenesis and neoplasia (Carlsson and Mahlapuu, 2002). Based on phylogenetic analysis, the FOX members are divided into 17 sub-families, named FOXA through FOXQ (Kaestner *et al.*, 2000).

The FOXO subfamily has been specifically implicated in the induction of apoptosis by inducing the transcription of the pro-apoptotic proteins FasL, Bim, Bcl-6, TRAIL and TRADD (Brunet *et al.*, 1999; Dijkers *et al.*, 2000; Modur *et al.*, 2002; Rokudai *et al.*, 2002; Schmidt *et al.*, 2002; Stahl *et al.*, 2002; Tang *et al.*, 2002). FasL and TRAIL are ligands for death receptors (Fas receptor and TRAIL receptor, respectively) that stimulate the activation of the extrinsic apoptosis pathway (see above). TRADD is one of the DED-containing adapter molecules recruited to an activated death receptor, which helps mediate caspase 8/10 auto-activation. Bim is a BH3-only protein that senses cell stress and triggers loss of mitochondrial membrane integrity (see chapter 3). Bcl-6 is a strong transcriptional repressor that negatively regulates the expression of the antiapoptotic protein Bcl-X_L (Burgering and Medema, 2003).

The FOXO subfamily is regulated in a post-translational manner by the PI-3K/Akt pathway (Burgering and Medema, 2003). Activation of PI-3K/Akt mediates the phosphorylation of the three members of the FOXO sub-family (FKHR, FKHRL1 and AFX) (Biggs *et al.*, 1999; Brunet *et al.*, 1999; Kops *et al.*, 1999). This phosphorylation leads to recruitment and binding of 14-3-3 proteins to FOXO, which, in turn, causes the translocation of FOXO from the nucleus to the cytosol (Burgering and Kops, 2002; Downward, 2004). Sequestration from the nucleus prevents FOXO from acting as a

transcription factor of the pro-apoptotic proteins described above. Once again, as *N. gonorroheae* infection and pilus retraction activate the PI-3K/Akt pathway, it is hypothesized that infection and/or mechanical stress of pilus retraction will in turn induce the phosphorylation of all three FOXO proteins, mediate FOXO association with 14-3-3-proteins, and subsequently stimulate FOXO relocalization from the nucleus to the cytosol.

Regulation of Inhibitor of Apoptosis Proteins (IAPs) (Figure 4-6, pathway 4)

IAPs are a family of anti-apoptotic proteins that function to block cell death by inhibiting the activity of caspase activation pathways (Gewies, 2003; Salvesen and Duckett, 2002; Schimmer, 2004). Eight human IAPs have been identified, based on the presence of 1-3 baculovirus IAP repeat (BIR) domains and a conserved zinc-binding region (Takahashi *et al.*, 1998). Some IAPs also contain a RING finger motif, which possesses E3 ubiquitin ligase activity (Gewies, 2003). This domain allows the IAPs to catalyze their own ubiquitination, targeting themselves and any associated proteins for proteasomal degradation (Huang *et al.*, 2000; Suzuki *et al.*, 2001; Yang *et al.*, 2000).

IAPs function by directly binding to and inhibiting the activity of caspases-3, -7 and/or -9 (Gewies, 2003; Salvesen and Duckett, 2002; Schimmer, 2004). Some IAPs, like XIAP, bind to and inhibit all three of these caspases (Deveraux *et al.*, 1998; Deveraux *et al.*, 1999). Other members of the IAP family bind only 1 or 2 of these caspases; cIAP1/cIAP2 and NAIP inhibit caspase-3 and -7 (Deveraux *et al.*, 1998), ML-IAP inhibits caspase-3 and -9 (Vucic *et al.*, 2000), ILP2 inhibits only caspase-9 (Richter *et al.*,

2001). The functions of the last two members of the IAP family, Survivin and Bruce, have not been characterized.

Activated PI-3K/Akt and ERK regulate a number of IAPs. Transcription of cIAP2, XIAP and Survivin are upregulated by activation of the PI-3 kinase pathway (Beierle *et al.*, 2005; Hu *et al.*, 2004; Wang and Greene, 2005). ERK and PI-3 kinase act in concert to stabilize XIAP levels, in part through the phosphorylation of XIAP at Ser-87 (Dan *et al.*, 2004; Gardai *et al.*, 2004). This stabilization prevents the auto-ubiquitination of XIAP, and subsequent proteasomal degradation. As *N. gonorrhoeae* infection induces the activation of both ERK and PI-3 kinase in a pilus retraction manner, it is hypothesized that infection with wild-type gonococci will lead to these effects on IAP transcription and stability.

Regulation by GIRE Genes

Eight of the GIRE genes identified in chapter 2 are known to be specifically anti-apoptotic. DTR, GRO1 and CSF2 modulate signaling through PI-3K/Akt and ERK (Kotone-Miyahara *et al.*, 2004; Reynolds *et al.*, 2002; Watson and Fan, 2004). DUSP1, Kip2, ATF3, HSP70 and GADD45B all inhibit JNK activation (Chang *et al.*, 2003a; Mizuno *et al.*, 2004; Nakagomi *et al.*, 2003; Papa *et al.*, 2004; Park *et al.*, 2002).

Of all anti-apoptotic GIRE genes identified however, Heat-shock protein (Hsp) 70 is perhaps the most important, in that the protein inhibits apoptosis at a number of critical points in the apoptosis cascade (Beere, 2004). Within the intrinsic pathway of apoptosis,

Hsp70 can cooperate with two additional Hsps to inhibit Bax translocation to the mitochondria (a function similar to that normally provided by Bcl-2) (Gotoh *et al.*, 2004). If mitochondrial membrane permeabilization does occur, Hsp70 can cooperate with Hsp40 to prevent the release of cytochrome c (Mosser *et al.*, 2000). Finally, if any cytochrome c release occurs, Hsp70 can function to inhibit the formation of a functionally competent apoptosome, either by inhibiting apoptosome oligomerization (Saleh *et al.*, 2000), or inducing an apoptosome conformation that is unable to recruit caspase 9 (Beere *et al.*, 2000). Within the extrinsic pathway of apoptosis, Hsp70 can prevent cleavage and activation of Bid in response to TNF binding to its cognate death receptor (Gabai *et al.*, 2002b). This vast array of anti-apoptotic signaling potential has caused some to call Hsp70 an effective 'multi-hit' suppressor of apoptosis (Beere, 2004). As this is one of the highest regulated genes identified in chapter 2, it is likely that this protein plays an important role in cytoprotective signaling during gonococcal infection.

The Nature of Pilus Retraction and Microcolony-Induced Mechanical Force

Other than the direct measurement of the force induced by a single pilus retraction event (Merz *et al.*, 2000), little is known about the nature of pilus retraction events from a single bacterium or an organized microcolony. Do multiple pili on a single bacterium retract at random time intervals, retract in unison, or retract in a coordinated manner? What about pilus retraction from a microcolony? Is the hypothesized "upward pulling force" the only force induced on the host cell membrane by a microcolony? These questions are important, as mechano-transduction within a cell is sensitive to the nature of the applied force. Constant membrane tension, random pulling events, periodicity and

the direction of force induction induce different cellular responses. By determining the nature of pilus retraction and force induction from a single bacterium and a microcolony, we will gain a more precise understanding of the host cell responses due to this applied force. Below is a discussion of preliminary data and experiments designed to answer these questions.

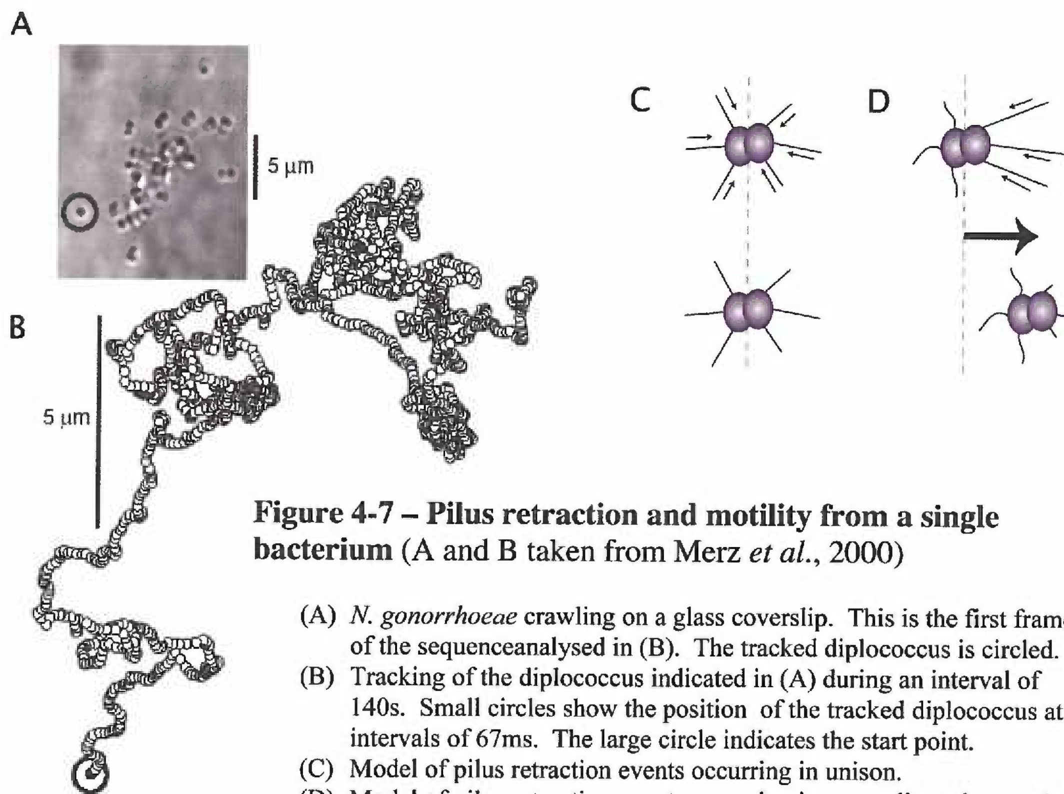

Figure 4-7 – Pilus retraction and motility from a single bacterium (A and B taken from Merz *et al.*, 2000)

(A) *N. gonorrhoeae* crawling on a glass coverslip. This is the first frame of the sequenceanalysed in (B). The tracked diplococcus is circled.
(B) Tracking of the diplococcus indicated in (A) during an interval of 140s. Small circles show the position of the tracked diplococcus at intervals of 67ms. The large circle indicates the start point.
(C) Model of pilus retraction events occurring in unison.
(D) Model of pilus retraction events occurring in a coordinated or random fashion.

Pilus Retraction: Random Events or a Coordinated Process

From video microscopy studies, single bacteria can be seen twitching along the surface of a glass coverslip (Merz *et al.*, 2000). While not necessarily a directional movement, the bacteria are still making forward progress; i.e. they do not remain "stuck" in the same place (Figure 4-7A and B). Pili appear to cover the entire surface of the gonococcus.

Thus, if all of the pili on a bacterium were retracting in unison, there would be a net force vector of zero; i.e. the pulling forces from pilus retraction on one side of the diplococcus would cancel out the pulling forces from pilus retraction on the other side of the diplococcus, resulting in no net movement (Figure 4-7C). If, however, retraction is coordinated such that only the pili on one side of the diplococcus retract, then the net force vector would be in the direction of this retraction, and the bacterium would move forward (Figure 4-7D).

However, as gonococci do not move solely in one direction, this cannot be the whole story. Either different sections of the diplococcus must "take turns" retracting their pili, or pilus retraction occurs over the entire diplococcus in a random, but non-unison manner. As retraction events from a single pilus are spaced by ~20 seconds, both mechanisms are possible. Moreover, given the tendency for long stretches of movement in one direction, followed by a period of "tumbling-like" movement (see Figure 4-7B), it is possible that both of these mechanisms are coordinately used to effect motility.

Given the ambiguity of the mechanism of pilus retraction from a single diplococcus, the mechanism of retraction from within a microcolony becomes even more complicated. In addition to the variables of retraction from individual diplococci, a group of diplococci in a microcolony structure can either undergo pilus retraction as individual entities, or may undergo pilus retraction in a globally coordinated manner. Microscopy studies have provided evidence that microcolonies, like individual diplococci, can travel substantial distances over a solid surface (Higashi and So, unpublished results). This suggests that

retraction must be somehow coordinated (in a non-unison manner), as discussed above for a single bacterium. As discussed in Chapter 2, the nature of the host cell response to pilus retraction may give some clue as to the nature of pilus retraction from a microcolony. Extended, repetitive mechanical force increases the expression of genes encoding cytoprotective heat shock proteins and lowers the number of apoptotic cells in a culture (Barkhausen *et al.*, 2003). Suppression of apoptosis requires permanent membrane tension or rhythmic, pulsatile forces (Graf *et al.*, 2003), which are thought to allow the cell to adapt to new environmental conditions. These observations provide additional evidence that pilus retraction is not random within the microcolony structure.

In order to test the hypothesis of coordinated pilus retraction from an individual gonococcus and a microcolony, retraction events from all sides of the bacterium and/or microcolony must be able to be assayed simultaneously. To this end, we are designing a microscopic "retraction detector" in collaboration with Dr. Jack McCarthy (Oregon Graduate Institute), to detect such retraction events (Figure 4-8). The detector is composed of 0.5 x 1 x 7μm PDMS cantilevers arranged in a fence-like pattern, to enclose a 5μm x 5μm area (for detection of pilus retraction from single diplococci, Figure 4-8A and B), and a 15μm x 15μm area (for detection of pilus retraction from a microcolony, Figure 4-8C and D). The ends of the cantilevers are coated with Au-Pd, to give high contrast in the optically transparent wells. Individual bacteria and/or microcolonies are allowed to fall into the "fenced" area. We expect pili to then bind to the cantilevers. Pilus retraction would result in cantilever bending. Deflection, or bending of a cantilever can be detected using light microscopy, with a 3μm deflection corresponding to a force of

~500pN (Jack McCarthy, personal correspondence). Deflections of lesser magnitude would, in turn, represent retraction events of lesser force. Thus, this device will allow us to monitor the nature of retraction events (i.e. coordinated vs. random), and the relative force of such events. By understanding how pilus retraction occurs in a bacterium and/or microcolony, we can better understand how *N. gonorrhoeae* move, and how retraction influences the infection process.

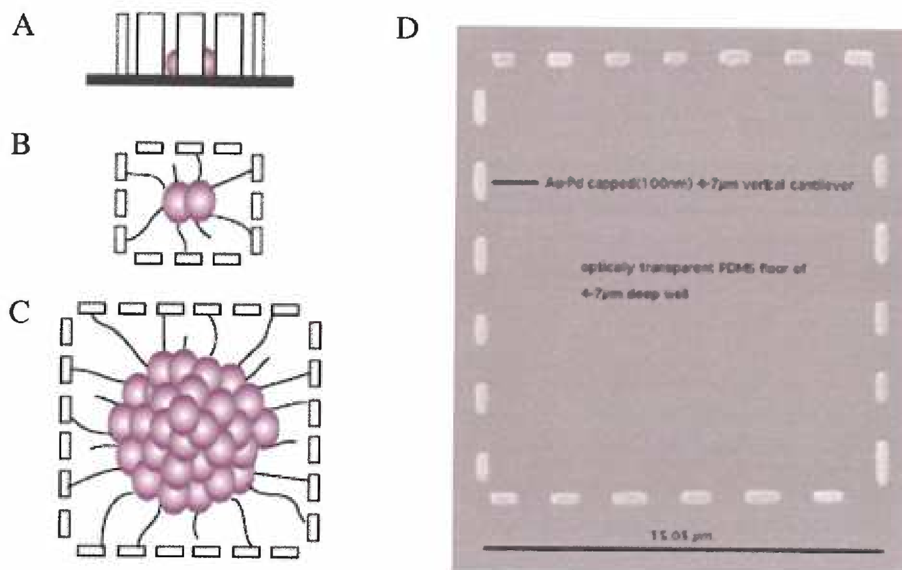

Figure 4-8 – Pilus retraction "detector"

(A) Side view of the fenced in area of the 5μm x 5μm detector, with relative location of a single diplococcus.
(B) Aerial view of the 5μm x 5μm detector with relative location of a single diplococcus.
(C) Aerial view of the 15μm x 15μm detector with relative location of a microcolony.
(D) Actual SEM of a manufactured fence.

Pushing and Pulling Forces

As discussed in Chapter 1, pilus retraction from a microcolony is hypothesized to induce a circumferential upward pulling force on the host cell membrane in the range of 1.9×10^4 – 3.3×10^4 pN. While no direct evidence of such force has been demonstrated, the results

presented in this thesis and elsewhere together provide compelling evidence that the host cell does indeed experience some form of applied force. This force has been hypothesized to be in the upward direction at the radial circumference from the center of the microcolony (Lee, 2003)

Appendix 2 provides further evidence that an upward pulling force induces host cell responses to infection. As stated earlier, retraction of a single pilus can exert forces up to 20–100 pN on its substrate (Maier *et al.*, 2002; Merz *et al.*, 2000). Forces of lesser magnitude can promote cytoskeleton rearrangements and protein clustering (Choquet *et al.*, 1997; Sheetz *et al.*, 1998), such as cortical plaque formation during infection. Pilus retraction has therefore been speculated to induce cortical plaque formation by exerting mechanical tension on the membrane (Merz and So, 2000). The data presented in Appendix 2 demonstrate that, indeed, an upward force of as little as 30pN could induce actin recruitment to the site of force application (Figures A2-1, A2-2 and A2-3). This recruitment is more robust if the amount or duration of applied force is increased (Figure A2-4 and A2-5). These results provide further evidence that cortical plaque formation is mediated by mechanical stress.

Importantly, this upward pulling force may not be the only force mediated by the gonococcal microcolony. In fact, the presence of an upward pulling force *requires* there be an opposing force of equal intensity. By re-examining the "model of radial membrane tension" we can assume that this force is directed downward, through the tangent point where the microcolony contacts the host cell (see Figure A2-6). Thus pilus retraction

from a microcolony structure should induce *both* an upward "pulling" force at the circumference of the microcolony, and a downward "pushing" force directly underneath the microcolony structure. Such distribution of force may explain why some cortical plaque constituents are preferentially recruited directly underneath the microcolony, while others are generally recruited to the microcolony circumference during infection.

Indeed, experiments presented in Appendix 2 demonstrate that different cortical plaque constituents are recruited to the site if bacterial attachment by each type of force (Figure A2-7). An upward pulling force induces a small amount of cortical actin rearrangement, and an intense recruitment of ezrin to the site of infection. Conversely, a downward pushing force induces robust actin accumulation, but no ezrin recruitment. These data suggest that, as hypothesized, circumference-localized proteins are recruited specifically by upward tension, whereas proteins localized directly below the bacterial microcolony are likely recruited specifically by the opposing pushing force.

Given the evidence that these two equal but opposite forces are induced during infection, it is tempting to speculate that other host cell responses, such as the activation of signaling cascades, are affected differently by each type of force. Indeed, the microarray results from Chapter 2 may already provide preliminary evidence of this. CYR61, one of the identified GIRE genes, is well known for its ability to be upregulated by mechanical stress (Lau and Lam, 1999; Leu *et al.*, 2002; Tamura *et al.*, 2001). However, CYR61 gene expression could not be recapitulated by use of the "upward force" inducing magnetic bead assay. While, many explanations may exist for this aberrant result, it is

possible that CYR61 expression is induced by a downward pushing force, rather than the upward force induced by the magnet. How this force-specific activation of signaling cascades and recruitment of proteins ultimately affects the infection process remains to be seen.

Conclusions

The data presented in this thesis demonstrate that Tfp retraction is sensed by the host cell as a mechanical force. The host cell responds to retraction mediated force by activating mechano-sensitive signaling cascades and rearrangement of the cortical cytoskeleton and other constituents of gonococcal induced cortical plaques. The ultimate outcome of these events is the establishment of a cytoprotective environment for the bacterium and its host.

Importantly, these data implicate bacterial-mediated mechanical stress as a new virulence determinate of gonococcal infection. Without this force, few piliated *N. gonorrhoeae* are capable of invading the host cell. Those that do invade must struggle to survive and replicate within a hostile environment. Thus if one can identify chemical(s) that inhibit pilus retraction, they may provide a potential non-antibiotic therapeutic treatment for infections caused by *Neisseria gonorrhoeae*, *Neisseria meningitidis* and other bacteria that use Tfp during infection.

Given the number of bacteria that harbor retractile Tfp, the implications for mechanical force involvement in infectious disease are broad. For example, Tfp retraction plays a role in *Pseudomonas aeruginosa* infection, which causes lung infections in cystic fibrosis

patients and burn victims. Tfp retraction is also involved in *Actinobacillus actinomycetemcomitans* pathogenesis, which can lead to periodontal disease. Thus, the results presented in this thesis will provide important insights into the infection process and host response to infection of numerous pathogens.

Appendix 1

Lutenizing Hormone Receptor: A Potential Mechanosensor of Type IV Pilus Retraction

Summary

Mechanotransduction is the process by which eukaryotic or prokaryotic cells translate mechanical signals into downstream signaling events. These mechanical signals are typically detected by mechanically-sensitive proteins (mechano-sensors), which in turn direct the appropriate down-stream signaling events. Previously, we provided evidence that host cells sense mechanical stress from pilus retraction during *N. gonorrhoeae* infection, and subsequently alter a number of mechano-sensitive signaling pathways. Here we examine one possible mechanosensor of *N. gonorrhoeae* type IV pilus retraction, luteinizing hormone receptor (LHr). We show that LHr mRNA and protein are expressed in a number of cell lines typically used to study *N. gonorrhoeae* infection. Additionally, we demonstrate that LHr is recruited to the site of bacterial attachment. Finally, we provide evidence suggesting that at least one G-protein coupled receptor, possibly LHr is activated during infection, as shown by the recruitment of β-arrestin-2 to cortical plaques. Together, these data provide important insights into how pilus retraction-induced mechanical stress may be transduced during *N. gonorrhoeae* infection.

Introduction

Neisseria gonorrhoeae Type IV pili (Tfp) are retractile appendages that facilitate adhesion and invasion of the host epithelium. Retraction of a single gonococcal pilus can exert forces between 20–100 pN on its substrate (Maier *et al.*, 2002; Merz *et al.*, 2000). A number of recent studies have provided indirect evidence that this retraction-mediated force is directly transferred to the host cell membrane as a mechanical signal. Pilus retraction has been implicated in cytoskeletal rearrangements (Merz *et al.*, 1999), calcium signaling (Ayala *et al.*, 2005), PI-3 kinase/AKT signaling (Lee *et al.*, 2005), gene expression changes, and MAPK activation (Howie *et al.*, 2005). Importantly, all of these signaling events can be induced by mechanical forces of much less magnitude than that of a single pilus retraction event (Choquet *et al.*, 1997; Feng *et al.*, 1999; Glogauer *et al.*, 1995; McCormick *et al.*, 2003; Ohki *et al.*, 2002; Sheetz *et al.*, 1998; Wasserman *et al.*, 2002; Wu *et al.*, 1999).

The observation that the host epithelium can respond to mechanical stress by activating mechano-sensitive signaling pathways suggests that these cells possess a sensor capable of transducing such mechanical perturbations into downstream biochemical signaling events. A number of potential cellular mechanosensors have been identified, including stretch activated ion channels, integrins, G-protein coupled receptors (GPCRs), receptor tyrosine kinases (RTKs) and caveoli (Li and Xu, 2000). As infection with *N. gonorrhoeae* is known to induce signaling via all of these receptors, it remains unclear

what the specific mechanosensor(s) are. Here we describe one potential mechanosensor for *N. gonorrhoeae* pilus retraction; Luteinizing Hormone Receptor (LHr).

LHr is a member of the rhodopsin/ β2-adrenergic receptor subfamily of GPCRs. The receptor was first identified as a constituent of gonadal tissues (testis and ovaries), where it functions as a receptor for the gonadotropins luteinizing hormone (LH) and human chorionic gonadotropin (hCG) (Ascoli *et al.*, 2002; Rao, 2001). In both males and females, LH stimulates the secretion of sex steroids (i.e. testosterone and estrogen) from the gonads (Catt and Dufau, 1991). In females, an LH "surge" induces ovulation of mature follicles in the ovary, while hCG functions in maintaining the uterine lining during pregnancy (Catt and Dufau, 1991; Richards and Hedin, 1988).

While originally thought to be expressed solely in gonadal tissues, there is increasing evidence of extensive non-gonadal expression of the receptor. To date, LHr has been found to be expressed in fallopian tubes, gametes, oviducts, uterus, placenta, cervix, fetal membranes, umbilical cord, brain, spinal cord, neural retina, skin, bladder, bone, prostate, seminal vesicles, sperm and breast tissue (Ascoli *et al.*, 2002; Rao, 2001). The role of LHr mediated signaling in non-gonadal tissue is unknown in most cases.

Expression of LHr in the fallopian tubes has been shown to vary with the menstrual cycle (Lei *et al.*, 1993; Segaloff and Ascoli, 1993). Minimal surface LHr levels are seen in the proliferative phase due to receptor mediated endocytosis caused by the mid-cycle LH surge. Surface LHr levels then increase through the secretory phase, as LH levels

decrease. Importantly, epidemiological studies have provided evidence that fluctuations of hormones during the menstrual cycle might influence the susceptibility of these tissues to gonococcal invasion, as the incidence of gonococcal pelvic inflammatory disease (PID) is clustered during the first week of menses (Eschenbach and Holmes, 1979; Sweet *et al.*, 1986). Thus, the increased expression of LHr immediately proceeds the temporal increase observed with gonococcal PID.

Despite these observations, the importance of LHr in *N. gonorrhoeae* infection has remained an issue of controversy. LHr was shown to be a receptor for non-piliated *N. gonorrhoeae* on Hec-1B cervical carcinoma cells (Spence *et al.*, 1997). In a subsequent study, it was shown that LHr was activated by infection, an event which ultimately promoted the trancytosis of the gonococci across the polarized epithelium (Spence *et al.*, 2002). However, LHr expression on Hec-1B cells has not been definitively shown, and the presence of the receptor on other cell types used for the study of *N. gonorrhoeae* infection remains speculative. Here we examine the mRNA and protein expression of LHr in a number of cell types, and demonstrate that the receptor is expressed in T84 cells, conjunctival cells, and Hec-1B cells. Moreover, we show that LHr is recruited to the site of bacterial attachment, and is activated in a pilus retraction dependent manner. These results pave the way for future studies of the role of LHr during Neisserial infection.

Results

Expression of LHr in Cells Used to Model Gonococcal Infection

The previous identification of LHr as a receptor for *N. gonorrhoeae* was largely based on indirect evidence; the addition of increasing amounts of hCG to the culture media was capable of inhibiting adhesion of the gonococcus to a number of cell lines derived from the reproductive tract (Gorby *et al.*, 1991; Spence *et al.*, 1997). As LHr was classically thought to be expressed only on gonadal tissues, critics of this study questioned the presence of the receptor on the epithelial cells used. Recent studies however have begun to identify LHr as being expressed in a wide variety of tissues (Ascoli *et al.*, 2002; Rao, 2001). Moreover, the microarray experiment described in Chapter 2 of this thesis, was able to detect LHr transcript from both infected and uninfected T84 cells (data not shown). To further address this issue of LHr expression, we examined LHr mRNA and protein levels in a number of cell lines used for the study of *N. gonorrhoeae* pathogenesis.

Total RNA was isolated from T84, A431, Hec1B, End-1, Conjunctival, Cos-7 (negative control) and granulosa cells (positive control). LHr mRNA levels were examined by use of both semi-quantitative RT-PCR, and real-time RT-PCR. Both methods detected LHr transcript in the conjunctival, and granulosa cells. The more sensitive real-time RT-PCR assay also detected LHr transcript in T84 and Hec-1B cells. Neither method detected transcript in the Cos-7 negative control. Next, immunoblots were performed on total cell lysates from a culture of each cell line, to determine the presence or absence of LHr protein in each type of cell. LHr protein was detected in T84, Conjunctival and Hec-1B

cells. Finally, LHr expression was examined by using indirect immunofluorescence microscopy in T84 and Hec-1B cells. As with the immunoblot analysis, LHr signal was detected in both cell lines (Figure A1-1). Together, these results indicate that LHr is expressed in some of the cell lines used for *N. gonorrhoeae* research, including the Hec-1B cells used in the original study that identified LHr as a gonococcal receptor. However, not all cell lines infected by *N. gonorrhoeae* express LHr (see Table A1-1 for a summary of expression data).

	A431	T84	End1	Conj	Hec-1B	Cos-7	Granul
Semi-quant RT-PCR	-	-	-	+	-	-	+
Real-time RT-PCR	-	+	-	+	+	-	+
Immunoblot	-	+	-	+	+	-	N/A
Immunofluorescence	N/A	+	N/A	N/A	+	N/A	N/A

Table A1-1 – LHr expression in cell lines used for *N. gonorrhoeae* infection

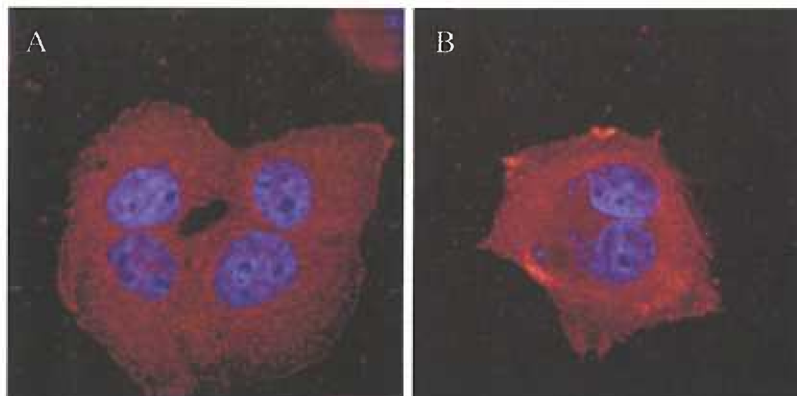

Figure A1-1 – Immunofluorescence detection of LHr in epithelial cells

(A) LHr expression in Hec-1B cells
(B) LHr expression in T84 cells

Localization of LHr During Infection

A number of receptors utilized by *N. gonorrhoeae* during the infection process are recruited to the cortical plaques underneath the site of bacterial attachment. We therefore examined the location of LHr in Hec-1B cells following a 3hr infection using indirect

immunofluorescence microscopy (Figure A1-2). In uninfected cells, LHr was distributed over the surface of the entire cell. In wild-type infected cells, the same pattern of staining was present, with the exception of an absence of signal in the area directly underneath bacterial attachment. An examination of the epitope recognized by the antibody revealed it to be in the binding pocket of the receptor. Thus, this antibody would not stain LHr bound to its ligands hCG, LH and likely *N. gonorrhoeae*. The absence of an LHr signal underneath the site of bacterial attachment therefore suggests that the bacteria do in fact bind this receptor and make the epitope inaccessible to antibody. Interestingly, this negative staining pattern is not present in *pilT*-infected cells, suggesting that the association between LHr and the gonococcus is dependent on pilus retraction.

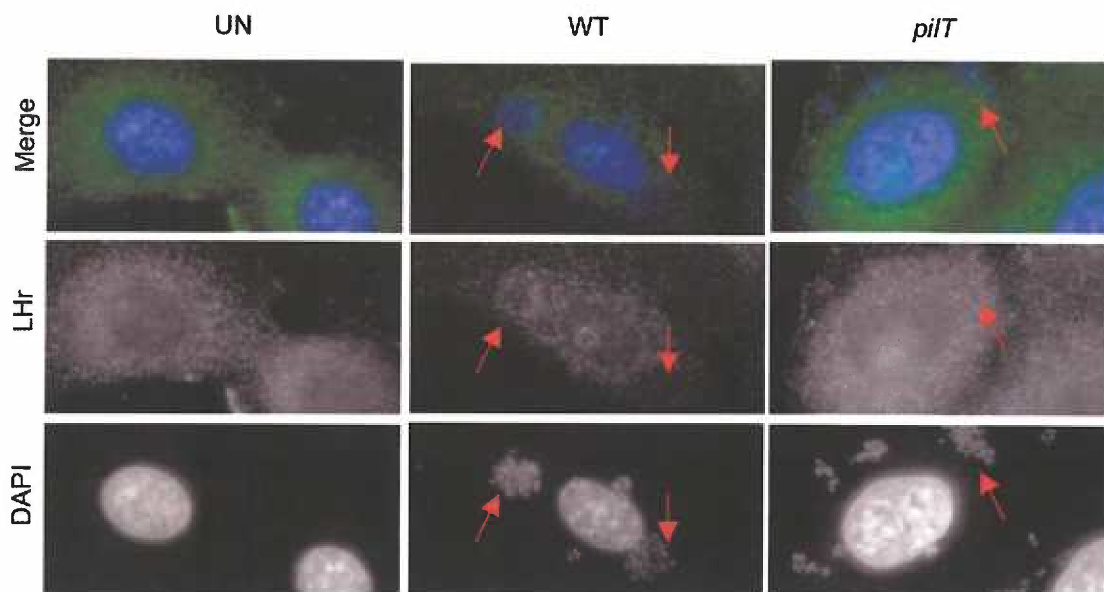

Figure A1-2 – LHr localization during infection with *N. gonorrhoeae*

Immunofluorescence microscopy showing LHr staining in uninfected Hec-1B cells and Hec-1B cells that have been infected for 3 hr with either wild-type *N. gonorrhoeae* (WT), or a *pilT* mutant (*pilT*). Red arrows indicate the sites of bacterial microcolonies.

As an alternative approach to study LHr location within infected cells, we used cells transfected with a myc-tagged LHr. This construct contains a myc tag at the N-terminus

of the receptor, and allows for the detection of the protein via an antibody specific to myc. Previous studies using this construct have demonstrated that the receptor traffics normally within the cell, and retains its function when exposed to hCG (Min and Ascoli, 2000; Min *et al.*, 2002). For our studies, myc-LHr was transfected into Hec-1B cells, and these cells were then either left uninfected, or infected with wild-type or *pilT* gonococci (Figure A1-3). As above, in uninfected cells the myc signal was evenly dispersed over the cell surface (data not shown). However, in wild-type infected cells, LHr appeared to cluster at the site of bacterial attachment. Importantly, like most other cortical plaque constituents, this clustering was dependent on pilus retraction, as infection with a *pilT* mutant did not induce clustering of LHr.

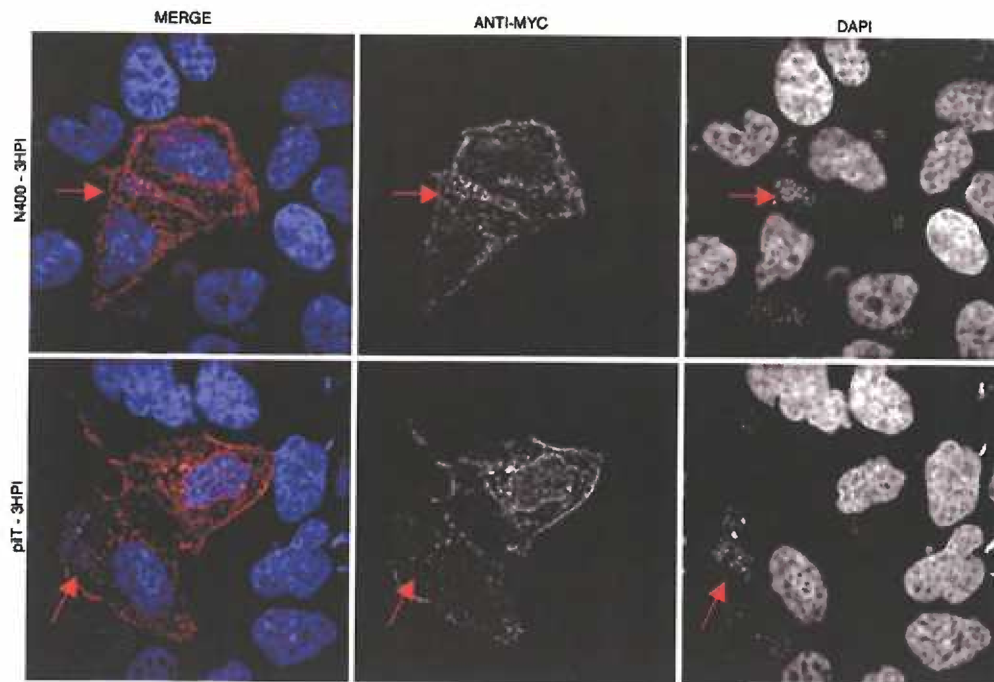

Figure A1-3 – Localization of myc-tagged LHr during infection with *N. gonorrhoeae*

Immunofluorescence microscopy showing myc-LHr staining in Hec-1B cells transfected with a myc-LHR construct that have been infected for 3 hr with either wild-type *N. gonorrhoeae* (WT), or a *pilT* mutant (*pilT*). Red arrows indicate the sites of bacterial microcolonies.

LHr Activation During Infection

G-protein coupled receptors, like LHr, become activated when bound by their cognate ligand. With regards to LHr, this activation can lead to the stimulation of a number of downstream signaling pathways such as cAMP/PKA, PKC, and MAPK (Ascoli *et al.*, 2002; Rao, 2001). One of the most immediate signaling events is the recruitment of β-arrestin-2 to the activated receptor. β-arrestin-2 works in conjunction with G-protein coupled receptor kinases (GRKs) to mediate receptor desensitization, ultimately limiting the duration of receptor activation (McDonald and Lefkowitz, 2001).

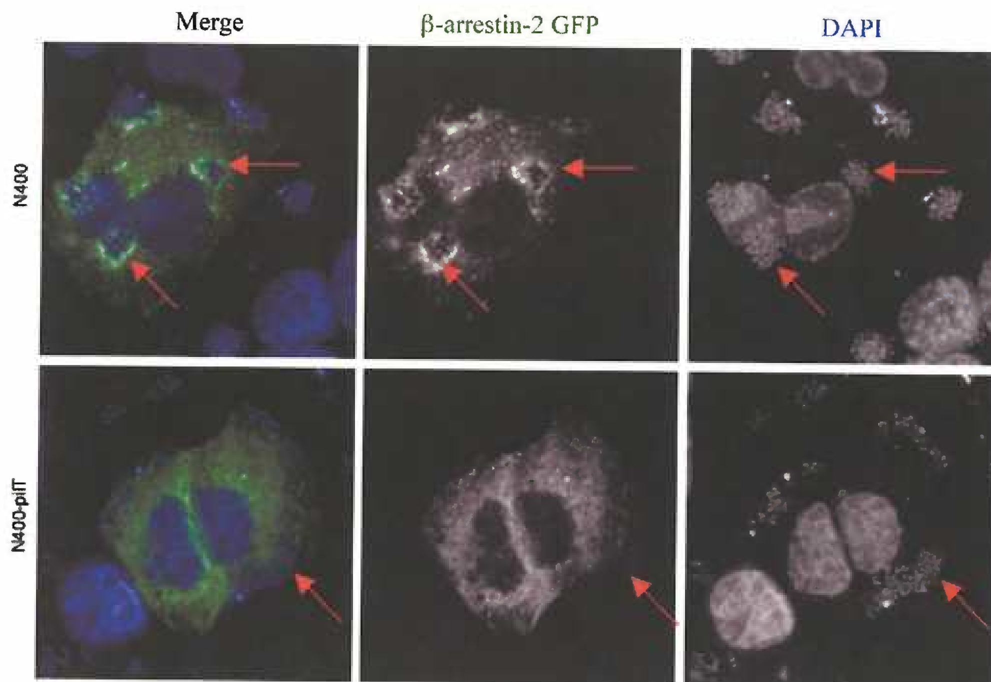

Figure A1-4 – Localization of GFP-tagged β-arrestin-2 during infection with *N. gonorrhoeae*

Immunofluorescence microscopy showing GFP staining in Hec-1B cells transfected with a β-arrestin-2-GFP construct that have been infected for 3 hr with either wild-type *N. gonorrhoeae* (WT), or a *pilT* mutant (*pilT*). Red arrows indicate the sites of bacterial microcolonies.

Given the evidence that *N. gonorrhoeae* binds LHr in a manner similar to hCG, we hypothesized that this interaction would activate the receptor to mediate downstream signaling events. To test this, we transfected β-arrestin-2-GFP into Hec-1B cells, and examined the localization of the protein in uninfected and infected cells (Figure A1-4). In uninfected cells, the GFP signal was evenly dispersed throughout the cell (data not shown). In wild-type infected cells, β-arrestin-2-GFP was dramatically recruited to the bacterial microcolony. This recruitment was absent when cells were infected with a *pilT* mutant. These data suggest that infection activates a G-protein coupled receptor, and that this activation is dependent on pilus retraction.

To better understand the relationship between LHr and β-arrestin-2, we co-transfected Hec-1B cells with myc-LHR and β-arrestin-2-GFP. Indirect immunofluorescence microscopy was performed on wild-type infected cells to determine if the two proteins co-localize following recruitment to the cortical plaque (Figure A1-5). Both myc-LHr and β-arrestin-2 –GFP were recruited to the area of bacterial attachment following a 3 hr infection. Specifically, myc-LHr appears to be recruited throughout the entire area directly underneath the bacterial microcolony, whereas β-arrestin-2 –GFP appears to be recruited to the circumference of the microcolony. Qualitative analysis of colocalization identified areas of overlapping pixels, which are represented as a white color in Figure A1-5. In order to specifically quantitate the amount of colocalization, the image was imported into CoLocalizer Pro, and the Manders overlap coefficient (MOC) was calculated. This coefficient gives values between 0 and 1, with values closer to 1 indicating perfect colocalization. Uninfected parts of the image had a MOC of 0.4988,

while areas containing a microcolony had an MOC of 0.749 ± 0.042. Thus, the MOC for infection is ~50% higher than that of the uninfected areas of the cell. Together, these data indicate that some of the myc-LHR and β-arrestin-2-GFP colocalize, however it is far from a complete phenomenon.

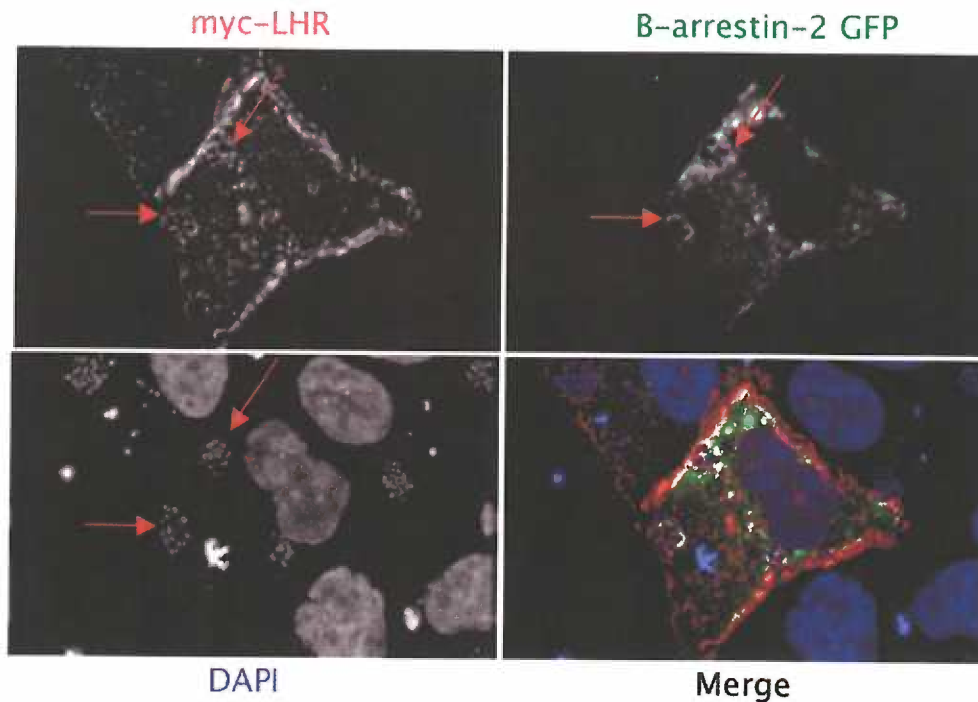

Figure A1-5 – colocalization of myc-LHR and β-arrestin-2-GFP during infection with *N. gonorrhoeae*

Immunofluorescence microscopy showing myc (red) and GFP (green) staining in Hec-1B cells co-transfected with a myc-LHR construct and a β-arrestin-2-GFP construct that have been infected for 3 hr with wild-type *N. gonorrhoeae.* Red arrows indicate the sites of bacterial microcolonies.

Discussion

Evidence provided in this thesis suggests that the host cell senses mechanical stress from pilus retraction during *N. gonorrhoeae* infection. The mechanism by which this force is sensed and transmitted to the intracellular milieu is unknown. Here we examined one possible mechanosensor of *N. gonorrhoeae* type IV pilus retraction, the GPCR luteinizing hormone receptor (LHr). Initial studies examining LHr have shown the protein to be a receptor for non-piliated *N. gonorrhoeae* on Hec-1B cervical carcinoma cells (Spence *et al.*, 1997).

Here we have shown that LHr mRNA and protein are expressed in a number of cell lines typically used to study *N. gonorrhoeae* infection (Table A1-1 and Figure A1-1). Additionally, we have demonstrated that LHr is recruited to the site of bacterial attachment in a *pilT* dependent manner (Figure A1-3). Previous studies of LHr demonstrated that the receptor is activated by gonococcal infection (Spence *et al.*, 2002). Indeed, we provide evidence that LHr activation occurs during infection, as β-arrestin-2 is recruited to gonococcal induced cortical plaques, and at least partially colocalizes with LHr (Figure A1-4 and A1-5). Not all of the β-arrestin-2 colocalizes with LHr however, indicating that other GPCRs may be activated during gonococcal infection as well.

Together these data have provided evidence of a potential mechanosensor for gonococcal Tfp retraction. While this study is not yet complete, it demonstrates that LHr is expressed in cell lines used to study gonococcal infection in the lab. Additionally, it suggests that LHr and possibly other GPCRs are activated by pilus retraction.

Materials and Methods

Reagents

Antibodies against LHR were purchased from Biogenesis (Poole, England); antibodies against c-myc were purchased from Invitrogen. Myc-LHR was a gift from Dr. M. Ascoli (University of Iowa). The β-arrestin-2-GFP construct was a gift from Dr. M. Caron (Duke University).

Cell lines, bacterial strains and infections

T84 human colonic epidermoid cells (ATCC) were maintained in DMEM-F-12 plus 5% heat-inactivated, filter-sterilized fetal bovine serum (FBS) at 37°C and 5% CO_2. End1 endocervical epithelial cells (ATCC) were maintained in EpiLife media with Keratinocyte growth supplement and 0.4mM $CaCl_2$ (Cascade Biologics). Conjunctival cells were a gift from Dr. R. Bonnah, and were maintained in EpiLife media with Keratinocyte growth supplement and 0.4mM $CaCl_2$ (Cascade Biologics). A431 human epidermoid carcinoma cells, Hec-1B human endometrial adenocarcinoma cells and Cos7 monkey kidney fibroblasts were maintained in DMEM plus 10% heat-inactivated filter-sterilized fetal bovine serum (FBS) at 37°C and 5% CO_2. Granulosa cells were a gift from S. Borman (OHSU), and were isolated as described (Molskness *et al.*, 1991). T84, End1, A431, Hec-1B, and Cos-7 cells were all purchased from the American Type Culture Collection (Rockville, Md) For all experiments, cells were seeded into tissue culture dishes and allowed to become sub-confluent prior to infection. GC strains N400, and N400*pilT* (Wolfgang *et al.*, 1998b) were used for all infections, and maintained on GCB agar plus Kellogg's supplements at 37°C and 5% CO_2. Piliation and Opa

phenotypes were monitored by colony morphology. Only piliated, Opa^- bacteria were used. For infection experiments, bacteria were resuspended in GCB liquid medium and added to the epithelial cells at an MOI (multiplicity of infection) of 50.

Transfections

Transfections of myc-LHr and β-arrestin-2-GFP were performed using FuGene 6 reagent (Roche-diagnostics) as per manufacturer's directions.

Immunoblotting

Cultures of the indicated cell lines were lysed with 150μl 1X SDS lysis buffer (62.5mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50mM DTT, 0.1% w/v bromophenol blue), scraped into eppendorf tubes, vortexed for 15 seconds and immediately stored at -20°C. Samples were boiled for 5 minutes at 100°C, then separated by SDS-polyacrylamide gels and transferred onto nitrocellulose sheets. Membranes were probed with anti-LHr antibodies as per the manufacturer's protocol.

RNA isolation

Culture media was aspirated and replaced with buffer RLT (+beta-mercaptoethanol). Cells were scraped off of plates, and transferred to qiashredder columns (Qiagen, Inc) to homogenize each sample. Samples were then stored at -80°C until further processing. After all samples had been frozen down, total RNA was isolated using the Qiagen RNeasy kit (Qiagen, Inc).

Semi-quantitative RT-PCR

1-5μg of total RNA (as isolated above) was reverse-transcribed to generate cDNA, using the iScript cDNA synthesis kit (BioRad). As a control, parallel samples were run in which reverse transcriptase was omitted from the reaction mixture. Nested PCR was then performed using the following primers (Lin *et al.*, 1994): Set 1; LHr forward 5'-GCATCTGTAACACAG GCATC-3' LHr reverse 5'-CATCTGGTTCAGGAGCACAT-3' Set 2; LHr forward 5'-GCAGAAGATGCACAATGGAG-3' LHr reverse 5'-CTCTC AGCAAGCATGGAAGA -3'. GAPDH transcript was used as a control and amplified with the following primers: GAPDH forward 5'-CACTGCCACCCAGAAGACTGT-3' and GAPDH reverse 5'-GGA AGGCCATGCCAGTGA-3'. Each PCR reaction was run using the following parameters, 95°C/1 min, 56°C/1 min, and 72°C/1 min, for 30 cycles. PCR products were then run on a 1% agarose gel to visualize samples.

Real time RT-PCR analysis

1-5μg of total RNA (as isolated above) was reverse-transcribed to generate cDNA, using the iScript cDNA synthesis kit (BioRad). As a control, parallel samples were run in which reverse transcriptase was omitted from the reaction mixture. Quantitative real-time PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Amplification was carried out using Taqman master mix, and pre-designed TaqMan probes (GAPDH, Hs99999905_m1; LHr, Hs00174885_m1) according to the manufacturer's instructions (Assays on Demand, Applied Biosystems). Reactions were performed in triplicate in a 20μl volume, with the following cycle parameters: 95°C/10min enzyme activation, 95°C/15 seconds, 60°C/1min for 40 cycles. Data analysis

was performed using the comparative Ct method (Applied Biosystems) to determine relative expression levels.

Immunofluoresence microscopy

Cell cultures were grown on coverslips to 50% confluency and were infected with N400, N400*pilT* or treated with GCB media alone for 3hr. The media was then aspirated, and the cells fixed for 15 minutes at room temperature in 4% paraformaldehyde. Cells were blocked and permeabalized in isotonic PBS containing BSA (3%, w/v) and saponin (0.02% w/v) for 1 hour at room temperature, followed by staining with the indicated antibodies and stained with DAPI. Samples were rinsed extensively in PBS before mounting in Fluoromount-G (Fisher Scientific). Images were obtained with a Deltavision Restoration Microscope (Applied Precision Instruments, Inc) fitted with a Nikon 60x oil immersion objective, and processed at a Silicon Graphics workstation with accompanying API software. The images were subsequently exported to Adobe Photoshop (version 7.0) and Adobe Illustrator (version 11.0) for manuscript preparation.

Quantitative colocalization analysis

Colocalization of antigens was evaluated quantitatively using CoLocalizer Pro (CoLocalizer Pro Software, Boise, ID). Manders overlap coefficient (MOC) was used to evaluate colocalization. Manders overlap coefficient is a generally accepted measure of colocalization. It indicates an overlap of the signals and thus represents the true degree of colocalization. Values of the MOC are defined from 0 to 1.0. If an image has an overlap coefficient equal to 0.7, it implies that 70% of both its components overlap with the other part of the image. A value of zero means that there are no any overlapping objects. Background was corrected using "average contrast and fluorescence" mode for all

channels to remove background and noise levels completely. Then, scatter grams were created and analyzed. Scatter grams estimated the amount of each detected antigen based on colocalization of myc-LHr (red, y-axis) and β-arrestin-GFP (green, x-axis). Colocalized pixels of yellow color were located along the diagonal of the scatter gram. Three samples from each experiment were analyzed. Colocalized pixels are represented as a white color in the merged image.

Statistics

Statistical analysis was performed using standard t-test analysis with SPSS version 11.0 unless otherwise stated.

Appendix 2

Force Generation by Type IV Pilus Retraction Induces Recruitment of Cytoskeletal Proteins

Summary

Mechanical forces can act as signals to mammalian cells and cause rearrangement of cytoskeletal components. Retraction of the *Neisseria gonorrhoeae* type IV pilus induces similar rearrangements, leading to the formation of cortical plaques underneath the site of bacterial attachment. Here, we use magnetic force to mimic mechanical stresses induced by type IV pili, to determine the nature of cortical plaque formation. We applied magnetic force to aggregates of coated magnetic beads seeded onto epithelial cells. We found that relatively small forces were able to induce the accumulation of actin and/or ezrin below 'bead clusters'. Moreover, higher forces and longer exposures to magnetic force resulted in increased levels of protein recruitment. Additionally, recruitment depended on the type of force exerted; a downward pushing force recruited actin, while an upward pulling force recruited ezrin. Together, these data suggest that cortical plaque formation during *N. gonorrhoeae* infection is the consequence of the interplay of pulling and pushing forces exerted on the epithelial cell membrane by pilus retraction.

Introduction

Neisseria gonorrhoeae Type IV pili (Tfp) are retractile appendages that facilitate microcolony formation and bacterial adhesion to various substrates. During attachment to the host epithelium, such microcolonies stimulate the formation of cortical plaques—structures in the cell cortex containing high concentrations of transmembrane receptors, nonreceptor tyrosine kinases and their anchors, and components of the cortical cytoskeleton (Merz *et al.*, 1999; Merz and So, 2000). This process requires pilus retraction via a functional PilT (Merz and So, 2000).

Retraction of a single gonococcal pilus can exert forces up to 80–100 pN on its substrate (Maier *et al.*, 2002; Merz *et al.*, 2000). Forces of lesser magnitude can promote cytoskeleton rearrangements and protein clustering (Choquet *et al.*, 1997; Sheetz *et al.*, 1998). Pilus retraction has therefore been speculated to induce cortical plaque formation by exerting mechanical tension on the membrane (Merz and So, 2000). Here we recapitulate the effects of pilus retraction by use of two independent magnetic bead assays to induce artificial force on epithelial cells. These results provide further evidence that cortical plaque formation is mediated by mechanical stress.

Results

Artificial Force Application Induces Actin Recruitment

To generate mechanical stress in a manner similar to that of pilus retraction, we first employed the modified magnet-based force assay described in Chapter 2 of this thesis. In this assay, the magnet generates an upward force of 4 pN per bead or approximately 20–100 pN per cell. Magnetic force was applied for 1 hr, and then cells were stained with phalloidin to visualize actin localization (Figure A2-1). In the presence of magnetic force, actin concentrated in the cell cortex around membrane-coated beads (Figure A2-1, top panel). In contrast, actin did not cluster underneath the beads in the absence of the magnet (Figure A2-1, bottom panel). Thus, the force generated by this magnet system was sufficient to recruit actin to the site of the attached beads.

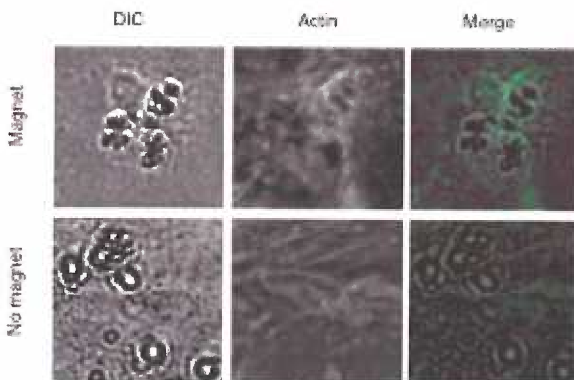

Figure A2-1 – Permanent magnet-induced clustering of actin beneath magnetic beads.

CPP-coated beads were seeded onto T84 cells and exposed to the magnet for 1 h (top panels) or left untreated (no magnet, bottom panels). Differential interference contrast images (left panels) reveal the location of the beads; phalloidin staining (middle panels) shows the presence of actin at the same site. Right panels show the two previous images merged.

Actin localization was also examined using electromagnet-based magnetic tweezers. This system uses a metallic tip wound with wire, to generate a magnetic field gradient. Calibration of this system demonstrated a maximum force/bead of ~300pN, with the force decreasing exponentially as a function of distance from the tip. Beads attached to

cells that were very near the magnet tip experienced high pulling forces, and induced the robust accumulation of actin to the site of bead attachment (Figure A2-2, top panel). Conversely, cells that were furthest from the magnetic tip experienced little, if any force, and in these cells there was no noticeable recruitment of actin (Figure A2-2, bottom panel). Interestingly, actin recruitment in this assay was much more pronounced than in the assay using the permanent magnet, suggesting that higher forces induce more actin accumulation underneath attached beads.

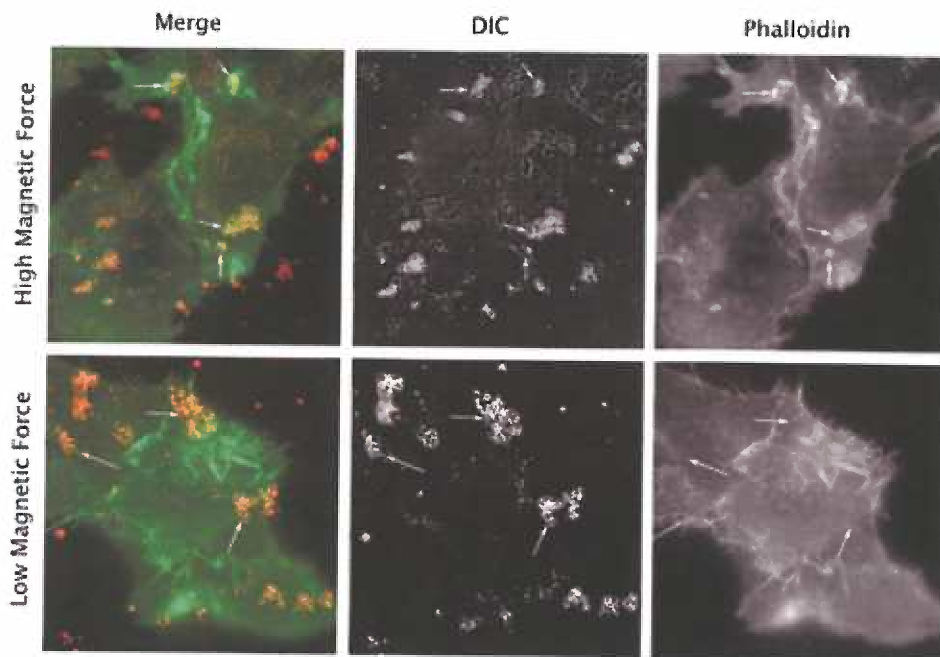

Figure A2-2 – Electromagnet-induced clustering of actin beneath magnetic beads

CPP-coated beads were seeded onto Hec1B cells and exposed to the magnet for 1 h. Top panels show cells in close proximity to the magnet tip, while the bottom panels show cells at a great distance from the magnet tip. Differential interference contrast images (middle panels) reveal the location of the beads; phalloidin staining (right panels) shows the presence of actin at the same site. Left panels show the two images merged.

Effect of Relative Force and Force Duration on Actin Clustering

Based on the differences in actin accumulation revealed by two magnet-based assays, we next examined the recruitment of actin relative to the amount of force applied to a bead. Figure A2-3 shows bead location (and subsequent actin accumulation) with respect to the location of the electromagnet tip. Interestingly, beads closest to the tip did not induce actin recruitment. Only beads that were located at a distance ≥30μm were capable of recruiting actin. The most-intense actin staining is seen at this distance, with a decrease in staining intensity apparent at greater distances. The absence of actin recruitment near the magnet tip may be due to excessive heat from the electricity running through the tip, or may be due to an aberrant magnetic field at this position. Beads located at a distance greater than 30μM from the tip do not appear to be adversely affected by the magnet.

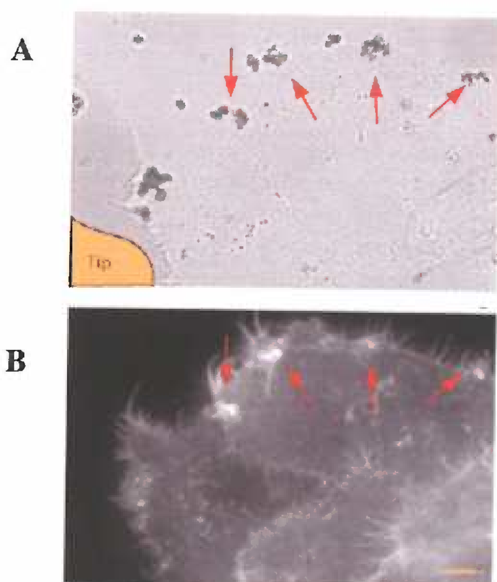

Figure A2-3 – Qualitative evidence of force-intensity-dependent actin recruitment

(A) Transmitted light image of cells and beads with respect to the tip of the magnetic device (orange spot).

(B) Corresponding fluorescent image showing phalloidin staining following 45 minutes of force application
Bar = 10μM

To quantitate the relative actin accumulation, we defined a region of interest by drawing a line around the bead clusters in the fluorescence image. The mean intensity of actin fluorescence was calculated in each slice of a z-stack, normalized to an adjacent

background region, and plotted as a function of force (Figure A2-4). At forces less than 30pN the ratio of the actin intensity to the background is 1.2, suggesting that even in the absence of external force, a small amount of actin accumulates around the beads. Forces exceeding 30pN significantly increased actin accumulation. Maximum actin recruitment was observed at an average force of 200-250pN per bead. Forces greater than 250pN did not induce actin recruitment. As above, this may be due to the proximity of the tip. And the associated heat from the electromagnet in this area.

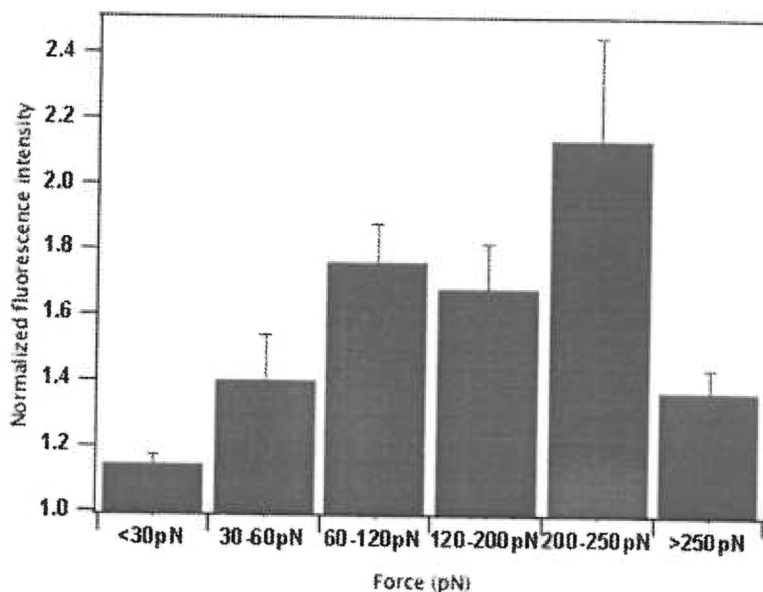

Figure A2-4 – Actin recruitment as a function of relative force

The average intensity of a selected region in a fluorescence image was normalized by an adjacent background region. In a z-stack the slice with the highest intensity ratio was selected for each 'bead colony'. Bars represent the average of over 80 aggregates in 3 samples, ± Std dev.

Next we determined if the duration of force application would affect actin recruitment. Forces between 200-250pN were applied to beads over a timecourse of 1hr. Cells were subsequently stained with phalloidin to visualize actin localization. As above, relative

actin accumulation was quantified by defining a region of interest, and calculating the mean intensity in each slice of a z-stack. These values were then normalized to an adjacent background region, and plotted as a function of force (Figure A2-5). Once again, beads which were not exposed to force were able to stimulate actin recruitment at a basal level. Actin recruitment increased with the time of the force application, as expected. Thus, the longer the force is applied, the more recruitment occurs.

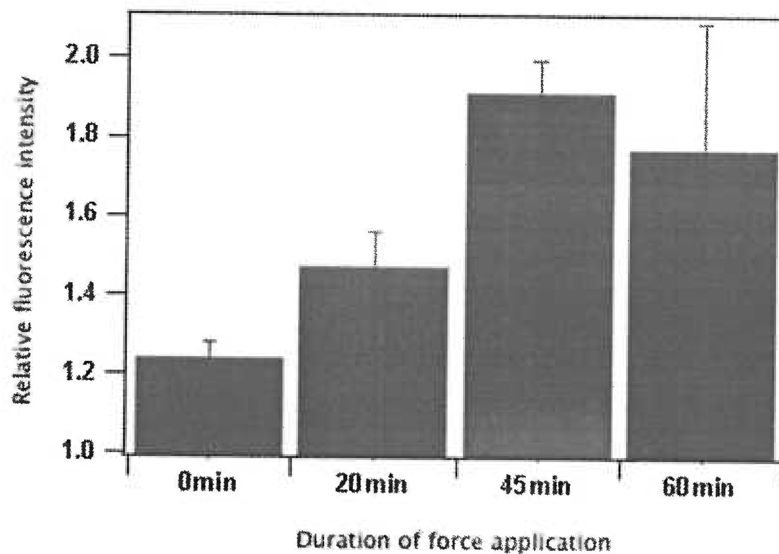

Figure A2-5 – Actin recruitment as a function of force duration

The average intensity of a selected region in a fluorescence image was normalized by an adjacent background region. In a z-stack the slice with the highest intensity ratio was selected for each 'bead colony'. Bars represent the average of over 80 aggregates in 3 samples, ± Std dev.

Recruitment of Cortical Plaque Components in the Presence of Downward "Pushing" vs. Upward "Pulling" Forces

As discussed in the introduction to this thesis, pilus retraction is hypothesized to generate an upward tension at a radial circumference from the center of the microcolony (Lee,

2003). The presence of an upward pulling force requires an opposing force of equal intensity. By re-examining the "model of radial membrane tension" we can assume that this force is directed downward, through the tangent point where the microcolony contacts the host cell (Figure A2-6). Thus pilus retraction from a microcolony structure should induce *both* an upward "pulling" force, and a downward "pushing" force.

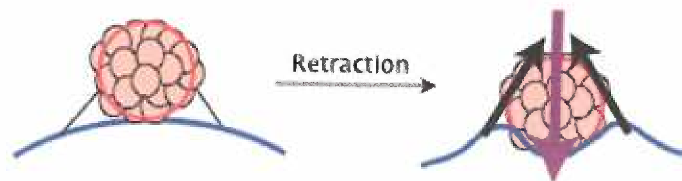

Figure A2-6 – Model of pushing and pulling force

A gonococcal microcolony with extended pili contains an anchor point from which retraction generates substantial amounts of membrane tension, radially extended from the sphere of the microcolony. A corresponding opposing force of equal intensity is hypothesized to be directed downward through the tangent point where the microcolony contacts the host cell.

In order to determine if the "pushing" and "pulling" forces have unique effects on recruitment of cortical plaque components, we used the permanent magnet bead assay described in Chapter 2 of this thesis, concurrently with a "reverse magnetic bead assay" in which the magnet was placed 10mm *below* the bottom of cultured cells harboring bound CPP-coated beads. At this distance, the magnet induces the exact same force/bead, but in the downward direction. Following force application, cells were fixed and stained for two cortical plaque constituents, actin and ezrin (Figure A2-7).

The pulling force induced substantial recruitment of ezrin, and a slight amount actin recruitment. This level of actin recruitment is consistent with that seen in figure A2-1, but is significantly attenuated compared to that seen during infection with wild-type *N. gonorrhoeae*. Under magnet-applied pushing force however, no ezrin recruitment was visible, but actin recruitment was increased considerably. This amount of actin rearrangement closely resembles that seen during infection. These data therefore suggest that actin is recruited to cortical plaques through a downward force, while ezrin recruitment requires an upward force. Further experiments are required to determine the type of force necessary to induce the recruitment of other cortical plaque constituents.

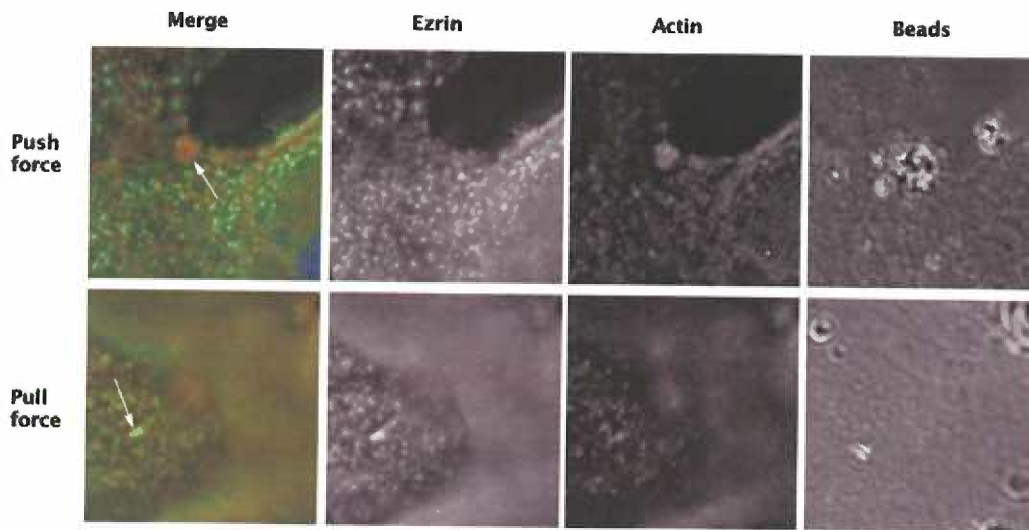

Figure A2-7 – Actin and ezrin recruitment require different types of force

CPP-coated beads were seeded onto Hec1B cells and exposed to the magnet for 1 h. Top panels represent cells exposed to a "pushing" force, while bottom panels represent cells exposed to a "pulling" force. Arrows point to the specific bead cluster for each image upon which conclusions were drawn.

Discussion

Retraction of the *N. gonorrhoeae* Tfp during infection induces the formation of cortical plaques; rearrangements of the cortical cytoskeleton and recruitment of signaling proteins to the site of bacterial attachment (Merz *et al.*, 1999). We tested the hypothesis that cortical plaque formation was induced specifically by retraction mediated membrane tension. When external force was applied to CPP-coated beads seeded onto epithelial membranes, actin and ezrin were recruited to the site of bead attachment (Figures A2-1, A2-2 and A2-7). The level of recruitment was dependent on the magnitude and duration of the applied force (Figures A2-4 and A2-5). Moreover, recruitment of ezrin appeared to be dependent on an upward "pulling force" while actin recruitment was most robust in the presence of a downward "pushing force" (Figure A2-7).

These experiments suggest that cortical plaque formation during *N. gonorrhoeae* infection is mediated specifically by pilus retraction induced mechanical force on the host cell membrane. While the effect of force magnitude and duration were only examined with respect to actin relocalization, it is likely that other cortical plaque components respond similarly, given the time-dependence of cortical plaque formation during infection. While protein clustering underneath the site of bacterial attachment is visible within 30 minutes of infection, fulminate plaques do not appear until 3-4 hours post infection. Thus, by this time, pilus retraction has been mechanically pulling on the epithelial membrane for an extended period of time, and with an ever-increasing amount of force due to the growth of the microcolony (and subsequently an increase in the number of pilus-retraction events).

The idea of a downward "pushing force" from a microcolony has not previously been investigated. Moreover, the constituents of cortical plaques that respond specifically to an upward force vs. a downward force remain to be investigated in more detail. Interestingly, some cortical plaque proteins like ezrin and β-arrestin-2 are consistently recruited around the circumference of a microcolony, the region that is thought to be under an upward tension. Conversely, other plaque proteins, like actin and LHr are typically relocalized to the area directly underneath the microcolony, which is the region predicted to be exposed to the downward "pushing force." Thus it is likely that circumference-localized proteins are recruited specifically by upward tension, whereas proteins localized directly below the bacterial microcolony are likely recruited specifically by the opposing pushing force. How this force-specific recruitment of proteins and signaling events affects the infection process remains to be seen.

Progress Towards Manuscript

Permanent magnet induced clustering of cortical plaque constituents (Figure A2-1)

This experiment has been repeated numerous times to examine actin relocalization to the point of force application. However, other cortical plaque constituents have not been examined using this method, and no quantitative calculations have been made using the data obtained.

Electromagnet induced clustering of cortical plaque constituents (Figure A2-2 to A2-5)

This experiment was repeated 2-3 times within a 1-week period. As above, only actin recruitment was assessed; no other cortical plaque constituents were examined. Quantitative analysis needs to be repeated, as was only performed on one sample (error bars within the quantitative data represent numerous observations within the same sample).

Effects of pushing force vs. pulling force on recruitment of cortical plaque constituents (Figure A2-7)

This experiment has only been performed once, and at that time only actin and ezrin recruitment were analyzed. This needs to be repeated, and other cortical plaque constituents examined.

Materials and Methods

Reagents.

Mouse anti-ezrin antibody was purchased from BD Biosciences (San Jose, Ca). Alexa-Fluor 594 phalloidin was purchased from Molecular Probes (Eugene, Oregon). Neodymium iron boron (NdFeB) magnets (Eneflux Armtek Magnetics, Bethpage, New York, United States) measured 2 in. in diameter by 1 in. thick and were grade 30 (MGOe).

Cell lines

T84 human colonic epidermoid cells (American Type Culture Collection, Manassas, Virginia, United States) were maintained in DMEM-F-12 plus 5% heat-inactivated, filter-sterilized fetal bovine serum at 37 °C and 5% CO_2. Hec-1B human cervical carcinomas cells (American Type Culture Collection, Manassas, Virginia, United States) were maintained in DMEM plus 10% heat-inactivated, filter-sterilized fetal bovine serum at 37 °C and 5% CO_2.

CPPs and bead coating.

N. gonorrhoeae CPPs were generated from piliated, Opa⁻ gonococci. Bacteria were scraped from overnight cultures (grown on plates) into HBSS and vortexed for 2 min, followed by centrifugation at 14,000*g* for 5 min. Supernatants were removed, quantitated by spectrophotometric analysis, and stored at -80 °C until use. Pili preparations were

assayed for the presence of pili via indirect immunofluorescence microscopy and immunoblot, using anti-pilin antibody (data not shown). Bio-Mag Plus carboxy-modified paramagnetic microspheres (Bangs Laboratories, Fishers, Indiana, United States), were activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (EDAC), and incubated with piliated *N. gonorrhoeae* CPPs or BSA as per the manufacturer's instructions. Bead coating was confirmed by immunoblotting using antibodies to BSA (ICN Biomedicals, Irvine, California, United States) and pilin (antibody SM1; data not shown).

Permanent magnet assay/Immnofluorescence microscopy.

T84 or Hec-1B cells were grown on coverslips to 50% confluency and incubated with either BSA-coated or CPP-coated magnetic beads for 15 min. Unbound beads were washed off and the magnet placed at a distance of 10 mm from the cell surface for 1 h. The medium was then aspirated, and the cells fixed for 15 min at room temperature in 4% paraformaldehyde. Cells were blocked and permeabilized in isotonic PBS containing BSA (3%, w/v) and saponin (0.02% w/v) for 1 h at room temperature, followed by staining with mouse anti-ezrin primary antibody. Samples were then washed, and stained with anti-mouse Alexa-Fluor 488 secondary antibody, and Alexa-Fluor 594 phalloidin (Molecular Probes, Eugene, Oregon, United States). Samples were rinsed extensively in PBS before mounting in Fluoromount-G (Fisher Scientific, Hampton, New Hampshire, United States). Images were obtained with a Deltavision Restoration Microscope (Applied Precision, Issaquah, Washington, United States) fitted with a Nikon (Tokyo, Japan) 60x oil-immersion objective and processed at a Silicon Graphics (Mountain View,

California, United States) workstation with accompanying API software. The images were subsequently exported to Adobe Photoshop (version 7.0) and Adobe Illustrator (version 11.0) (Adobe Systems, San Jose, California, United States) for manuscript preparation.

Magnetic Tweezers assay/Immnofluorescence microscopy.

Hec-1B cells were grown on coverslips to 50% confluency and incubated with CPP-coated magnetic beads for 15 min. Unbound beads were washed off, and the tip of the magnet inserted into the culture dish. The magnetic field was applied for the time points specified. The medium was then aspirated, and the cells fixed for 15 min at room temperature in 4% paraformaldehyde. Cells were blocked and permeabilized in isotonic PBS containing BSA (3%, w/v) and saponin (0.02% w/v) for 1 h at room temperature, followed by staining with Alexa-Fluor 594 phalloidin (Molecular Probes, Eugene, Oregon, United States). Samples were rinsed extensively in PBS before imaging.

Calculation of magnetic force (permanent magnet).

To quantify the amount of force that the magnet exerts per magnetic bead, the change-in-mass method (Glogauer and Ferrier, 1998) was used. Briefly, the mass of a known number of dry beads (0.12 g) was measured on an electronic balance in the presence and absence of the magnet. Given the mean bead diameter of 1.5 μm and the bead density of 2.5×10^3 kg/m^3 (Bangs Laboratories), the number of beads in this sample was calculated to be 1.2×10^{10}. The change in mass of the beads in the presence of the magnet was

entered into the equation: force = Δmass x acceleration (with acceleration being equal to gravity, or 9.81 m/s^2) to give a value for the force. Change-in-mass measurements were taken at varying distances from the magnet to determine force as a function of distance.

Calculation of magnetic force (permanent magnet).

To quantify the amount of magnetic force that the electromagnet exerts per magnetic bead, Stoke's law was applied. Under Stoke's law, the force due to viscous drag (F_{vis}) = $6\pi\eta rv$ where η = liquid viscosity, r = the radius of the bead and v = the velocity at which the bead is moving. To this end, a small volume of beads of 1μm diameter were dispersed in glycerol, with a known viscosity coefficient. The magnet was then turned on, and bead velocity was then calculated with respect to the distance from the magnet tip.

Appendix 3

Mechanical force calculations

Forces mediated by a single gonococcus

- An average gonococcus contains roughly 10 pili

- Each pili is capable of retracting with a force of ~100pN

10 pili x $100pN =$ **$1000pN$ or 10^3 pN**

- Diameter of a gonococcus is ~$1\mu m$

- Area $= \pi r^2 = \pi(.5\mu m)^2 = .79\mu m^2$ or approximately $1\mu m^2$

10^3 pN / $1\mu m^2$ = **$0.1N/cm^2$**

Forces induced by a small microcolony (non-circumferential force)

- A small microcolony contains approximately 100 bacteria

- We can approximate that roughly 1/3 of these bacteria are in contact with cell

1/3 of 100 = 33 bacteria

33 bacteria x 10 pili each x 100pN = **$3.3x10^4$ pN**

- Area underneath microcolony $= \pi r^2 = \pi(3\mu m)^2 = 28.27\mu m^2$

$3.3x10^4$ pN / $28.27\mu m^2$ = **$0.12N/cm^2$**

Forces induced by a small microcolony (circumferential force)

- The diameter of a microcolony containing 100 gonococci can be approximated by:

$V = 4/3\pi r^3$ $\quad$ $100\mu m^3 = 4/3\pi r^2$ $\quad$ r = 2.88 or 3 gonococci

- The bacteria around the circumference of the microcolony can be approximated by:

$C = \pi d$ $\quad$ $6\pi = 19$ gonococci

19 bacteria x 10 pili each x $100pN =$ **$1.9x10^4$ pN**

- The area in which the gonococci retract is a $1\mu m^2$ area around the circumference of the microcolony. This can be estimated by taking the area of a $9\mu m^2$circle and subtracting it from the area of a $6\mu m^2$ circle:

$A = \pi r^2$ $\quad$ $A(6) = \pi(3\mu m)^2 = 28.3\mu m^2$ $\quad$ $A(5) = \pi(2.5\mu m)^2 = 19.6\mu m^2$

$28.3\mu m^2 - 19.6\mu m^2 = 8.7\mu m^2$

- As the Tfp retraction force is proposed to be distributed in this area, the estimated circumferential force can be calculated as:

$1.9x10^4$ pN / $8.7\mu m^2 =$ **0.218 N/cm^2**

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