THE IgA PROTEASE OF PATHOGENIC *NEISSERIAE:*

Transcription, Targets and Types

by

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A DISSERTATION

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To Daddy.
Thanks for the roots
and the wings.

INTRODUCTION

The Gram-negative bacteria in the genus *Neisseria* exist within humans both as harmless commensals and as horrific pathogens. How and why individual strains are able to maintain both roles remains a mystery. As we learn more about the biology of these species, and how they interact with their host, we begin to understand how this phenomenon is possible, as well as how these diseases might be prevented.

Gonorrhea

Gonorrhea, the disease caused by *Neisseria gonorrhoeae*, is the second most prevalent sexually transmitted disease in the world and is one of the leading causes of infertility in developed countries (Lightfoot and Gotschlich, 1974; Ruden *et al.*, 1986; Ruden *et al.*, 1993; Weir *et al.*, 1994). Hundreds of thousands of cases are reported in the U.S. each year. Gonococci (or GC) infect the epithelium of the urethra and endocervix, causing inflammatory pain and discharge. Several infections, however, do not induce symptoms at this initial site and progress to invade the bloodstream (Disseminated Gonococcal Infection or DGI) or ascend the female genital tract, inducing inflammation in the fallopian tubes and pelvic cavity of women (Pelvic Inflammatory Disease or PID). The latter often results in scarring and adhesions throughout the upper genital tract, hindering ovum transit and thus, fertility. Anorectal inflammation and conjunctivitis may also be caused by *Neisseria gonorrhoeae*.

Although these diseases can still be easily treated with antibiotics, the bacterium remains a major public health problem due to asymptomatic carriage and the severity of

invasive and ascending infections. Strikingly, in a recent study of a stratified population in Baltimore, 5.3% of persons ages 18-35 were shown to be harboring gonococci without symptoms. This study, possible due to the advent of nucleic acid amplification tests (a GC-specific PCR assay on urine samples), was the first of its kind to address the prevalence of asymptomatic STD carriers in the population (Turner *et al.*, 2002).

Meningococcal meningitis

Neisseria meningitidis (or MC) is one of the three leading causes of non-neonatal bacterial meningitis, along with *Streptococcus pneumoniae* and *Haemophilus influenzae*. The incidence of meningococcal infection in the U.S. is 2500-3500 persons per year, with major outbreaks occurring decades apart. The last major outbreak in the U.S. was in 1994, when 4.6 cases occurred per 100,000 persons in Oregon and Washington (as reported by the CDC). Although there are incidence peaks for infants and the elderly with poor immune defenses, approximately 25% of cases occur in teens and young adults. This is often attributed to the frequency of close contacts and dense living situations; college freshmen living in dormitories, new military recruits, and inmates are groups known to have a relatively high frequency of meningococcal outbreaks.

Meningococcal disease is quite prevalent worldwide. There is a region of relatively high endemic levels of disease in a region of savannah extending from Ethiopia to Senegal often referred to as the "meningitis belt". Outbreaks here typically involve 500-1000 cases per 100,000 persons; the largest ever recorded involved over 150,000 people in 1996 (Rosenstein *et al.*, 2001). The disease is treatable by antibiotics, but the

disease progression is often so swift, morbidity and mortality are high. At least 10% of meningococcal patients die in all regions. For this reason, much effort is focused on finding a reliable means of prevention. However, the current available vaccine (against serogroup A, C, and Y) does not protect against serogroup B strains; serogroup B meningococci were responsible for 68% of reported cases in Europe between 1993 and 1996 (Rosenstein *et al.*, 2001). Vaccine development continues.

Carriage

Although hundreds die each year from the infection, meningococci colonize approximately 15% of the population as a harmless resident of the nasopharyngeal flora. It is not known whether these carriers may become susceptible to the disease, or if they are simply responsible for the passage of the organisms within our host species. Carriers do have antibodies against several meningococcal antigens, but it is not known if these can actually prevent the disease.

Neisseria gonorrhoeae is also known to colonize humans without causing disease as mentioned above. This is so true that many call *Neisseriae* "accidental pathogens". Reasonably so – there is no selective or proliferative advantage for gonococci to make their host less likely to transmit it sexually, nor for meningococci to kill their host. Why some go on to cause disease anyway remains a mystery.

GC and MC are very closely related. Genetically, they are >85% identical. Although their diseases are quite different, they still rely on many of the same

mechanisms in order to cause infection. For this reason, we use them interchangeably in the lab for some studies. Both begin by asymptomatically colonizing a mucosal epithelium and must pass through this barrier for disease to occur.

The steps to disease

Physiological pathogenesis

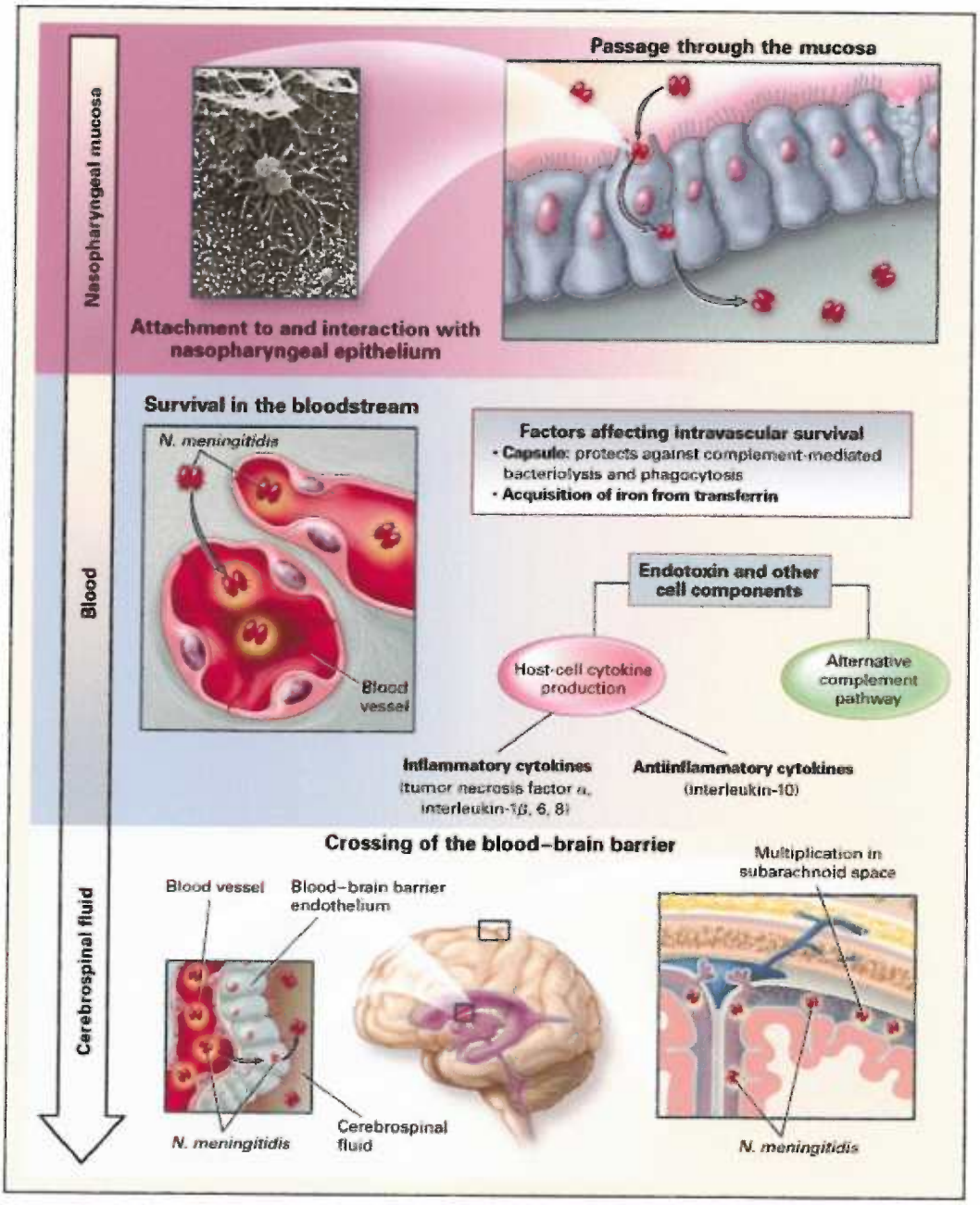
Both meningococci and gonococci are passed from person to person via mucosal secretions. Respiratory droplets carry MC from one nasopharynx to another. Gonococci transmit via genital secretions. Neither is known to be passed via fomites, and no other animal can act as their host.

The pathway of meningococcal infection is not entirely clear. We do know that they colonize and are shed from the nasopharyngeal mucosa, and that they do not cause upper respiratory disease. Bacteria may infect the bloodstream, the meninges, or both. An accepted model of how they proceed to enter the bloodstream and/or the cerebrospinal fluid compartment (it is not clear which comes first) is depicted in this figure from a recent review, taken from (Rosenstein *et al.*, 2001):

Model for Colonization of Neisseria meningitidis in the Nasopharynx and Entry into the Bloodstream and Cerebrospinal Fluid. (figure below)

"N. meningitidis enters the nasopharynx and attaches to nonciliated epithelial cells, probably through the binding of the pili to the CD46 receptor (a membrane cofactor protein) and the subsequent binding of opacity-associated proteins, Opa and Opc, to the CD66e (carcinoembryonic antigen) and heparan sulfate proteoglycan receptors, respectively. The attached organisms are engulfed by the cells, enter phagocytic vacuoles, and may then pass through the cells. IgA1 protease (an outer-membrane protein) cleaves lysosome-associated membrane protein and may promote the survival of N. meningitidis in epithelial cells. PorB (another outer-membrane protein) crosses the cell membrane and arrests the maturation of the phagosome. In the bloodstream, the organisms release endotoxin in the form of blebs (vesicular outer-

membrane structures) that contain 50 percent lipooligosaccharide and 50 percent outer-membrane proteins, phospholipids, and capsular polysaccharide. The endotoxin and probably other components stimulate cytokine production and the alternative complement pathway. N. meningitidis crosses the blood–brain barrier endothelium by entering the subarachnoid space, possibly through the choroid plexus of the lateral ventricles." (Rosenstein *et al.*, 2001)



Alternative hypotheses of the invasion pathway exist. One theory is that meningococci enter the bloodstream during routine disruption of nasopharyngeal epithelium and capillaries (e.g. nose blows) and transfer to the meninges at the site of CSF production, the choroid plexus. Another possibility is that the bacteria may enter the meninges directly by ascending through the porous cribriform plate of the skull, through which olfactory nerves descend from the brain.

The disease progresses rapidly and has been known to kill victims within hours of the earliest symptoms. Like gonococci, and as with many other infectious diseases, it is not necessarily the bacterium itself that induces the disease syndrome. It is the host response to the very presence of the invader which mediates the damaging inflammatory responses. The meninges are the membranes which closely associate with the brain and form a protective sac around it and the spinal cord, which is normally filled with sterile fluid. For meningococcal disease, a fulminant inflammatory response occurs within these membranes in response to the meningococcal invasion. If this progresses to brain edema, survival is rare. Bacteria within the bloodstream weaken the endothelial integrity of capillaries and small blood vessels, resulting in a recognizable purpuric "rash" appearance on the skin of patients. Loss of circulatory stability often results in poor blood flow to the extremeties and is a major reason why meningcoccal patients require amputation following recovery.

The pathway of infection for *Neisseria gonorrhoeae* is fairly well understood. The bacteria first adhere to mucosal epithelia. Here they can reside for long periods of time without disturbing the host. The gonococci may later invade through this epithelium into the substratum where an inflammatory reaction occurs and disease symptoms begin.

Even if they colonize their host without invading (i.e. without causing symptoms), they may still be transmitted to close contacts via mucosal secretions.

Once the bacteria are beneath the protective epithelium, the host mounts an inflammatory response. The innate immune armament, including neutrophils, complement, and cytokine signals are the first to attack the invaders. Gram stains identifying GC in genital exudates or MC in centrifugated CSF most commonly demonstrate the bacteria within polymorphonuclear cells (PMNs).

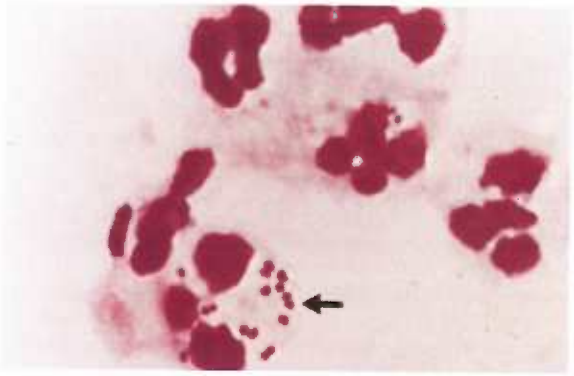
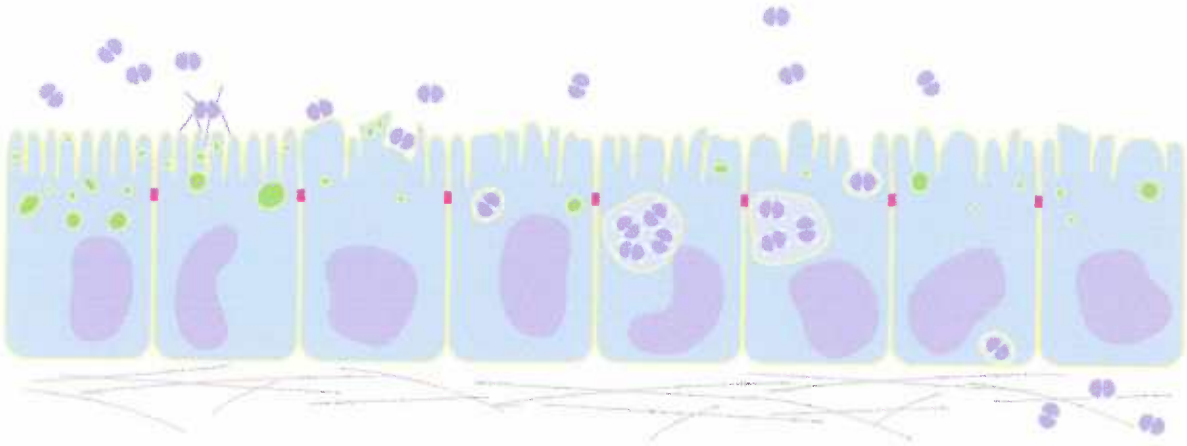


Image: CSF PMNs with intracellular meningococci (arrow). (Rosenstein *et al.*, 2001)

Histological pathogenesis

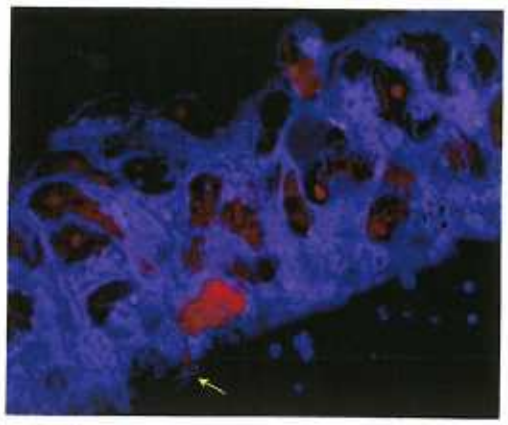
Both *Neisseriae gonorrhoeae* and *meningitidis* must colonize the mucosa, invade cells, and survive within and transcytose through the epithelium in order to cause disease. They survive and replicate in the substratum and within the bloodstream, and may pass through the endothelium.

The studies discussed in this thesis focus primarily on the initial interactions of the bacteria with the epithelium. Following is an abbreviated model, from a histological point of view, of this process. The model is derived from several observations made in both cellular and organ cultures with *in vitro* infections.



(figure above) First, bacteria attach and form close contacts with the epithelial surface. For the first few hours, *Neisseria* are seen in clusters, or microcolonies, on the cell surface. They attach to cells of the mucosa which are seen at this step to alter their ciliated morphology. The bacteria dispurse on the apical membrane and begin to invade. These events don't occur evenly throughout cells in a population; cells chosen for colonization and invasion are selected for undefined reasons. In some cell types, such as primary cervical cells, membrane ruffling around gonococci have been observed (Edwards *et al.*, 2001).

The most current view is that both meningococci and gonococci invade the cell one diplococcus at a time, although it has yet to be observed in real-time. Once inside the cell, *Neisseria* resides within its vacuolar comparment; escape to the cytoplasm has been hypothesized but never proven. Either the bacteria replicate within this endosome or several invasosomes aggregate within the cell, as large compartments containing multiple diplococci have been observed. Many also recycle back to the apical surface of the epithelium. This mechanism probably plays a role in bacterial evasion of the immune system and carriage. The intracellular compartment transcytoses and fuses with the



Gonococci exit the basal surface of a polarized monolayer. T84 colorectal epithelial cells were polarized and infected with wild-type MS11A for 22 hours, fixed, and stained for total membranes (blue) and DNA (red). Images were acquired by confocal microscopy on the X-Z axis. Arrow points to a cluster of gonococci exiting the monolayer. (Vasquez, unpublished)

basolateral membrane of the epithelial cell, permitting exit of its intracellular visitors. *Neisseriae* are not seen trafficking across the epithelium paracellularly.

Biochemical pathogenesis

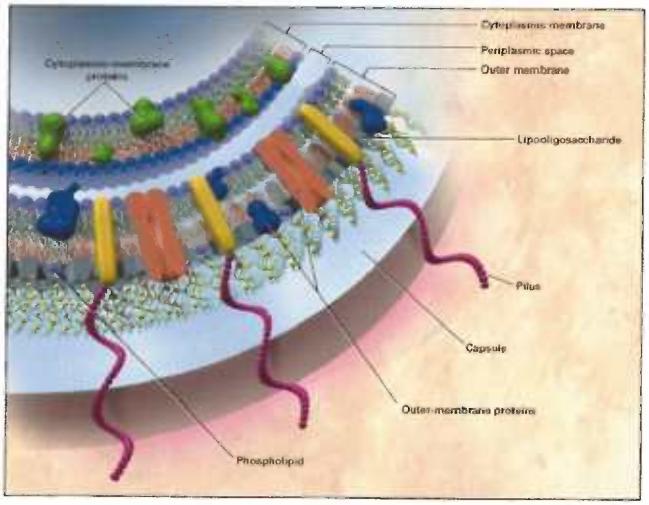
Gonococci and meningococci have many ways of evading the immune system. They are experts at changing their identity such that a slight alteration in one's make-up may go on to cause disease in a seemingly-immune host. The pathogens employ both antigenic (changing variable regions of a component) and phase (on/off) variation to avoid being recognized. Vaccines against these chameleonic masters are largely unsuccessful.

This molecular masking probably also plays a role in regulating expression of bacterial virulence factors and switching their properties in order to signal to the cell. Cells are manipulated such that the bacteria can survive on and within the epithelial cell without killing it or triggering host immune defenses. Recent studies have shown that *Neisseriae* alter the host cytoskeleton upon colonization, recruiting actin and several receptors and signaling molecules to the site of attachment (Merz and So, 1997; Merz *et al.*, 1999; Merz and So, 2000). Phosphorylation, calcium, and perhaps membrane tension or rupture are each known to occur early in the infection process. These signals have the

potential to alter gene expression, protein localization and trafficking mechanisms of the cell. It is still unknown what all of the specific triggers of these signals are – both from the bacterial as well as the cellular perspective – but this is a field of intense investigation, for *Neisseriae* as well as other pathogens.

Virulence factors

Virulence factors are the tools which bacteria use to achieve these steps in infection. *Neisseria gonorrhoeae* and *meningitidis* share many of their virulence factors, which are outlined in the following figure:



Transferrin Binding Protein (TBP), Lactoferrin Binding Protein (LBP), Hemoglobin Receptor (Hmbr) – Bind to human iron-binding proteins. Responsible for *Neisseria* iron acquisition. Factor in limiting host tropism to humans.

Lipooligosaccharide – Major outer membrane component of *Neisseria*. Key component in the inducing inflammation.

Capsule – Antiphagocytic polysaccharide coating of *N. meningitidis*

Porin – Outer membrane pore-forming protein. May conduct ions in the absence of ATP. Has been shown to insert into mammalian cell membranes spontaneously. Anti-apoptotic vs. pro-apoptotic actions are actively debated today.

Pili – Type IV pili contributes towards adhesion to the host cell. Also actively signals the host cell by binding of receptor(s), and possibly by retraction force on the cell membrane.

Opa – "opacity protein". Outer membrane protein. Has been shown to bind to heparan sulfate proteoglycans on the surface of host cells. Aids in invasion; may also contribute to adhesion. Our studies focus primarily on Opa- *Neisseriae*.

IgA1 Protease – the focus of this thesis. **Described below.**

Where and when virulence factors are expressed is important. Many bacteria regulate their genes based on environmental cues such that necessary proteins are

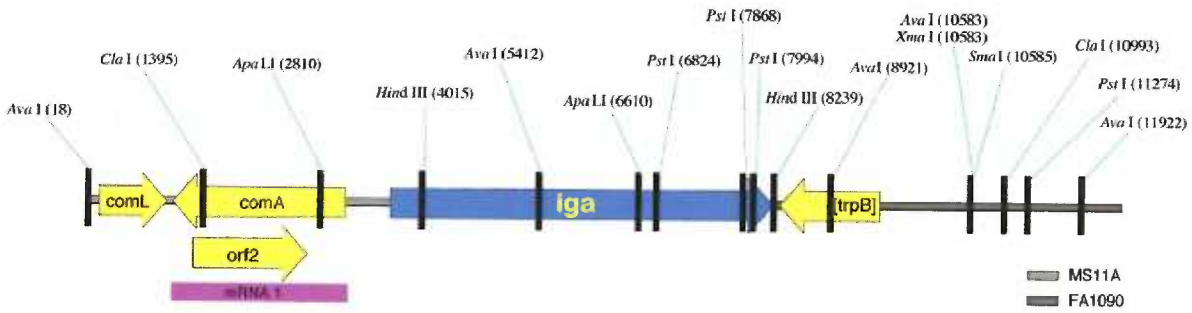
produced only at the right time and place. This includes metabolic genes as well as virulence factors. One key regulator of these systems is iron availability. Ferric uptake regulator (Fur) protein is one such regulator protein that controls the expression of several genes, both positively and negatively regulating gene transcription (Genco and Desai, 1996; Sebastian *et al.*, 2002; Serkin and Seifert, 2000; Stojiljkovic *et al.*, 1994). It was first characterized as the protein responsible for repressing the proteins necessary for iron uptake (e.g. Tfbp, Lfbp, Fbp, etc.) when iron was in sufficient supply. Iron binds to Fur and the protein conforms to bind a specific sequence in bacterial promoters.

Some virulence factors have also been identified as being regulated by Fur. Toxins, including the RTX toxin of *N. meningitidis* undergo this regulation. It was recently published that the promoters upstream of Opa proteins in gonococci are bound by Fur (Sebastian *et al.*, 2002). The first manuscript of this thesis explores Fur regulation of the neisserial IgA1 protease. The significance of this regulation in virulence, for *Neisseriae* or for other pathogens, has yet to be well defined.

IgA1 Protease

IgA1 protease is a virulence factor employed by several mucosal pathogens. Besides the Neisseriae, Streptococcus pneumoniae, and Haemophilus influenzae also secrete this factor, which was so named because of its specific cleavage of the human IgA1 proline-rich hinge. Although each of these bacteria are mucosal pathogens and overlap in their scope of disease (e.g. they are the top three causes of bacterial meningitis), genetic comparison suggests that their iga genes evolved convergently.

They are only ~35% homologous, and they each have different specificities within the IgA1 hinge (Mulks and Shoberg, 1994; Simpson et al., 1988). Recent discoveries of other substrates for the neisserial IgA1 proteases have not been shown as cleavable by these other proteases, as well.



MS11A-FA1090 iga region

12379 bp

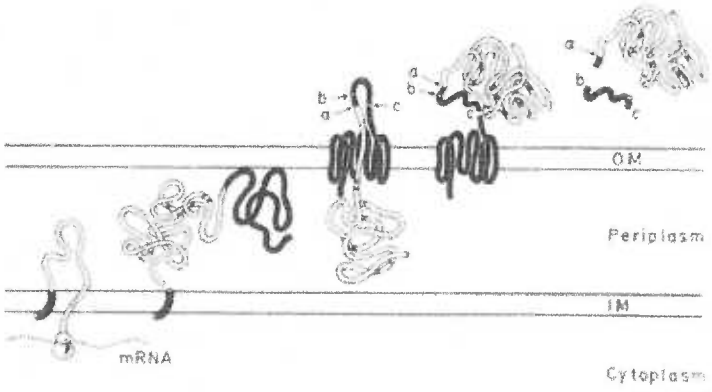
Chromosomal region of iga gene in *N. gonorrheae*. Hybrid of MS11A and FA1090 database sequences were spiced *in silico* for complete analysis of flanking regions of iga. The gonococcal genes in this region occur in the same order within the meningococcal chromosome, with varying homology between genes and intergenic regions. (sequence and graphic created using Vector NTI© software)

Sequence, structure and secretion of IgA1 protease

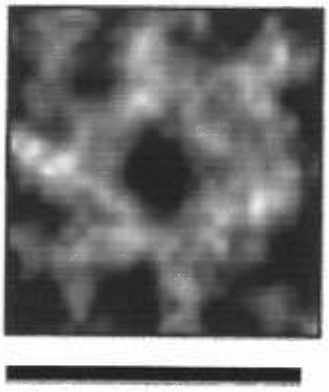
IgA1 protease occurs in *Neisseriae* as two alleles, Type 1 and Type 2. Each strain encodes the gene for only one type and cannot switch between them. The two types of IgA1 protease were so named because they have two different cleavage specificities within the human IgA1 hinge. They share > 95% identity at the genetic level, and have not, until now, been distinguished by sequence differentiation.

Fifteen years ago, a manuscript in *Nature* described the first model of IgA protease secretion (Pohlner *et al.*, 1987b). This autotransporter was the first Type I secretion protein to be characterized. Several outer membrane proteins of the Gram-

negative bacteria have since been described to secrete by this process including VacA of *Helicobacter pylori* and the Hap protein of *Haemophilus influenzae* (Veiga *et al.*, 2002). The sequences of these preproteins include an N-terminus signal sequence targeting it to insert in the inner membrane and be translated into the periplasmic space. The C-terminus of the protein encodes a beta-sheet with 14 transmembrane domains, which inserts into the outermembrane and translocates the middle domain (including the protease) outside of the cell. IgA1 protease may then autoproteolytically release itself from the beta-domain into the environment.



The original model of IgA1 Protease secretion. From: (Pohlner *et al.*, 1987a)



Oligomerization of the IgA1 protease β subunit. (Veiga *et al.*, 2002) Bar = 10 nm

The original model above depicted a single molecule acting as its own transporter (Pohlner *et al.*, 1987a). Very recently, a manuscript was published in EMBO which appears to have more clearly resolved the autotransporter mechanism of IgA1 protease (Veiga *et al.*, 2002). The beta-domains oligomerize to form a pore in the outer membrane of the bacterium, translocating the protease through a 2nm pore after oligomerization. At least 6 and perhaps as many

as 12 subunits are in each ring. The pore was able to transport folded even prefolded proteins, the size of the mature IgA1 protease. This is somewhat contradictory to prior work which showed the autotransporter could not secrete a pre-folded protein. More work will be needed to fully characterize this mechanism.

IgA1 protease in disease pathogenesis

All pathogenic *Neisseriae* species secrete IgA1 protease. The gene is uniquely present amongst those that are virulent, whereas their commensal relatives do not have it 90. IgA1 protease is a host-targeted product of the bacterium; it cleaves molecules within the host. No bacterial substrates have been identified with the exception of the pre-protease itself. These reasons and others have led the scientific community to accept that IgA1 protease is a crucial virulence factor in *Neisseriae* diseases.

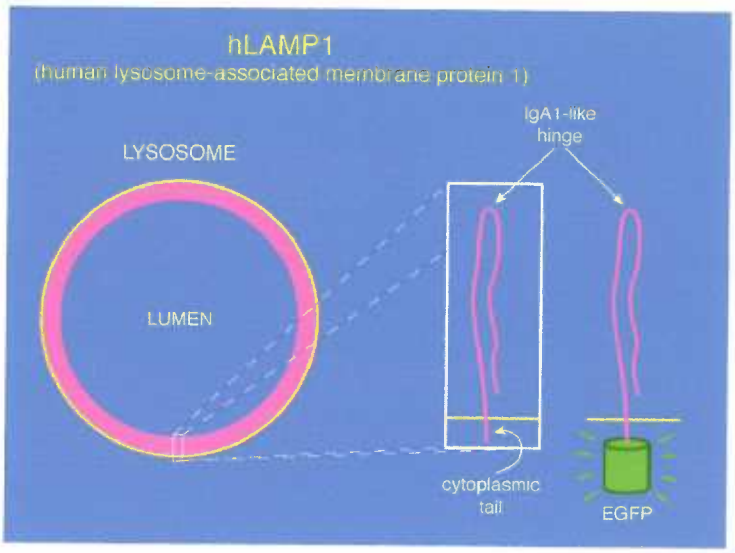
Several findings over the last several years have begun to paint a picture of the role of IgA1 protease in disease progression. IgA1 protease appears to function at all three levels of *Neisseria* pathogenicity.

IgA1 protease role in molecular pathogenesis

The IgA1 protease of pathogenic *Neisseriae* has been shown to have several substrates other than hIgA1. *In vitro*, it has been shown to be able to cleave the SNARE, synaptobrevin, leading authors to believe it may play a role in halting vesicular fusion within infected cells (Binscheck *et al.*, 1995). The protease also cleaves the TNF receptor (II) on the surface of monocytes, possibly contributing to immune evasion by

disabling activation of these phagocytes, though it prevents their apoptosis (Beck and Meyer, 2000). Human chorionic gonadotropin has also been shown as a substrate for the Type I IgA1 protease. This has implications towards bacterial interaction with the host cell receptor for HCG, as the L12 bacterial surface protein has been shown to compete for this receptor (Senior *et al.*, 2001).

In 1997, the first work from our lab was published which showed the major lysosomal associated membrane protein, Lamp1, to be an additional substrate for IgA1 protease cleavage (Lin *et al.*, 1997). It has a proline-rich hinge, very similar to that of hIgA1. The protease was shown to cleave at this hinge, accelerating the degradation of Lamp1 in host cells. Consequently, this led to a reduction in other lysosomal markers, such as Lamp2, lysosomal acid phosphatase (LAP), and CD63 (Ayala *et al.*, 1998).



Concept illustration of Lamp1 and the construction of a Lamp1-EGFP fusion protein (see manuscript #3).

IgA1 Protease role in histological pathogenesis

A strong reduction in host cell lysosomes led our lab to investigate the implications for the intracellular life of *Neisseriae*. Results demonstrated that bacteria which produced IgA1 protease survive better intracellularly than their *iga*- counterparts, in which a deletion of the gene encoding the protease was engineered (Lin *et al.*, 1997).

The same bacteria were also compared in their ability to pass through a polarized epithelial monolayer, a model for mucosal epithelium transcytosis. Indeed, the *iga*- mutant did not traffic across this monolayer as well as gonococci which produced the protease (Hopper *et al.*, 2000a).

IgA1 Protease role in physiological pathogenesis

IgA1 protease was so named because it cleaves human IgA1 on the mucosal surface. Evidence of cleavage fragments have been found in infections (Hedges *et al.*, 1998). This may contribute to the mucosal tropism of the *Neisseriae* as IgA1 is primarily encountered in these sites. However, it does not appear to play as strong a role in immune evasion, as once believed. Although it does cleave hIgA1 Fab fragments from their Fc domain, disabling opsonization for professional phagocytes, these Fabs still bind to bacterial antigens and can neutralize microbial surface structures. The cleavage of TNFRII (TNF receptor II) on the surface of monocytes most likely plays a role in evasion of immune clearance. In contrast, the a-fragment of the IgA protease pro-protein has been shown to induce the expression of proinflammatory cytokines, which is consistent with the fact that inflammation is the key disease process once the bacteria crosses the epithelial barrier.

The cleavage of Lamp1 by IgA1 protease has strong implications for physiological process of disease. Since bacteria are more likely to invade through cells and survive if they express the protease, it follows that expression of the protease may correlate with disease invasiveness. A recent study supports this hypothesis, demonstrating a correlation between protease expression levels in clinical isolates and

occurrence of disease by meningococci (Vitovski *et al.*, 1999). This thesis will explore the role of IgA1 protease in disease invasiveness, as well as the significance of Lamp1 cleavage in this process further.

A major obstacle to the study of *Neisseriae* pathogenesis in the lab is the fact that humans are their only host. These bacteria only bind to human receptors and only utilize human iron sources. Both an estradiol-treated murine model and an infant rat model have been developed for the testing of GC cervicitis and (meningococcemia), respectively, but they lack human factors known to influence infection (e.g. hTf, hIgA1, CD46, carcinoembryonic antigens, and HCG). A volunteer male urethral challenge study has been employed, but has failed to distinguish differences for mutants lacking many known virulence factors such as IgA1 protease and Opa. Gonococcal infections may also be tested in a human fallopian tube organ culture (FTOC) model, but the availability of organs limits its utility for broad genetic studies.

Two tissue culture cell lines have been widely used and studied in our lab and will be described in this thesis. Others, such as the recently-developed immortal vaginal, endocervical and ectocervical cell lines for gonococci offer promise for future investigations to be closer to physiologically-relevant. Due to the uncertainty of the pathway of meningococcal infection, as well as their difficulty in handling, endothelial cells were not used in our studies.

This thesis explores three distinct aspects of IgA1 protease and its pathogenic role within the host. The first manuscript looks at regulation of IgA1 protease by iron. In the

presence of high iron, expression of the protease is transcriptionally repressed by the ferric uptake regulator protein, Fur. In low iron environments, then, *iga* expression is higher. We also show that this regulation has a significant effect on the pathogenesis of gonococci through polarized epithelial monolayers. In the presence of human transferrin, *iga* is repressed such that the bacteria transcytose at the same rate as an *iga* null mutant. Bacteria expressing more protease (i.e. in medium without added transferrin) transcytose at a significantly faster rate.

Next, we explored IgA1 protease access to the substrate Lamp1. The second manuscript by Ayala, et al. was the first to explore this concept. We discovered that protease can access the proline-rich hinge of Lamp1 on the cell surface. It accomplishes this by signaling via pili, which stimulate an intracellular release of calcium. This induces the exocytosis of Lamp1-containing lysosomes to the surface, where the IgA1 protease may cleave it.

Further work characterized the dynamics of this process. We constructed a Lamp1 fusion to EGFP, which enabled visualization of lysosomal movement in real time. We discovered that pili and other component of gonococcal outer membranes could actually direct the exocytosis of Lamp1 compartments to the site of their attachment. A two-step calcium-dependent process of compartment relocalization and fusion is outlined. This work demonstrates how *Neisseriae* possess the ability to drastically manipulate host cellular processes.

Lastly, we compared the two alleles of the *iga* gene and their contribution to *Neisseria* pathogenesis. Based on the sequencing of the Type 1 *iga* gene, we developed a simple PCR assay by which isolates could easily be "typed". This method to analyze the *iga* locus of 68 gonococcal isolates. We discovered that IgA1 protease type correlated well with invasiveness of disease. To test the contribution of Lamp1 cleavage in this process, Lamp1 hinge mutants were made in transfected cell lines. These cell lines could then be used to test the invasiveness of *iga* isogenic strains from each allele. Our findings indicate both the specificity of IgA1 protease types within the Lamp1 hinge, as well as projecting the contribution of Lamp1 cleavage in *Neisseria* invasiveness.

MANUSCRIPT 1:

IgA1 protease repression by iron and its significance in *Neisseria* pathogenesis

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SUMMARY

IgA1 protease plays a multitude of roles in the virulence of pathogenic *Neisseriae*. While it has long been assumed that the protease is constitutively expressed, the regulation of the *iga* gene had yet to be investigated. Recent results linking protease activity with severity of disease (Vitovski *et al.*, 1999; Vitovski *et al.*, 2002), suggest that variations in the level of the protease may, itself, participate in the virulence of these pathogens. We identified a potential Fur (Ferric uptake regulator)-regulated region in the promoter of the *iga* gene in both *Neisseria gonorrhoeae* and *Neisseria meningitidis*. We show that *iga* transcription is less within an iron-rich environment than under iron-poor conditions. Protease activity itself, however, is not affected by iron. Studies with GFP promoter fusion constructs demonstrate that mutations in the putative Fur-interaction sequence abolish iron repression of *iga*. Finally, we address a role this regulation may play in *Neisseria* pathogenesis. Previous work from our group (Hopper *et al.*, 2000a) showed that IgA1 protease is important in the trafficking of gonococci across a polarized epithelial monolayer; an *iga*- mutant is compromised in its ability to transcytose. In this work, we show that with the addition of human transferrin, wild-type gonococci behave identically to their *iga*- isogenic counterparts in epithelial transcytosis. Taken together, our results clearly demonstrate the iron regulation of IgA1 protease and suggest the importance of this regulation in *Neisseria* pathogenesis.

INTRODUCTION

The IgA1 protease of pathogenic *Neisseria gonorrhoeae* and *Neisseria meningitidis* has long been considered an important factor in their virulence. The protease was originally identified on the basis of its hydrolytic activity on the human IgA1 hinge (Plaut *et al.*, 1975), but recent reports have revealed additional substrates for the enzyme. It cleaves hLamp1 *in vitro and in vivo*, a reaction that leads to a reduction of functional lysosomes within infected cells (Ayala *et al.*, 1998; Hauck and Meyer, 1997; Lin *et al.*, 1997). IgA1 protease has also been shown to cleave the SNARE protein synaptobrevin *in vitro* (Binscheck *et al.*, 1995), to cleave human chorionic gonadotropin (Senior *et al.*, 2001), and to induce the expression of proinflammatory cytokines (Lorenzen *et al.*, 1999; Tsirpouchtsidis *et al.*, 2002).

The gene encoding IgA1 protease, *iga*, has long been considered to be constitutively expressed (Pohlner *et al.*, 1987a), although its regulation has never been examined. Recent studies have demonstrated a correlation between IgA1 protease activity and severity of disease (Vitovski *et al.*, 1999; Vitovski *et al.*, 2002). These observations suggest that variations in the level of the protease may influence the virulence of these pathogens, and imply that IgA1 protease expression may be regulated. *Neisseriae* reside solely in the human host and, during the infection process, they are subject to several changes in local environment. Both species undergo periods of exposure to conditions on the surface of a mucosal epithelium, within host cellular compartments, in the epithelial substratum, within the bloodstream, or in the presence of harsh bodily fluids such as urine and CSF. Like other infectious microbes, the pathogenic *Neisseriae* must recognize changes in their environment in order to

differentially regulate their genes, to ensure that each gene is being expressed in the right place at the right time. This regulation is probably most important for metabolic "survival" genes, for the bacteria to adapt to its new environment. It has also been shown to play a role in the regulation of virulence genes, enabling expression of site-specific weaponry against host defenses.

One well-studied environmental regulator of bacterial gene expression is iron. The genes of several proteins, including many virulence factors, have been shown to be transcriptionally regulated by the Ferric uptake regulator (Fur) protein (Chai *et al.*, 1998; Stojiljkovic *et al.*, 1994). In the presence of iron, Fur interacts with a specific sequence in the promoter region of many genes, thereby repressing transcription. In iron-poor conditions, Fur interaction is inhibited and transcription of these genes proceeds. Transcriptional activation by Fur has also been reported (Dubrac and Touati, 2000).

Unfortunately, it has not been as simple to study the Fur regulon in *Neissseriae* as it has been in other Gram-negative bacteria. A *fur* knockout strain in *Neisseria* is not viable. *E. coli* Fur is only ~50% homologous to the gonococcal protein, thus it is unlikely to interact with most Fur-regulated promoters in GC and MC (Berish *et al.*, 1993). A mutant Fur was isolated in gonococci using manganese selection; however, its utility in identifying genes in the neisserial Fur regulon is limited to those affected by the specific mutation and not necessarily by Fur altogether (Thomas and Sparling, 1996). A recent study identified several new Fur-regulated genes by an *in silico* search for homology to a known Fur-interaction sequence and was able to show gonococcal Fur binding to these sequences *in vitro* (Sebastian *et al.*, 2002). It is clear from these studies

that Fur plays an essential role in titrating the expression of multiple genes within *Neisseria*, although the *in vivo* significance of this activity remains to be elucidated.

We have identified a potential Fur-interaction sequence in the promoter of *iga*, the neisserial gene encoding the IgA1 protease. This sequence is present in the promoter sequences upstream of both *iga* alleles (encoding Type 1 and Type 2 proteases), in both *Neisseria gonorrhoeae* (gonococci, or GC) and *Neisseria meningitidis* (meningococci, or MC). We therefore wished to test the hypothesis that the *Neisseriae iga* genes are iron-regulated. Cleavage of hIgA1 (human IgA1) by culture supernatants showed that IgA1 protease activity is decreased in conditions incubated with excess iron in the growth medium. Activity of protease itself, however, was not affected by iron within the cleavage reaction. RNase protection assays and GFP promoter fusions showed that iron regulation occurred at the transcriptional level. IgA1 protease production, then, was repressed under high-iron, and induced under low-iron conditions. Regulation of *iga* requires the putative Fur-interaction sequence, as point mutations in this region of P_{iga} abolished iron-responsiveness. Previous work from our group (Hopper *et al.*, 2000a) showed that IgA1 protease modulates the trafficking of gonococci across an epithelial monolayer; an *iga*⁻ mutant is compromised in its ability to transcytose compared to IgA1 protease-producing wild type bacteria. In our present studies, we therefore also addressed the role that iron regulation of *iga* may play in *Neisseria* transcellular trafficking. Results from transcytosis experiments indicate that wild-type gonococci behaved identically to their isogenic *iga*⁻ counterparts in the presence of human transferrin (i.e., high iron conditions). These results support the earlier transcytosis findings and illustrate the utility of the GFP-P_{iga} fusions in probing iron levels in the

intracellular environment of *Neisseria*. Taken together, our studies show that *Neisseria iga* expression responds to iron levels and that this regulation likely involves the interaction of Fur with its cognate sequence in the *iga* promoter. They provide a starting point for understanding the iron levels available to the pathogenic *Neisseria* during the infection process as the bacteria move from one micro-environment to another. The *iga* gene was recent demonstrated by microarray analysis to be upregulated within meningococci during infection of epithelial cells (Dietrich *et al.*, 2003). Our results on Fur regulation of *iga* provide a molecular basis for this upregulation. Many other bacterial pathogens also produce IgA protease, but the regulation of these *iga* genes has not been examined. To our knowledge, this is the first demonstration that *iga* genes are transcriptionally regulated in response to environmental iron.

EXPERIMENTAL PROCEDURES

Sequence analysis

DNA sequences were downloaded from GenBank and analyzed using VectorNTI Suite software for Mac from Informax.

Bacterial strains

MS11A (Pili+, Opa-) gonococci produces a Type 2 IgA1 protease from an *iga* gene that has been sequenced and well characterized (Halter *et al.*, 1984; Pohlner *et al.*, 1987b). *Neisseria gonorrhoeae* strains GCM740 (Pili+, Opa-, *iga*+) and GCM740Δ4 (Pili+, Opa-, *iga*-) are previously described (Hopper *et al.*, 2000a; Shoberg and Mulks, 1991). *N. meningitidis* strain 8013.6 is a piliated isolate of serogroup C (Nassif *et al.*, 1993).

In vitro cleavage of hIgA1

MS11A gonococci were cultured in GCB liquid medium for six hours at 37°C, 5%CO_2. IgA1 protease-containing supernatants were then collected following centrifugation. Either samples from these supernatants or purified IgA1 protease (Type 2, Roche) were incubated with purified human IgA1 (from colostrum, Calbiochem) at 37°C for 2-3 hours. Samples were analyzed by SDS-PAGE stained with Coomassie Blue. Images of these gels were obtained by a BioRad GelDoc 1000, and densitometry analysis of Coomassie Blue bands was performed using NIH Image, v.1.62.

RNase Protection Assay

A plasmid to transcribe an RNA anti-sense probe for *iga* was made by isolating a fragment of the gene by PCR from MS11A, and cloning into the MCS of pBluescriptIIKS+. This fragment was transcribed in the presence of ^{32}P-labelled UTP (ICN) using the Maxiscript T3/T7 Kit (Ambion). The probe was isolated following gel electrophoresis, and used immediately for RNase Protection according to the protocol with the RPAIII kit from Ambion. Total RNA for analysis was isolated from MS11A (RNAEasy; Ambion) from 6-hour GCB liquid cultures in the presence of Desferal (100μM; Ciba-Geigy) or various concentrations of Supplement II (5 mg/ml $Fe(NO_3)_3$ (Mallinckrodt).

Construction and transformation of GFP promoter fusions

A plasmid encoding GFP following a *tac* promoter with flanking sequence homologous to the gonococcal cryptic plasmid was a kind gift from V. Clark. Plasmid pJAL151 encodes GFP ("mutant 2") following a promoterless multiple cloning site and its own RBS; this sequence is flanked by the same DNA from the gonococcal cryptic plasmid (JP Dillard) (pJAL151 constructed by J. Larson, manuscript in preparation). 80 bp of promoter sequence from *iga* (upstream of the RBS) was amplified by PCR and cloned into the MCS of pJAL151, yielding pJAL153a (P_{iga}GFP). Site-directed mutagenesis was performed in order to mutate the promoter sequence (as in Figure 1) by long range PCR (in a Strategene Gradient Robocycler 96), followed by DpnI digestion and transformation into *E.coli* DH5α. Mutagenesis was confirmed by sequence

determination by the OHSU-MMI Research Core Facility (http://www.ohsu.edu/core) on a model 377 Applied Biosystems Inc. automated fluorescence sequencer.

These plasmids were transformed into MC8013.6 as described previously (Hopper *et al.*, 2000a). Of note, P_{iga} GFP transformation required passage of meningococci on high-iron (20μg/ml $Fe(NO_3)_3$) plates before, during, and following transformation (see Discussion). Cloramphenicol-resistant recombinants were selected by plating on GCB containing 3μg/ml and 20μg/ml $Fe(NO_3)_3$. GFP expression was confirmed by immuno-dot blot of lysates spotted on to nitrocellulose filters using a monoclonal αGFP antibody (Clontech; data not shown).

GFP expression assays

Meningococcal strains to be tested (P_{tac}GFP, P_{iga}GFP, and mutP_{iga}GFP) were struck from frozen glycerol stocks on to GCB agar plates containing 10x Supplement II (=10μg/ml $Fe(NO_3)_3$) and grown at 37°C, 5% CO_2 for 10-16 hours. Colonies from these plates were resuspended in GCB liquid medium with 1x Supplement I and divided into two tubes, one of which was then supplemented with 10x Supplement II. Samples from these cultures were aliquoted in quadruplicate to sterile black 96-well microtiter plates (Packard), then incubated for two hours at 37°C, 5%CO_2. Fluorescence was measured using a fluorimetric plate reader (Spectramax Gemini XS, Molecular Devices). Relative GFP expression was calculated by subtracting the background fluorescence of GCB medium alone from each of the measurements, then dividing this fluorescence in the low-iron cultures by the fluorescence in the iron-supplemented wells. Numbers expressed are

the average of three separate experiments with the average standard deviation from these means.

Cell culture and Transcytosis Assay

T84 colorectal carcinoma cells were obtained from the American Type Tissue Collection (ATTC). They were maintained in DMEM-F12 medium (Gibco BRL) supplemented with 5% FCS (Gibco BRL). Cells were polarized on Transwell filters with 3μm pores (Costar). Cultures were maintained, assayed for polarization, infected and assayed for transcytosis as previously described (Hopper 2000), with the exception that 5μM human Transferrin (Intergen) was supplemented in the medium for transcytosis assays in this study.

RESULTS

The *iga* promoter in *Neisseriae* contains a putative Fur-interaction sequence

We began our studies on the regulation of *Neisseria iga* genes by examining their promoter sequences. The *iga* sequences from the following strains were compared: gonococcal isolate 32819 (Type 1 producer; Vasquez, Mulks and So, manuscript in preparation, (Fishman *et al.*, 1985)), gonococcal strain FA1090 (Type 1 producer; GenBank Acc. No. AE004969), gonococcal strain MS11 (Type 2 producer; NGIGAPRO; GenBank Acc. No. X04835) and meningococcal strains MC58 and Z2491 (Type 1 producers; PIR ref. Nos. A81000 and A81775). Alignments of the P_{iga} sequences are shown in Figure 1. These comparisons demonstrate that the *iga* promoters are nearly identical from –50 to the start codon. They also reveal a putative Fur-interaction sequence, the so-call "Fur box", located between –41 and –23 and overlapping the assumed RNA polymerase binding at the designated "–10 site" or TATA box.

Several Fur-regulatory sequences have been described for *Neisseriae* (Sebastian *et al.*, 2002) and other Gram-negative bacteria (Stojiljkovic *et al.*, 1994). From these studies, a consensus 19bp Fur box sequence in Gram-negative bacteria was deduced. This consensus sequence is shown in Figure 1B, together with the "acceptable" permutations at each position (i.e. substitutions that do not alter Fur regulation activity.) The putative *Neisseria iga* Fur-interaction sequence is identical to the consensus sequence in 18 of the 19 positions. The other residue, at position –12, is adenosine, which is considered an "unacceptable" permutation. The putative P*iga* Fur-box sequence is also in good agreement with the consensus Neisseria Fur-box sequence (Genco and

Desai, 1996) and with the Fur-box sequences of other recently identified Fur-regulated Neisseria genes (Sebastian *et al.*, 2002).

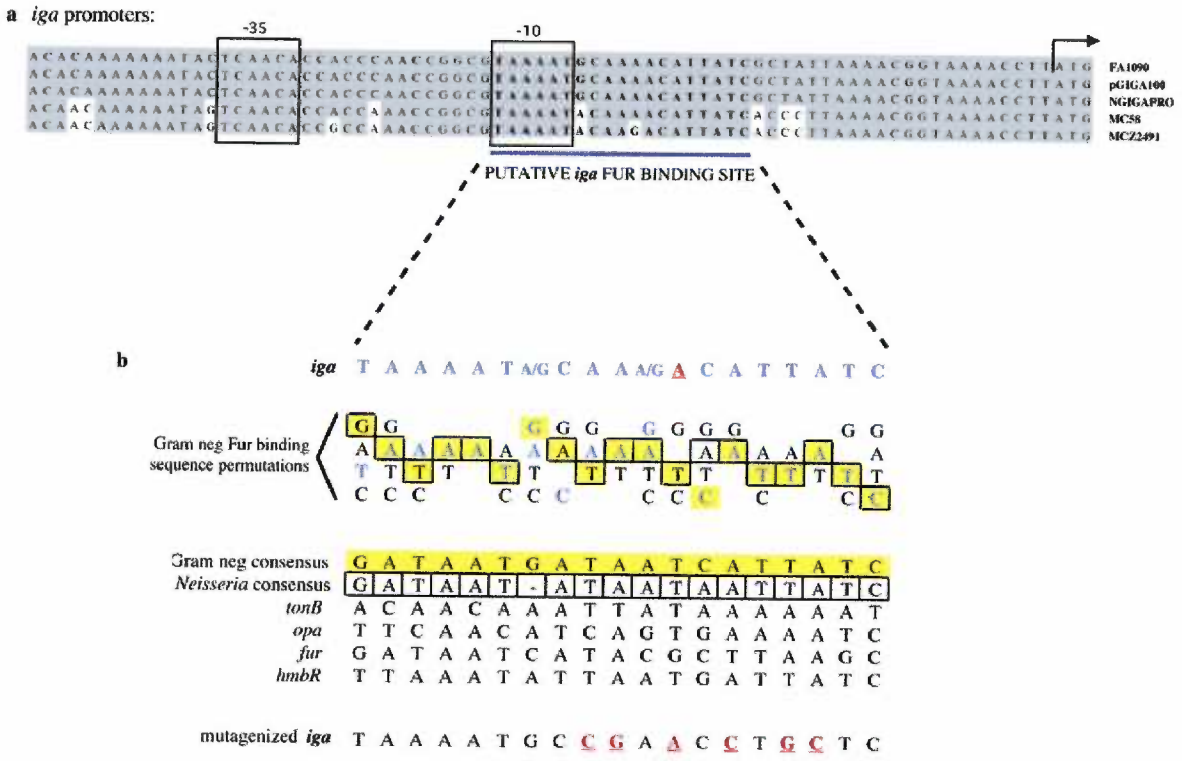

Figure 1. Alignment of *iga* promoter and known "fur box" sequences. a. *Iga* promoters are well-conserved amongst both *Neisseria gonorrhoeae* and *meningitidis* strains. 75 bp of sequence upstream of the *iga* gene from five isolates aligned. " –10" and "–35" sequences are from annotations in the NGIGAPRO GenBank sequence. The putative Fur regulatory site is highlighted in bold. **b.** The putative Fur-interaction site is compared to other Gram-negative Fur recognized sequences (Stojiljkovic *et al.*, 1994), as well as other gonococcal sequences shown to bind Fur (Genco and Desai, 1996; Sebastian *et al.*, 2002).

Neisseria incubated under high iron conditions produces less IgA1 Protease

We next determined whether or not IgA1 protease production by gonococcal strain MS11A in liquid culture is influenced by iron levels. The neisserial IgA1 protease is completely autosecreted by a Type 1 secretion system, resulting in an absence of

protease activity on the bacterial cell surface or within the cytoplasm (Reinholdt and Kilian, 1997). IgA1 protease production was determined by assaying the bacterial supernates for proteolytic activity on hIgA1. Cleavage of hIgA1 by the neisserial IgA1 protease generates two products that migrate as a broad band at 25-35 kD in SDS-polyacrylamide gels (Mulks and Shoberg, 1994). For an accurate assessment of protease activity in these experiments, the level of full-length hIgA1 heavy chain remaining after incubation with supernate was determined by densitometry and expressed as a percent of the total level of input substrate (Figure 2, Panel A). The actual Coomassie Blue-stained full length hIgA1 remaining after incubation is shown in Figure 2, Panel B. Results show that IgA1 protease activity is inversely correlated with iron levels in the growth medium. Protease activity was highest in the supernates of cultures grown under low iron conditions, i.e., medium pre-treated with 10 μM desferal to chelate free iron. It is lowest in supernates of high-iron cultures, i.e., medium supplemented with $50^{\mu g}/_{ml}$ $Fe(NO_3)_3$.

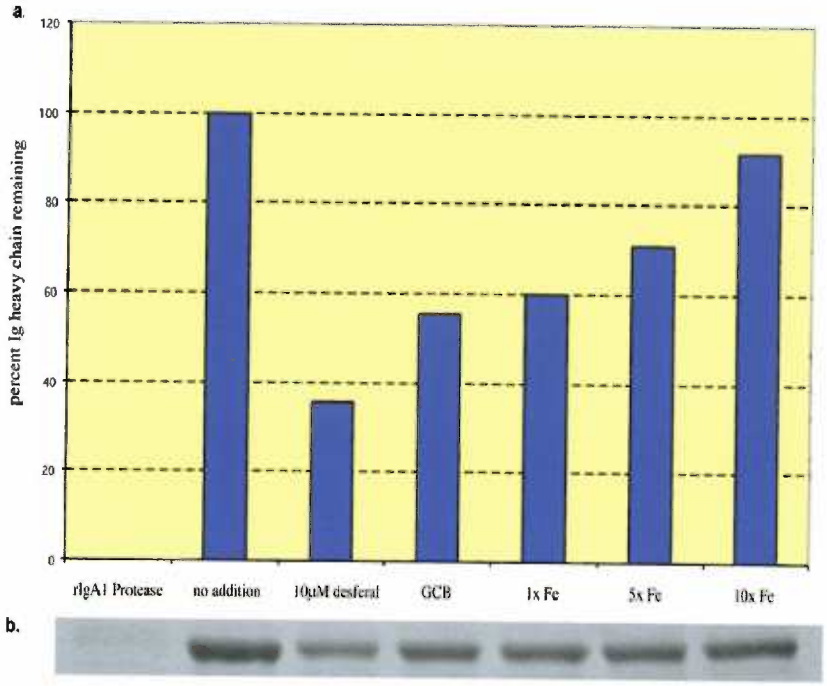

Figure 2. Iron affects IgA1 protease production by *Neisseria*. Supernatants from gonococcal cultures incubated with specified concentrations of iron or the iron chelator, desferal, were used to cleave human IgA1 in equal amounts. **a.** Concentration of α1 heavy chain remaining after protease cleavage, as analyzed by SDS PAGE. **b.** Coomassie Blue stain of full-length heavy chain present following cleavage reaction. 1x Fe = $5\ ^{\mu g}/_{ml}$ = 12.4μM $Fe(NO_3)_3$.

Controls for this experiment include incubation of excess recombinant IgA1 (rIgA1) protease with substrate, and substrate alone. This experiment indicates that a high level of free iron in the media reduces IgA1 protease activity on hIgA1.

IgA1 protease, a serine protease, was once believed to be a metalloprotease because of the inhibitory effects of some metal ion chelators on its enzymatic activity. To determine whether iron physically inhibits protease activity, we incubated rIgA1 with increasing concentrations of free iron and measured protease activity as described above (Figure 3). Protease activity did not vary with free iron levels. Taken together, these results strongly suggest that Neisseria *iga* expression is iron-regulated, and that high levels of free iron in the culture medium inhibits expression.

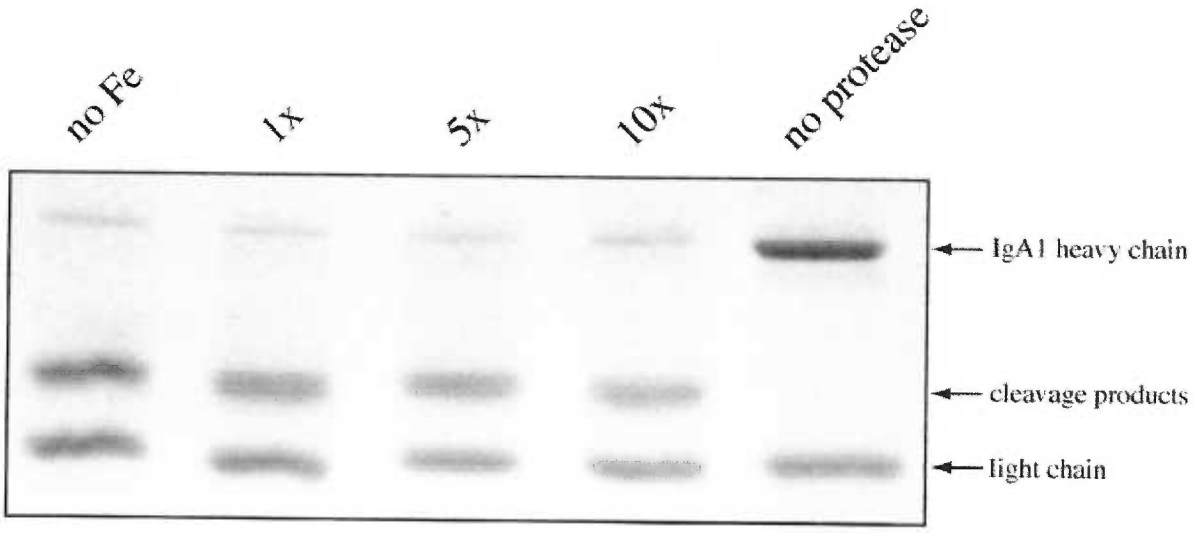

Figure 3. Iron concentration does not affect cleavage activity of IgA1 protease. hIgA1 was incubated with purified recombinant IgA1 protease and iron added, at specified concentrations, for 4 hours at 37°C. Products of cleavage were visualized by SDS-PAGE with Coomassie Blue. 1x Fe = 5 $^{\mu g}/_{ml}$ = 12.4μM $Fe(NO_3)_3$.

Transcription of *iga* is repressed under conditions of high iron

In most instances, iron regulation by Fur takes place by promoter interaction and inhibition of gene transcription. We therefore wished to confirm the effect of iron on transcription of the *iga* gene. Gonococcal strain MS11A was grown in liquid medium containing varying levels of $Fe(NO_3)_3$. RNA was extracted from these cultures and used for an RNase protection assay with an oligonucleotide probe encoding sequences within the *iga* open reading frame (Figure 4). Results show that *iga* transcript levels were highest in bacteria grown in desferal-treated medium, and lowest in bacteria grown in medium supplemented with 62μM Fe^{+3} ($50^{\mu g}/_{ml}$ $Fe(NO_3)_3$). They are consistent with the results from the preceding experiments, which demonstrated a clear reduction in active protease secretion when bacteria were grown under high iron conditions.

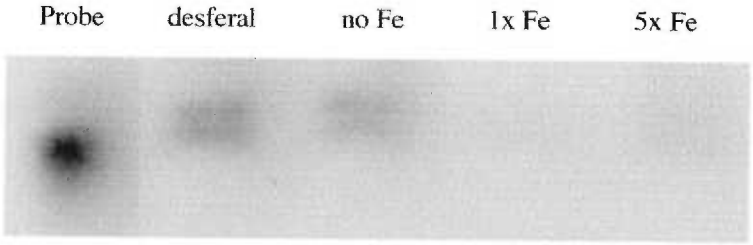

Figure 4. RNase protection analysis of *iga* transcribed under different iron conditions. RNA was isolated and subjected to RNAse protection analysis following gonococcal incubation in the appropriate iron-containing medium for six hours. RNAse protection conditions were as described in Materials and Methods. Probe lane is undigested probe. 1x Fe = $5\ ^{\mu g}/_{ml}$ = 12.4μM $Fe(NO_3)_3$.

Mutations in the putative Fur-inhibition sequence abolish iron regulation of *iga*

We next determined whether iron regulation of *iga* involved the P_{iga} region containing the putative Fur-interaction sequence. A fragment of DNA containing 50 bases of sequence upstream from the *iga* start codon was ligated to the promoterless open

reading frame encoding Green Fluorescence Protein (GFP). Flanking sequences within the plasmid permitted insertion into the wild-type *N. meningitidis* MC8013.6 chromosome by homologous recombination. (We were unable to stably express this construct in the gonococcal MS11A strain.) To further demonstrate the importance of this putative Fur-interaction sequence in iron regulation, a mutant P_{iga} was constructed by site directed mutagenesis. In this mutant P_{iga} (mutP_{iga}), five bases within the Fur-interaction sequence, but downstream of the "–10" RNA polymerase binding site, were exchanged for nucleotides that are not considered "acceptable" bases (Stojiljkovic *et al.*, 1994). A plasmid containing the common bacterial *tac* promoter (P_{tac}) upstream of GFP was constructed as a positive control of iron-independent expression of GFP.

Following transformation, the MC P_{iga}GFP and mutP_{iga}GFP and P_{tac}GFP strains were passed on GCB agar plates containing high amounts of iron in order to repress expression of GFP. Colonies from these plates were transferred to liquid medium without iron or containing 10x free iron (124 μM $Fe(NO_3)_3$) and GFP fluorescence was quantified by fluorimetry. New GFP expression was determined by calculating the ratio change in fluorescence between the two conditions after two hours incubation (GFP fluorescence no iron/GFP fluorescence high iron, corrected for background media fluorescence; Figure 5.)

P_{tac}GFP and mutP_{iga}GFP showed little difference in fluorescence between low- and high- iron cultures (0.97 +/- 0.13 and 1.02 +/-0.11, respectively). The P_{iga}GFP strain, however, expressed significantly more GFP in the absence of iron (2.08 +/- 0.22) (Figure 5). These results were derived from three independent experiments, each performed in triplicate.

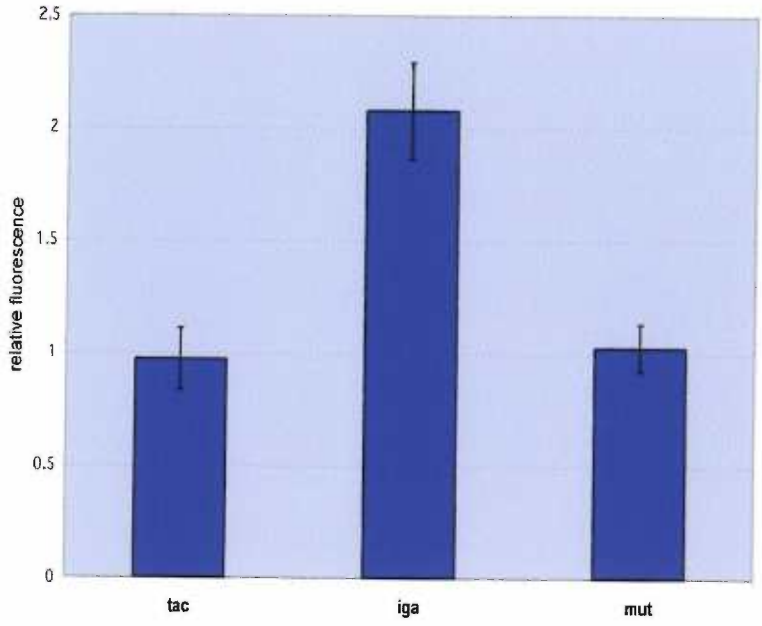

Figure 5. Sequence-specific iron repression of a P_{iga}GFP promoter fusion. Meningococci containing each promotor fusion to GFP on the chromosome were harvested from high-iron GCB plates into liquid medium with or without 124μM $Fe(NO_3)_3$ supplementation. Two hours following incubation in this medium, GFP expression was measured using fluorimetry. Represented are the fluorescence differences between cultures with no iron in the medium from cultures incubated with 10x Fe supplementation (124 μM).

Taken together, the above findings demonstrate that IgA1 protease production varies inversely with the levels of free iron in the bacterial environment. Iron regulation of IgA1 protease occurs at the transcriptional level, involving the Fur-interaction sequence which overlaps the –10 sequence in the *iga* promoter, (residues –41 to –23.)

Iron repression of *iga* hinders gonococcal transcytosis

The transcytosis model for *Neisseria* infection (Hopper *et al.*, 2000b; Merz *et al.*, 1996; Merz and So, 2000), has been used to study the role of several *Neisseria* virulence factors in transepithelial trafficking, including the IgA1 protease. Briefly, T84 human colorectal epithelial cells seeded onto a semi-porous synthetic membrane will "polarize" and form a barrier similar in architecture and function to other mucosal epithelia (Madara *et al.*, 1987; Merz *et al.*, 1996). Pathogenic *Neisseria* inoculated onto the apical region of polarized T84 monolayers will enter and traffic through the longitudinal axis of invaded

cells, and exit through the basolateral region of the cell into the bottom well. An *iga*⁻ mutant traffics across polarized T84 monolayers more slowly than the isogenic wild-type strain (Hopper *et al.*, 2000a). This defect of the *iga*⁻ mutant is consistent with its demonstrated decrease in intracellular survival (Lin *et al.*, 1997). These findings predict that gonococcal transcytosis would be responsive to iron levels in the medium, and furthermore, that withholding iron would increase the rate of gonococcal transepithelial trafficking.

We determined whether iron availability influenced gonococcal transcytosis through polarized T84 monolayers. *In vivo*, gonococci acquire iron from host iron-binding proteins, including human transferrin (Genco and Desai, 1996; Schryvers and Stojiljkovic, 1999). The ability of gonococci to utilize hTf is essential for the bacteria to initiate an infection in the urethra of men (Cornelissen *et al.*, 1998). Transcytosis assays were therefore performed in tissue culture medium (DMEM + 10% FBS) with or without hTf. The apical region of polarized T84 monolayers were inoculated with wild-type gonococcal strain GCM740, or GCM740Δ4, the *iga* null mutant (the isogenic strains used in our previous transcytosis experiments; (Hopper *et al.*, 2000a). At various times, the bottom wells were sampled for the presence of bacteria. For comparison, an *iga*⁻ mutant was assayed in parallel. In the presence of hTf (high iron conditions), both wild-type and *iga*⁻ strains trafficked across polarized monolayers within the same time period (Figure 6). However, in media lacking hTf (low iron conditions) wild-type bacteria always trafficked across the monolayers more quickly than the mutant (Hopper *et al.*, 2000a). These results suggest that iron regulation of *iga* plays a role in transcellular trafficking and confirms the importance of IgA1 protease in this aspect of infection.

DISCUSSION

The gene encoding the IgA1 protease (*iga*) of pathogenic *Neisseriae* has long been considered to be consitutively expressed. A comparison of a number of Neisseria *iga* promoters revealed that this region is highly conserved, with >87% sequence identity. The analysis also revealed a potential Fur-interaction site located between residues –23 to –41 upstream of the start codon, in which 18 of the 19 residues are identical to other Gram-negative Fur box sequences (Stojiljkovic *et al.*, 1994). In this report, we presented results of experiments that tested the hypothesis that Neisseria *iga* is transcriptionally regulated by the Fur system. We showed that supernatant IgA1 protease activity (Figure 2) and *iga* transcript levels (Figure 4) were highest when bacteria were grown in iron poor medium and lowest when they were grown in iron rich medium. The activity of protease was not affected by iron within the cleavage reaction itself (Figure 3). Finally, in cells containing the promoter fusion construct P_{iga}GFP, fluorescence intensity was significantly higher in cells grown in low iron medium than those grown in high iron medium; this variation was dependent on the putative Fur-interaction sequence (Figure 5). Taken together, these results demonstrate that the *Neisseria* IgA1 protease is transcriptionally regulated through the Fur regulon.

In this report, we demonstrated that iron also plays a role in *Neisseria* transcellular trafficking. Our previous results demonstrated that *iga*- bacteria transcytosed polarized T84 monolayers significantly more slowly than wild type bacteria (Hopper *et al.*, 2000a). In the present study, we found that wt bacteria had similar transit times similar to those of the *iga* mutant when the transcytosis experiments were performed in high iron conditions (Figure 6). These observations are consistent with our

previous findings that the IgA1 protease plays a role in gonococcal intracellular growth and transcellular trafficking (Hopper *et al.*, 2000a; Lin *et al.*, 1997). In this context, it is interesting to note that human challenge studies have illustrated the importance of iron acquisition to the ability of gonococci to initiate infection in the urethra of men (Cornelissen *et al.*, 1998). Thus, iron availability is an important modulator of *Neisseria* virulence at the mucosal surface.

Fur-regulated genes were originally considered part of iron-uptake regulons that are induced in response to iron starvation. The neisserial *tonB*, encoding an energy transducer required for iron uptake, and *fbpA*, encoding a ferric binding protein, are repressed by Fur in iron rich, and induced in iron-poor, media (Genco and Desai, 1996; Schryvers and Stojiljkovic, 1999; Sebastian *et al.*, 2002; Thomas and Sparling, 1996). Recent reports indicate that a number of genes that do not function in iron uptake are also Fur-regulated (Sebastian *et al.*, 2002; Thomas and Sparling, 1996). The Neisseria *iga* gene likely belongs to the latter category. The IgA1 protease plays a role in altering lysosomes and subsequently promoting bacterial intracellular survival and transepithelial trafficking. In this sense, it is a virulence factor. However, recent evidence suggests that the Type1 IgA1 protease may also play a role in iron acquisition. The β-subunit of the IgA1 protease, the transmembrane autotransporter subunit, oligomerizes in the membrane to form a 2 micron pore. Besides the secretion of the protease subunits, the function of this structure is unknown, but has been speculated to participate in transport of other solutes (Veiga *et al.*, 2002).

Several other mucosal pathogens secrete an IgA1 protease, including *Haemophilus influenzae*, pneumococci, and several oral streptococci (Kornfeld and Plaut,

1981; Qiu *et al.*, 1996). Aside from their ability to cleave human IgA1, the functions of these proteases are unclear. The regulation of these *iga* genes is also unclear.

Multiple attempts at transforming the P_{iga}GFP fusion into gonococci were unsuccessful. The fusion could be transferred into meningococci only by plating the tranformants on high iron agar plates (see Materials and Methods). Meningococci containing the P_{iga}GFP construct lost viability upon prolonged passage, even on high iron plates (i.e., Fur repressive conditions). They are therefore kept as frozen stocks and passaged only once on high iron plates before each experiment. The reason for decreased viability of the P_{iga}GFP construct is unclear. Green Fluorescent Protein is known to be toxic at high levels for some bacteria, including *Neisseriae* (Tsien, 1998). It is possible that the *iga* promoter may not be completely repressed under high iron conditions, allowing enough GFP to accumulate to reach toxic levels.

What role does iron regulation of *iga* play in *Neisseria* infectivity? Using cell culture models of infection, we have demonstrated that an IgA1 protease-producing strain survives better within epithelial cells and crosses polarized epithelial monolayers more quickly than an isogenic *iga* null mutant. We have also shown that IgA1 protease cleaves Lamp1, leading to a reduction in the total number of lysosomes within an infected cell (Hopper *et al.*, 2000a; Lin *et al.*, 1997). We hypothesized that this enzymatic activity of the protease is responsible for promoting bacterial intracellular survival and efficient trans-epithelial trafficking, as cells with fewer functional lysosomes should be less able to deal effectively with invading bacteria. The role of iron in regulating *iga* expression and *Neisseria* infectivity, therefore, may be to allow IgA1 protease production at the appropriate place and time, i.e., at the site of infection of the mucosal epithelium. Further

studies on the temporal expression of *iga* during infection, using the P_{iga}GFP fusion strain, will shed light on this model. As the availability and concentration of iron at various sites in the human body is not well defined, these studies may also yield information on the iron status of the immediate surroundings of the bacteria during infection.

MANUSCRIPT 2:

The pilus-induced Ca^{2+} flux triggers lysosome exocytosis and increases the amount of Lamp1 accessible to *Neisseria* IgA1 protease**

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ABSTRACT

The IgA1 protease secreted by the pathogenic *Neisseriae* cleaves Lamp1, a major integral membrane glycoprotein of lysosomes, and significantly reduces its steady state levels in an infected cell. IgA1 protease hydrolysis of Lamp1 is inefficient at the low pH of lysosomes, strongly suggesting that the enzyme is unlikely to reduce Lamp1 levels within lysosomes to any appreciable extent. We therefore explored the possibility that the protease may reach Lamp1 through an alternate route. We demonstrate that *Neisseria* pili induce a transient increase in the levels of cytosolic free Ca^{2+} in A431 human epithelial cells, as was demonstrated previously for ME180 cells. This Ca^{2+} flux triggers lysosome exocytosis, quickly altering the cellular distribution of Lamp1 and increasing surface Lamp1 levels. Finally, we demonstrate that surface Lamp1 is cleaved by IgA1 protease secreted by adherent bacteria. We conclude that the pilus-induced Ca^{2+} flux increases the amount of Lamp1 that is cleavable by the IgA1 protease.

INTRODUCTION

Like a number of other mucosal pathogens, *N. meningitidis* (MC) and *N. gonorrhoeae* (GC) secrete an IgA1 protease with specificity for the proline-rich hinge of human IgA1 (hIgA1;(Plaut *et al.*, 1975; Shoberg and Mulks, 1991)). Indirect evidence suggests that the protease cleaves hIgA1 *in vivo*. hIgA1 activity (Blake *et al.*, 1979), anti-hIgA1 antibodies (Hedges *et al.*, 1998) and hIgA1 fragments of the sizes predicted to result from IgA1 protease cleavage (Blake *et al.*, 1979) have been found in the mucous of infected individuals. The IgA1 protease may therefore promote bacterial colonization through cleavage of protective secretory antibodies.

The enzyme also cleaves Lamp1 (Hauck and Meyer, 1997; Lin *et al.*, 1997), a major lysosomal glycoprotein with a proline-rich hinge (Frangione and Wolfenstein-Todel, 1972; Viitala *et al.*, 1988). Protease cleavage of Lamp1 accelerates it degradation and reduces its steady-state levels in infected epithelial cells (Lin *et al.*, 1997). By cleaving Lamp1, the protease indirectly reduces the levels of several other lysosomal constituents, thereby altering the lysosomes of *Neisseria*-infected cells (Ayala *et al.*, 1998). A mutant with a defined deletion in the IgA1 protease gene (*iga*) is affected in intracellular growth (Lin *et al.*, 1997) and in the early stages of passage through polarized T84 cells (Hopper *et al.*, 2000a). These results strongly suggest that IgA1 protease-mediated alterations of lysosomes play a role in *Neisseria* intracellular survival and trafficking.

From other studies, the role of IgA1 protease in infection is less clear. Reductions in Lamp1 levels are detectable by immunoblotting only after prolonged infection (12 –18 hours; (Lin *et al.*, 1997), suggesting that the protease is ineffective in hydrolyzing Lamp1

and/or that its access to the substrate is limited. *In vitro*, IgA1 protease activity against Lamp1 is highest at pH 7.5, intermediate at pH 6.5 and low but detectable at pH 5.0 (Lin *et al.*, 1997), suggesting that the protease may not be active within the acidic lysosomal compartments. Finally, limited human challenge studies with a GC *iga* mutant indicate that the protease is not strictly required for the establishment of gonococcal urethritis in men (Johannsen *et al.*, 1999). Thus, the role of the IgA1 protease in *Neisseria* virulence and infectivity remains to be clarified.

In considering the significance of IgA1 protease interactions with Lamp1, an important issue is the accessibility of the enzyme to this substrate. Lysosomes are terminal degradative compartments in the endocytic route. They contain numerous acid hydrolases that degrade a wide variety of biological compounds and the lumen of this compartment is maintained at low pH by a membrane-associated proton-ATPase (Holtzman, 1989). The ~110,000 M_r Lamp1 is a major integral membrane glycoprotein in the lysosome (Carlsson *et al.*, 1988; Chen *et al.*, 1985). It consists of a short cytoplasmic tail, a single membrane-spanning domain and two heavily-glycosylated lumenal domains separated by an hIgA1-like hinge (Chen *et al.*, 1985). The majority of newly synthesized Lamp1 is delivered from the Golgi directly to lysosomes. A fraction, 5-20% depending on the cell type, travels from the Golgi to the plasma membrane before it is endocytosed and delivered to lysosomes (Akasaki *et al.*, 1995).

That a portion of Lamp1 cycles past the plasma membrane en route to lysosomes suggests one mechanism by which IgA1 protease may gain access to Lamp1, i.e., surface Lamp1 may be cleaved by IgA1 protease secreted at the cell surface by adherent bacteria. Consistent with this hypothesis is the fact that the proline-rich hinge of surface Lamp1 is

on the outer face of the plasma membrane and therefore exposed to bacterial products. Moreover, as mentioned above, protease activity against Lamp1 is highest at pH 7.5, the pH of many mucosal surfaces (Lin *et al.*, 1997).

Recent reports on the effect of the *Neisseria* type IV pilus on host cell signalling lend additional support to this hypothesis. Binding of piliated *Neisseria* or purified pili to epithelial cells transiently increases the levels of cytosolic free Ca^{2+} in ME180 cells. This response is thought to be mediated by CD46, the putative pilus receptor, as it can be blocked by aCD46 antibodies (Kallstrom *et al.*, 1998). The pilus-induced Ca^{2+} transient has a relatively long lag time of 10 minutes and is mediated by release of Ca^{2+} from intracellular stores. Ca^{2+} fluxes of similar magnitude have been demonstrated to induce extensive exocytosis of undefined compartments in Chinese Hamster Ovary (CHO) cells (Coorssen *et al.*, 1996) and lysosome exocytosis in normal rat kidney (NRK) fibroblasts (Rodriguez *et al.*, 1997; Rodriguez *et al.*, 1999). As Lamp1 is an integral component of the lysosomal membrane, and the proline-rich hinge is in the lysosomal lumen, fusion of the lysosome with the plasma membrane would result in an increase in Lamp1 levels at the cell surface and expose the IgA1 protease cleavage site to the extracellular milieu.

We therefore tested the hypothesis that the flux in cytosolic free Ca^{2+} levels induced by the *Neisseria* pilus triggers lysosome exocytosis and an increase in cell surface Lamp1 levels. We also tested the hypothesis that surface Lamp1 can be cleaved by IgA1 protease secreted by adherent bacteria. In this report, we show that *Neisseria* pili induce a transient increase in cytosolic free Ca^{2+} levels in A431 human epithelial cells, as was demonstrated previously for ME180 cells. This Ca^{2+} flux triggers lysosome exocytosis, quickly altering the cellular distribution of Lamp1 and increasing surface

Lamp1 levels. Finally, we demonstrate that surface Lamp1 is cleaved by IgA1 protease secreted by bacteria adhered to the cell surface. We conclude that adhesion to epithelial cells by piliated *Neisseria* increases the amount of Lamp1 that is accessible to secreted IgA1 protease shortly after infection.

MATERIALS AND METHODS

Cell culture

The A431 human epidermoid carcinoma cell line was obtained from S. Schmid. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco). The T84 human colonic epithelial-like cell line (American Type Culture Collection, ATCC) was propagated in T75 flasks (Falcon) in DMEM-F-12 + 5% FCS and polarized on Transwell™ semi-porous filters with 3 mM diameter pores (Costar) as described previously (Merz *et al.*, 1996).

Bacterial strains

All *Neisseria* strains used in this study are listed in Table I. Their piliation status was checked by colony morphology; pilin and Opa production and porin type were checked by immunoblotting using the appropriate mAbs (see below). *N. meningitidis iga* mutant 8013.6-500 was constructed from the WT strain 8013.6 (Nassif *et al.*, 1993) as previously described for construction of the *N. gonorrhoeae iga* strain MS11A-500 (Hopper *et al.*, 2000a). Transformants were selected on supplemented GCB agar with 100 mg/ml kanamycin. Chromosomes of the transformants were checked for correct allelic exchange events by Southern blotting using three different probes.

Confocal microscopy of infected polarized T84 monolayers

Polarized T84 monolayers with transepithelial resistance between 1,000 and 3,000 ohms/cm^2 were used for infection studies. Monolayers were infected with 8013.6 or

8013.6-500 for various lengths of time at an MOI of 50, then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), for 10 min at room temperature (RT). Samples were immunostained ON at 4°C with primary anti-Lamp1 monoclonal antibody (mAb) H4A3 (Pharmingen) and secondary Cy5-labelled goat anti-mouse antibody (Amersham). Following immunostaining, preparations were stained with 3 mg/ml propidium iodide (Molecular Probes) in PBS to stain DNA; and with 0.5 mg/ml FM™1-43 (Molecular Probes) in PBS to visualize membranes. After being washed with PBS, the filters were dissected away from the mounting structure. Each filter was folded in half, monolayer side out, and mounted in 90% glycerol with 5 mM Tris (pH 8.0) and 20 mg/ml n-propylgallate (Sigma), under a #1.5 coverslip. Images were acquired using a BioRad 1024 ES laser scanning confocal microscope (CLSM) on a Nickon Eclipse TE300 inverted fluorescent microscope. Cells were imaged at the fold of the membrane in order to acquire the best apical-basal (coronal) resolution, as reported for imaging tissue sections (Prince et al., 1993). Preparations were viewed trough a 100x N.A. 1.4 PlanApo objective, and cells were selected for imaging based solely on the appearance of their propidium iodide and FM™1-43 staining. Successive optical sections were obtained at 0.5 mm intervals. Images were processed using Adobe Photoshop v.5.0 software for Macintosh.

Antibodies and chemicals

The mAb 10H5.1.1 against the conserved SM1 epitope (Merz and So, 1997) was used for immunoblotting to detect pilin production. mAb 3.10 used to detect PI.A (Elkins *et al.*, 1994) and 3C8 to detect PI.B porin types (Tam *et al.*, 1982) were kindly provided

by P.F. Sparling. mAb 4B12 (from M. Blake) was used to detect Opa production. mAb H4A3 (anti-human Lamp1) was obtained from the Developmental Studies Hybridoma Bank and Pharmigen. Biotinylated mAb H4A3 used to quantitate surface Lamp1 was obtained from Pharmingen (San Diego) and was dialyzed against PBS before use. Streptavidin-POD conjugate was from Boehringer Mannheim (Germany). Thapsigargin and BSA were from Sigma Chemicals Co. (St Louis, MO). BAPTA-AM and Lucifer Yellow were from Molecular Probes, Inc (Eugene, OR); ^{3}H-Dextran (mol wt 70,000) was from Amersham Corp. (Arlington Heights, IL). QuantaBlu™ Fluorogenic Peroxidase Substrate Kit, goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP and Super Signal Chemiluminescence Kit were from Pierce. Fluo-3, AM was from Molecular Probes.

Monitoring fluxes in cytosolic free Ca^{2+} levels

A431 cells were plated in 6-well microtiter plates at 70% confluence and incubated overnight. Cultures were washed in prewarmed Ca^{2+}-free or Ca^{2+} replete medium, and loaded with 2 mg/ml Fluo-3, AM in HBSS supplemented with 5mM $MgCl_2$. Cells were maintained at 34°C throughout the experiment using the Bioptechs DTC3 culture dish heating system, including the stage adaptor and objective heater. Cells were then stimulated with the crude pili or with Tg and fluorescence changes in individual cells were captured using a DeltaVision Restoration Microscopy system (Applied Precision, Inc., Issaquah, WA) equipped with a 60x 1.2 N.A. Water immersion lens and a FITC filter set. Fluo-3, AM fluorescence was imaged in a single plane through the cells as a timelapse. Each acquisition was approximately 2.3 seconds apart. Data sets were

processed using DeltaVision Acquire 3D-software package to quantify fluorescence intensity from each frame through a small section of each individual cell. Intensities from two cells per sample were plotted on a graph (Figure 3) using Microsoft Excel.

Crude pili preparations

Crude pili preparations from piliated (CP-P^+) or nonpiliated (CP-P^-) derivatives were prepared using the procedure of Parge et al. (1990) as follows. Bacteria were grown on supplemented GCB agar at 37^0C, 5% CO_2 for ≤18 hours, then harvested and resuspended in Ca^{2+}-free DMEM or HBSS supplemented with 5 mM $MgCl_2$ for monitoring Ca^{2+} fluxes. Bacteria were vortexed vigorously for 30-45 sec then pelleted by centrifugation for 5 min at 11,000 x g. Supernatants containing the crude pili preparations were saved and their protein concentrations were adjusted to 20 mg/ml. The preparations were aliquoted and stored at –20°C until ready for use. For appropriate experiments, CP preparations were supplemented with 2 mM $CaCl_2$ immediately before use. Proteins in the CP preparations were separated by SDS-PAGE, transferred onto nitrocellulose and blotted with mAbs, 3.10, 3C8 and 4B12 (see above). Purified pili were prepared as dscribed by Parge et al. (1990).

Quantitation of surface Lamp1 levels

Surface Lamp1 levels were quantitated using iodinated or biotinylated mAb H4A3 as described (Warren *et al.*, 1998). Cultures were quickly chilled to 4^0C by addition of ice-cold DMEM and by placing the microtiter plates on an ice bath. Cultures were then incubated with ice-cold labeled mAb alone or in the presence of 750-fold

excess unlabeled mAb (to determine non-specific binding) at 4°C for 60 min. Cultures were then washed 5X in ice-cold DMEM and ^{125}I mAb counts in the cultures were determined using a gamma counter. Labeled cells were fixed in paraformaldehyde (4% in PBS) for 10 min at RT, blocked with BSA (2 mg/ml in PBS), incubated with Streptavidin-POD for 30 min at RT, then developed using the QuantaBlu Fluorogenic Peroxidase substrate according to manufacturer's instructions. Readings at t=0 (background) were subtracted from each sample. Then to determine specific mAb binding, counts bound in the presence of excess unlabeled mAb was subtracted from total counts bound.

Quantitation of fluid phase markers and β-hexosaminidase activity

For LY and ^{3}H-Dextran release experiments, 1.2 x 10^5 A431cells were seeded into each well of a 24 well microtiter plate and incubated at 37^0C, 5% CO_2 for 5 – 6 hours. LY (in DMEM-FCS) was added to each well at a final concentration of 1.5 mg/ml and the cultures incubated overnight (20 hours) or 1 h with 100 mCi/ml ^{3}H-Dextran. Cultures were then washed 3X with prewarmed DMEM and chased for 2 hr in the same medium before addition of CP preparations or other treatments. 100 ml of CP preparations were added to each well and the cultures were incubated for 15 min. Culture supernatants were then transferred to Eppendorf tubes and centrifuged for 5 min and supernatants were transferred to Nunc Maxisorp 96 well plates. To determine total fluorescence, cultures in parallel wells were lysed with NP-40 (1% in PBS), centrifuged and the supernatants diluted 1:10 for fluorescence readings. LY fluorescence was determined using a Fluostar

Spectrofluorimeter (BMG Lab Technologies). For ^{3}H-Dextran release experiments, cells were plated as described above and incubated overnight. They were washed in DMEM and loaded for 1 hour with ^{3}H-Dextran in DMEM at a final concentration of 100 mCi/ml. Loaded cultures were washed as described for the LY release experiments, and tritiated counts were determined using a Beckman LS500 TD scintillation counter. N-acetyl-b-D-glucosaminidase (β-hexosaminidase) activity was determined as described (Rodriguez *et al.*, 1997). Relevant calculations for LY, ^{3}H-Dextran and β-hexosaminidase release results were obtained after background levels at t=0 were subtracted from each sample. Students' Paired T Test was performed using MultiStat v.1.1. To release Ca^{2+} from the ER, Tg was added to cultures at 1 mM in Ca^{2+}-free DMEM. Prior to addition of CP preparations, specified cultures were pre-incubated for 15 minutes with BAPTA-AM (30 mM/ml resuspended in Ca^{2+} free HBSS for monitoring Ca^{2+} fluxes, or in DMEM for LY release experiments).

RESULTS

Neisseria rapidly reduces Lamp1 levels in infected cells

Past studies of IgA1 protease-Lamp1 interactions have used nonpolarized human epithelial cell lines at relatively late stages of infection (Hopper *et al.*, 2000a; Hopper *et al.*, 2000b; Lin *et al.*, 1997). We therefore wish to examine Lamp1 levels, as well as its cellular distribution, in individual polarized cells early after infection. Studies were performed by immunofluorescence confocal microscopy on T84 human colorectal carcinoma cells polarized on semi-porous membranes (Hopper *et al.*, 2000a; Hopper *et al.*, 2000b; Merz *et al.*, 1996). Unlike nonpolarized cells, polarized T84 monolayers mimic the architecture of native mucosal epithelia and are used as a model epithelial barrier for studying *Neisseria* infections, notably adhesion to the apical cell surface, changes in cell morphology, and bacterial transcytosis (Hopper *et al.*, 2000a; Hopper *et al.*, 2000b; Merz *et al.*, 1996; Pujol *et al.*, 1997; Wang *et al.*, 1998).

The apical region of polarized T84 monolayers were infected with wild type (WT) MC strain 8013.6 or its isogenic *iga* mutant 8013.6-500 (Table I) for various lengths of time. Figure 1 shows vertical sections of infected monolayers stained for DNA (red), cellular membranes (green), and Lamp1 (purple). In uninfected polarized monolayers, Lamp1 signals occurred throughout the cytosol, but heavily concentrated at the apical region of the cell. In control studies, fluorescence was not observed in cells stained only with the primary anti-Lamp1 monoclonal antibody (mAb) or only with Cy5-labelled secondary mAb (data not shown). Confocal images are typical of results from numerous experiments. It is interesting to note that in uninfected cells grown on solid substrates (unpolarized), Lamp1 compartments are heavily concentrated in the perinuclear region

(Carlsson and Fukuda, 1992; Lin *et al.*, 1997). Whether the apical distribution of Lamp1 seen here in polarized T84 cells is unique to this cell line or whether it is a general characteristic of native epithelia is unknown at present.

Table I. Relevant characteristics of the bacterial strains used in this study.

Strain	Piliation Status	IgA1 Protease Production	Comments	Ref.
N.meningitidis				
8013.6	+	+	8013 variant expressing high- adhesive pilin	(Nassif *et al.*, 1993)
8013.6-500	+	-	8013.6D*iga*	This study and (Hopper *et al.*, 2000a)
8013.6*pilE* deletion strain	-	+	8013D*pilE*	(Nassif *et al.*, 1993)
N.gonorrhoeae				
MS11A	+	+	P.IB, Opa$^-$	(Segal *et al.*, 1986)
MS11A-307	-	+	MS11AD*pilE1,pilE2*	(Merz *et al.*, 1996)
FA6612	-	+	MS11A expressing P.IA allele	(Carbonetti *et al.*, 1990)
FA6616	-	+	MS11A expressing P.IB allele	(Carbonetti *et al.*, 1990)
GCM740	+	+	P$^+$Opa$^-$ low passage clinical isolate	(Shoberg and Mulks, 1991)
GCM740-D4	+	-	GCM740D*iga*	(Shoberg and Mulks, 1991)

After 15 minutes of infection, bacteria had begun to adhere to the apical plasma membrane of polarized cells. At this time, the distribution of Lamp1 signals in infected cells had changed. In cells infected with either WT or *iga* mutant bacteria, a higher concentration of Lamp1 was observed at the apical region of the cell. At 30 minutes post-infection, Lamp1 signals were noticeably reduced in cells infected by WT MC. As expected, they were undiminished in cells infected with the *iga* mutant. By 120 minutes,

Lamp1 signals were very abundant in the cell cortex immediately beneath the adherent *iga* mutant, but nearly undetectable in cells infected with WT bacteria. Lamp1 levels appeared to be normal in uninfected cells adjacent to infected cells, although its signals also had an apical distribution (Figure 1, arrow). These results suggest that IgA1-protease-producing *Neisseria* can rapidly reduce Lamp1 levels in infected cells.

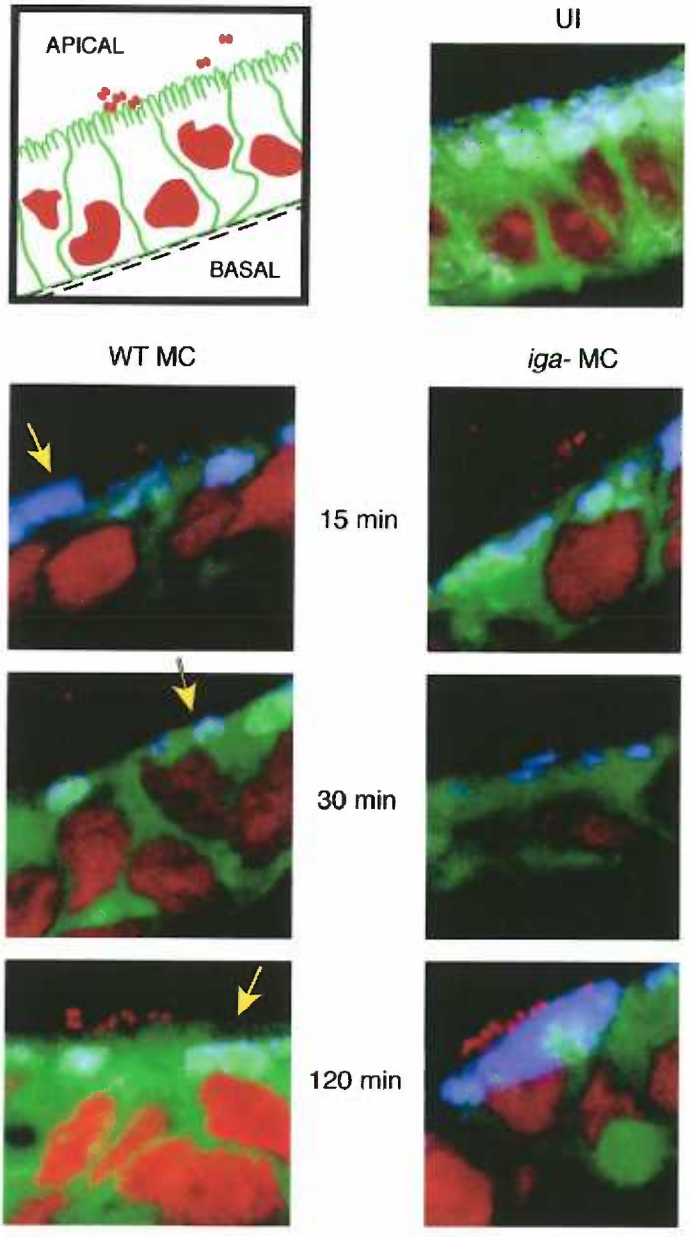

Figure 1. Representative vertical sections of *Neisseria* infected polarized T84 monolayers. Polarized T84 monolayers were infected with wild type MC strain 8013.6 (WT MC) or its isogenic *iga* mutant 8013.6-500 (*iga*-MC; Table I) at an MOI of 50, then fixed and stained for microscopy as described in Methods. Images were obtained using a BioRad 1024 ES laser scanning confocal microscopy system, 100X NA 1.4 objective. Red: DNA stained with propidium iodide (both bacteria and epithelial cell nuclei are detected). Green: total cell membranes stained with FM™1-43. Purple: Lamp1 detected by indirect immunofluorescence as described in Experimental procedures. UI: uninfected T84 monolayer. A cartoon demonstrating an infected polarized monolayer appears in the upper left hand. Arrows point to uninfected cells adjacent to infected cells.

Neisseria pili stimulate an increase in cytosolic free Ca^{2+} levels

Källstrom et al. (1998) have shown that treatment of ME180 cells with purified *Neisseria* pili increased cytosolic free calcium levels from 90nM to 450nM, using Fura-2 as the Ca^{2+} indicator. We determined whether preparations of crude pili (CP) could also trigger a Ca^{2+} flux in A431 cells, the human epidermoid carcinoma cell line that has been used to study IgA1 protease/Lamp1 interactions.

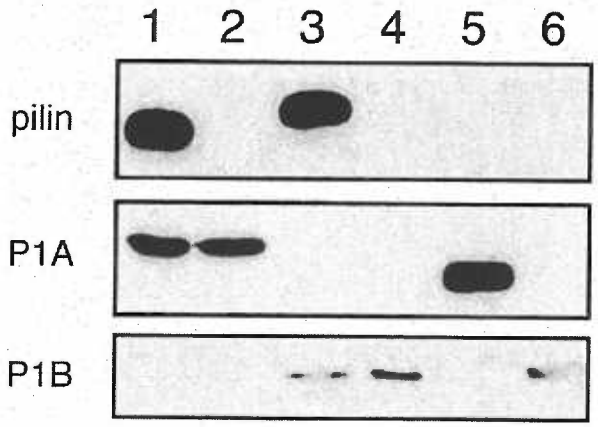

Figure 2. Crude pili (CP) preparations from 8013.6 (lane 1), 8013.6*pilE* (lane 2), MS11A (lane 3), MS11A-307 (lane 4), and non-piliated MS11A expressing P.1A (lane 5) or P.1B (lane 6) were separated by SDS-PAGE. Proteins were transferred onto nitrocellulose and blotted with mAbs anti-pilin, anti-P.1A or anti-P.1B.

CP were prepared from piliated (CP-P^+) and nonpiliated (CP-P^-) derivatives of MC and GC (Table I and Figure 2) in Ca^{2+}-free DMEM or HBSS (Hanks' Balanced Salt Solution). Coomasie Blue staining of proteins in CPs revealed numerous bands (data not shown). The presence of pilin and porin in these preparations were visualized by immunoblotting with the appropriate antibodies (Figure 2). None of the strains produced Opa (data not shown). A431 cells were washed in HBSS loaded with the fluorescent Ca^{2+} indicator Fluo-3 AM and exposed to CP preparations. Qualitative changes in cytosolic Ca^{2+} levels in individual cells (Figure 3) were monitored by fluorescence microscopy, as described in Experimental procedures. Changes in Ca^{2+} levels occurred in the cytosol of

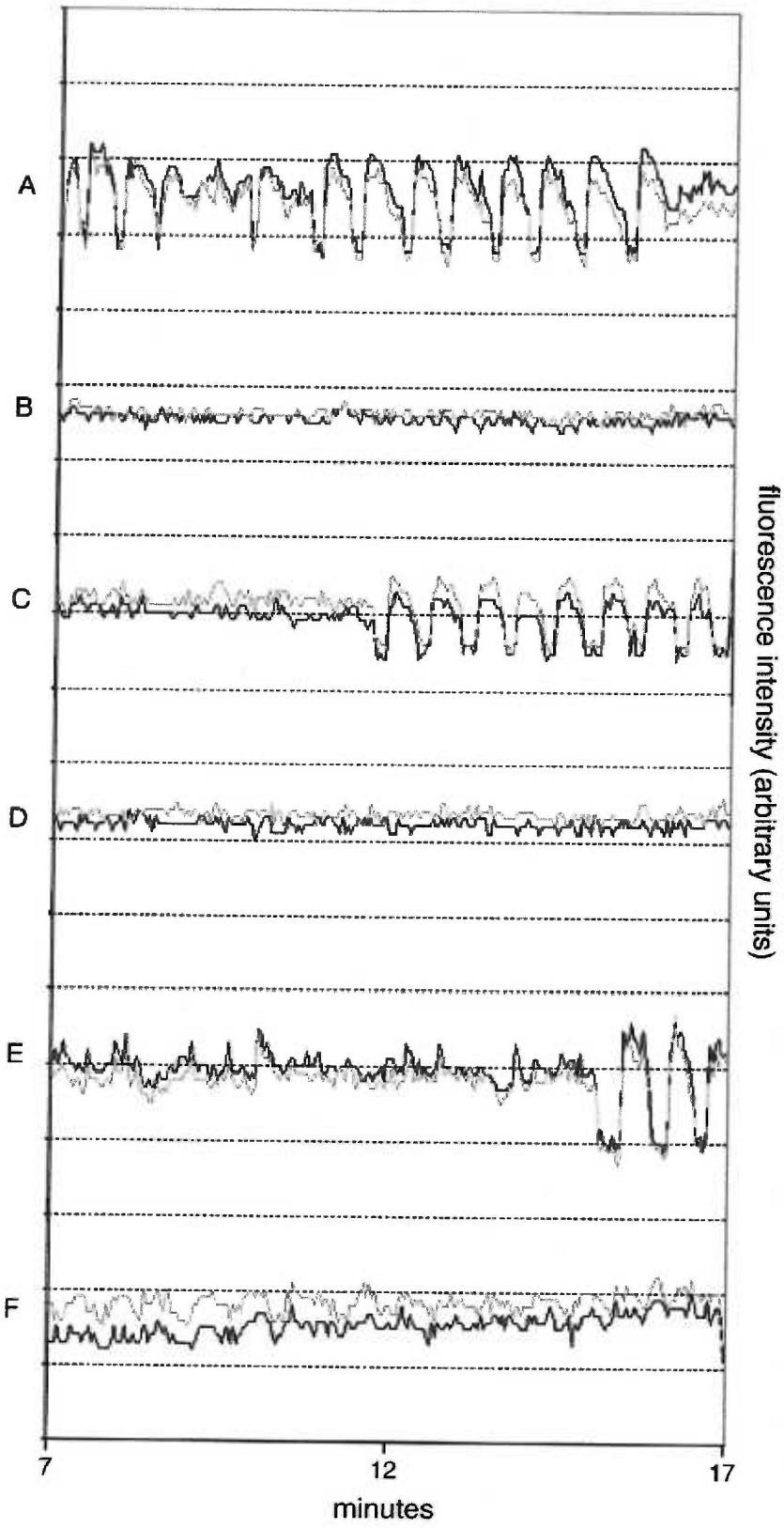

Figure 3. Ca^{2+} fluxes in A431 epithelial cells triggered by *Neisseria* crude pili . A431 cells were maintained at 34°C in HBSS in the absence of Ca^{2+} throughout the experiment. The cells were loaded with Fluo-3, AM then treated as indicated below. Images were acquired using an API DeltaVision microscopy system every 2.3 seconds for 15 minutes. Traces begin 7 min after addition of stimulus. Traces are of fluorescence intensity (arbitrary units) from a subset of image pixels from two individual cells in each sample. Cells were treated with 1 μM Thapsigargin (A), HBSS alone (B), GC CP-P^+ (C), GC CP-P^- (D), MC CP-P^+ (E) or MC CP-P^+ in the presence of 30 mM BAPTA-AM (F).

cells treated with CP-P^+ from MC and GC. As reported for the Ca^{2+} flux induced by purified pili in ME180 cells (Kallstrom *et al.*, 1998) this response in A431 cells had a relatively long lag time (12-14 minutes) and was inhibited in cells pretreated with 30 mM BAPTA-AM, a membrane-permeant chelator of free Ca^{2+} (Davletov *et al.*, 1998; Rodland *et al.*, 1990). In contrast, neither CP-P^- nor culture medium alone triggered the Ca^{2+} response. As expected, a similar Ca^{2+} response was induced in cells treated with 1 mM Thapsigargan (Tg), which causes the release of Ca^{2+} by inhibiting the endoplasmic reticulum (ER) ATPase, into the cytosol (Davletov *et al.*, 1998; Scheenen *et al.*, 1998). These results demonstrate that pili in CP preparations trigger fluxes in cytosolic Ca^{2+} levels in A431 cells. As the response occured in the absence of extracellular Ca^{2+}, this flux must be due to release of Ca^{2+} from intracellular stores.

The pilus-mediated Ca^{2+} transient induces lysosome exocytosis

Using multiple approaches, we next determined whether the pili-induced Ca^{2+} flux results in lysosome exocytosis. Cultures stimulated by CP preparations were first monitored for the release of fluid phase markers into the medium. Lysosomes of A431 cells were loaded overnight with the fluorescent marker Lucifer Yellow (LY) and chased with medium for 2 hours, then incubated for 15 minutes with CP-P^+ from 8013.6 and CP-P^- from 8013.6*pilE* (Nassif *et al.*, 1993) in the presence or absence of Ca^{2+}. The percentage of total cellular LY released into the supernatant was then determined (Figure 4A). Cultures stimulated with CP-P^+, in the presence or absence of extracellular Ca^{2+}, or with 1 mM Tg, released significantly more LY than control cultures incubated with medium alone ($P<0.03$, $P<0.035$, and $P<0.02$respectively). In contrast, cultures exposed

to CP-P⁻ released as much LY as unstimulated cultures. Preincubation of cultures with 30 mM BAPTA-AM prior to the addition of CP-P⁺ blocked LY release. These results demonstrate that the pilus-induced Ca^{2+} transient induces the release of LY from lysosomal compartments into the culture supernatant.

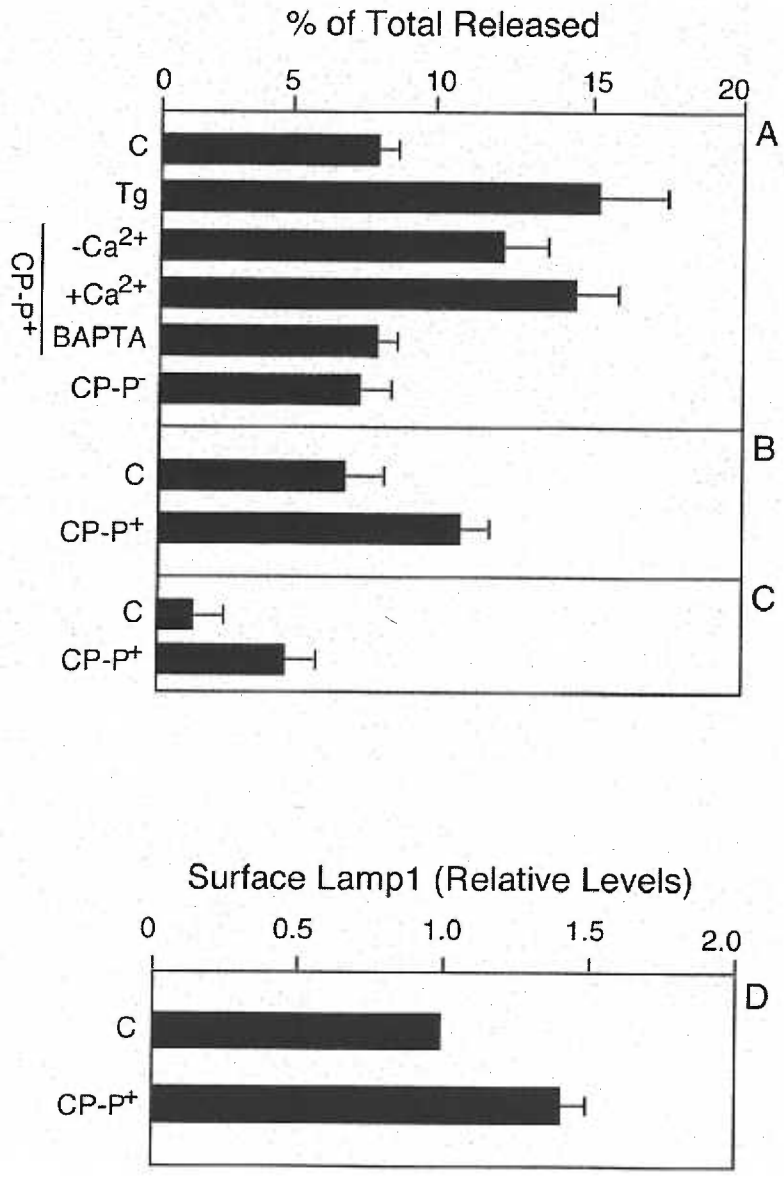

Figure 4. Stimulation of lysosome exocytosis in A431 cells by *Neisseria* crude pili. Panels A and B: Release of fluid phase markers Lucifer Yellow (LY) and ^{3}H-Dextran (^{3}H-Dex), respectively. Lysosomes of A431 cells were loaded with LY or ^{3}H-Dex, and the cultures stimulated as follows. Cultures were incubated for 15 minutes with Ca^{2+} free medium alone (C), or with Thapsigargin (Tg), or with crude pili from P⁺ (CP-P⁺) or P⁻ (CP-P⁻) bacteria, in the presence (+Ca^{2+}) or absence (-Ca^{2+}) of Ca^{2+}. BAPTA-AM: Cultures were pre-loaded with BAPTA-AM, a membrane permeable Ca^{2+} chelator, before addition of Ca^{2+} free CP-P⁺. Exocytosis was monitored by determining LY fluorescence or tritiated counts in the supernatants as described in Experimental procedures. Panels C and D: Cultures were incubated for 15 minutes with Ca^{2+} free medium alone (C), or with crude pili from P⁺ 8013.6 (CP-P⁺). Panel C: Release of lysosomal enzyme β-hexosaminidase (β-hex). Panel D: Surface Lamp1 levels.

Lysosome exocytosis was next monitored in cells loaded with ^{3}H-Dextran, another fluid phase marker (Figure 4B). As observed in the previous experiment, cultures incubated with Ca^{2+} free 8013.6 CP-P^{+} released significantly more ^{3}H-Dextran than control cultures exposed to medium alone ($P<0.025$). Identical results were obtained when the experiment was performed in Ca^{2+} supplemented medium (data not shown).

Lysosome exocytosis was also monitored by the release of β-hexosaminidase, an lysosomal enzyme, into the supernatant (Figure 4C;(Griffiths *et al.*, 1990; Rodriguez *et al.*, 1997; Rodriguez *et al.*, 1999)). Cultures were exposed to 8013.6 CP-P^{+} or medium alone (in the absence of Ca^{2+}) and enzyme activity in the supernatant relative to total activity in the culture was determined. β-hexosaminidase activity was significantly higher in supernatants of cultures exposed to CP-P^{+} than to medium alone. Thus, CP-P^{+} stimulated the release of β-hexosaminidase from A431 cultures. Identical results were obtained when experiments were performed in Ca^{2+} replete medium (data not shown).

Finally, plasma membrane Lamp1 levels were determined in cultures exposed to CP-P^{+} (Figure 4D). A431 cultures were incubated with Ca^{2+}-free 8013.6 CP-P^{+} for 15 minutes, then chilled to 4^{0}C to stop endocytosis. Surface Lamp1 was detected using an antibody-binding assay. Results show that surface Lamp1 levels in cells incubated with CP-P^{+} increased by approximately 37% compared to control cells incubated with medium alone ($P<0.025$), indicating that fusion of lysosomal membrane with the plasma membranes had occured. Identical results were obtained when experiments were performed in Ca^{2+} replete medium (data not shown). Taken together, these results indicate that the pilus-induced Ca^{2+} transient triggers lysosome exocytosis.

Porin in the CP preparations do not affect lysosome exocytosis

Porin can increase *Neisseria* attachment to epithelial cells (van Putten *et al.*, 1998), insert into eukaryotic membranes to form ion-gated channels (Rudel *et al.*, 1996; Weel and van Putten, 1991), and induce a Ca^{2+} flux and trigger apoptosis in monocytes (Muller *et al.*, 1999). In contrast to the pilus-induced Ca^{2+} flux (Kallstrom *et al.*, 1998), the porin-related Ca^{2+} response is rapid, occurring within 2 minutes, and is due to influx of extracellular Ca^{2+} (Muller *et al.*, 1999). Since our CP preparations have never been exposed to detergent, the porins in these preparations (Figure 2) may be found in their native conformation and may insert into plasma membranes. We therefore determined whether the porin in our CP preparations can influence the pilus-induced lysosome exocytic event. Lysosomes in A431 cultures were loaded with LY and incubated for 15 minutes with CP-P^+ and CP-P^- from GC strain MS11 and its derivatives (see below; Table I, Figure 5). Experiments were performed in the presence of extracellular Ca^{2+}.

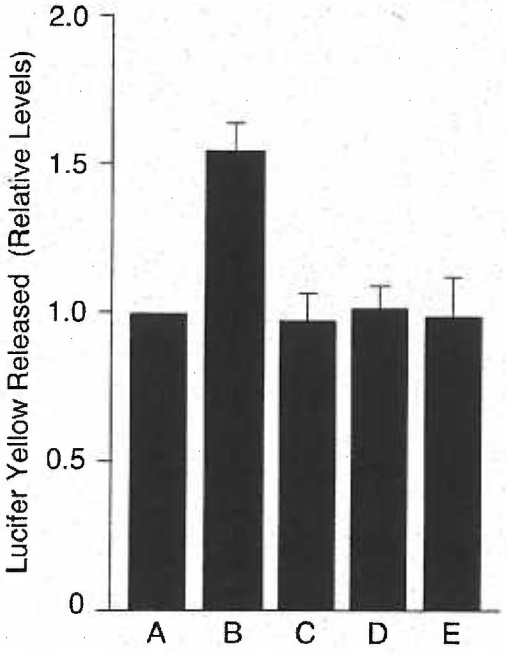

Figure 5. Effect of porin on pilus-induced lysosome exocytosis. Lysosome exocytosis was determined using the LY release assay as described above. Cultures were incubated for 15 minutes with medium alone (A), CP-P^+ from piliated MS11A (B), CP-P^- from MS11A-307 (MS11D*pilE*) (C), CP from non-piliated MS11A expressing PI.A porin (D), or PI.B porin (E). Experiments were performed in medium containing 2 mM Ca^{2+}.

Cultures incubated with MS11 CP-P^+ released significantly more LY than the control culture exposed to medium alone. In contrast, cultures incubated with MS11-307 CP-P^- released only background levels of LY. Cultures treated with CP-P^- from FA6612 or FA6616, expressing P.1A and P.1B respectively (Carbonetti *et al.*, 1990), also behaved like the negative control. Thus, porin in the CP preparations did not influence pilus-mediated lysosome exocytosis in the presence of extracellular Ca^{2+}.

Purified pili induce lysosome exocytosis

Next, purified pili were tested for their ability to trigger lysosome exocytosis. A431 cells were pre-loaded with LY or ^{3}H-Dextran as described above, or left unloaded, and incubated for 15 minutes with 20 mg/ml highly purified MS11 pili (Parge *et al.*, 1990) resuspended in DMEM in the presence of Ca^{2+}. The amount of LY, ^{3}H-Dextran and β-hexosaminidase in the media were then determined. As expected, treatment of A431 cultures with purified pili induced the release of all three lysosomal constituents into the media, compared to cultures treated with medium alone (Table II).

Table II. Stimulation of lysosome exocytosis by highly purified *N. gonorrhoeae* MS11 pili.

	% of Total Released		
	Lucifer Yellow	^{3}H-Dextran	β-hexosaminidase
Control[1]	6.90±0.90	7.02±0.38	0.90±0.10
Pili[2]	11.67±2.70	9.06±0.45	2.54±1.06

[1]Medium alone
[2]Pili were resuspended at a concentration of 20 mg/ml in DMEM plus 2 mM $CaCl_2$.

Surface Lamp1 is cleaved by IgA1 protease secreted by adherent bacteria

Finally, we determined whether surface Lamp1 can be cleaved by IgA1 protease secreted by adherent bacteria (Figure 6). A431 cultures were infected for 4 hours with WT GC strain GCM740 or GCM740-D4 its isogenic *iga* mutant (Shoberg and Mulks, 1991), then chilled at 4^0C to stop endocytosis. Surface Lamp1 levels were determined using an antibody binding assay. The strains used in this study do not invade cells until ~7 hours post-infection (data not shown). This experimental protocol therefore allows the detection of cell surface Lamp1 levels exclusively. Cultures infected with WT bacteria had reduced levels of Lamp1 on their plasma membranes compared to uninfected controls (relative levels, 0.65 +/- 0.1; $P<0.05$). In contrast, cultures infected with the *iga* mutant had surface Lamp1 levels comparable to those on uninfected cultures (relative levels, 0.97 +/- 0.1; $P<0.074$). These results indicate plasma membrane Lamp1 can be cleaved by IgA1 protease secreted by adherent bacteria.

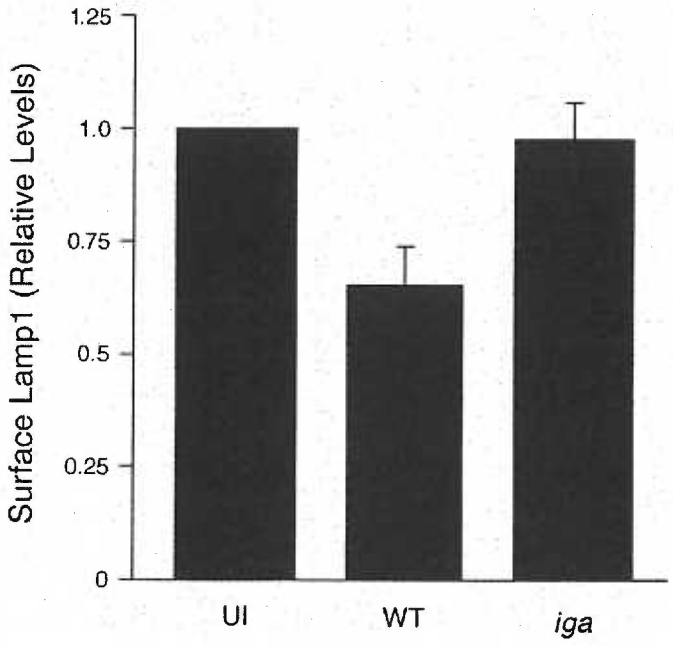

Figure 6. Cleavage of surface Lamp1 in A431 cells by *Neisserial* IgA1 protease. Cultures were incubated with medium alone (C) with WT, IgA1 protease-producing GC strain GCM740, or with its isogenic *iga* mutant GCM740-D4. Plasma membrane Lamp1 levels were determined using an antibody binding assay as described in Experimental procedures (relative Lamp1 levels for WT-infected cultures: 0.65 +/- 0.1; $P<0.05$; for *iga* mutant infected cultures: 0.97 +/- 0.1; $P<0.04$; Paired Student's T Test.)

DISCUSSION

IgA1 protease cleavage of Lamp1 accelerates its rate of degradation and decreases its total steady state levels in epithelial cells (Ayala *et al.*, 1998; Hopper *et al.*, 2000a; Lin *et al.*, 1997). In our previous studies, reductions in Lamp1 levels were observed after 12 hours of infection (Lin *et al.*, 1997). This long lag time between addition of bacteria and a noticeable decrease in cellular Lamp1 raised the question of how IgA1 protease might reach Lamp1 compartments during an infection.

In this report, we tested the hypothesis that the pilus-mediated transient increase in cytosolic free Ca^{2+} triggers lysosome exocytosis and increases the amount of cell surface Lamp1 that is cleavable by IgA1 protease. We provided microscopy evidence that reductions in Lamp1 levels in polarized T84 monolayers occur as early as 30 minutes post-infection (Figure 1). We demonstrated that pili in CP preparations trigger a flux in cytosolic Ca^{2+} in A431 cells, and that this flux is due to release of Ca^{2+} from intracellular stores (Figure 3). Our results are in agreement with an earlier study using purified pili and ME180 cells (Kallstrom *et al.*, 1998). We presented multiple lines of evidence that the pilus-induced Ca^{2+} flux induces lysosome exocytosis and the recruitment of additional Lamp1 to the cell surface (Figure 4). We also showed that porin in the CP preparations did not influence the pilus-mediated lysosome exocytosis event observed in these studies (Figure 5). Finally, we demonstrated that cell surface Lamp1 can be cleaved by IgA1 protease secreted by adherent bacteria (Figure 6). Taken together, our results strongly suggest that pilus-mediated adhesion of pathogenic *Neisseria* to epithelial cells leads to an initial rapid reduction in cellular Lamp1 levels.

The apparent discrepancy in results regarding when Lamp1 reductions are observable is likely to be due to differences in experimental protocols. In the previous study, Lamp1 levels were measured for an entire infected culture. As *Neisseria* do not infect all cells in a culture, a decrease in Lamp1 levels may not be detectable by immunoblotting after only a short period of infection. In this study, immunofluorescence confocal microscopy revealed a rapid and noticeable reduction in Lamp1 levels in individual infected cells. It also revealed a cellular redistribution of this lysosomal constituent early after *Neisseria* infection (Figure 1).

Trypanosoma cruzi infection of NRK cells also results in Ca^{2+} regulated lysosome exocytosis, and this process is apparently required for subsequent cell entry by the protozoan parasite (Rodriguez *et al.*, 1995; Rodriguez *et al.*, 1996). Only a small fraction of lysosomes is triggered to exocytose by the parasite-induced Ca^{2+} transient. This and other observations on Ca^{2+} regulated trafficking (Coorssen *et al.*, 1996; Kasai *et al.*, 1999; Xu *et al.*, 1998) has led to the hypothesis that lysosomes are heterogeneous and only a subpopulation reach the cell surface in response to a Ca^{2+} flux (Coorssen *et al.*, 1996; Dunn and Holz, 1983; Rodriguez *et al.*, 1997). The finding that the pilus-mediated Ca^{2+} transient induces the release of only a fraction of the internalized fluid phase markers and of total cellular β-hexosaminidase activity (Figure 4A-C), is consistent with this hypothesis. Whether the pilus-mediated Ca^{2+} flux also triggers exocytosis of other subcellular compartments such as endosomes is unclear. We recently reported that *Neisseria* infection results in a redistribution of the epithelial cell transferrin receptor (TfR) as well as a reduction in the rate of TfR internalization and mRNA levels (Bonnah

et al., 2000). It will be interesting to determine whether altered TfR trafficking and lysosome exocytosis are related processes.

The *Neisseria* porin has been shown to trigger a transient flux in cytosolic Ca^{2+} levels in monocytic cells (Muller *et al.*, 1999). Unlike the pilus-induced Ca^{2+} response, the porin-induced response is rapid and occurs only in the presence of extracellular Ca^{2+}. The pilus-mediated Ca^{2+}-induced exocytic event reported here is unlikely to be affected by porin in our own preparations (Figure 2). In the presence of Ca^{2+}, CP preparations containing PI.A or PI.B porin but not pili failed to trigger LY release (Figure 5).

Only a portion of lysosomal Lamp1 is exposed to the plasma membrane in response to the pilus-mediated Ca^{2+} flux (Figure 4); a large fraction remains within the cell. Yet, microscopy data indicate that Lamp1 levels in individual cells can be significantly reduced by 120 minutes post-infection (Figure 1), suggesting that additional mechanisms besides cleavage of surface Lamp1 may act to reduce Lamp1 levels in these cells. One explanation for these results is that infection by live bacteria may cause a more extensive exocytic event than that induced by crude pili preparations. Indeed, infection by an *iga* mutant (which preserves Lamp1 signals in an infected cell) causes the redistribution of a large fraction of intracellular Lamp1 to the apical region of the cell (Figure 1, lower right hand panel), suggesting that extensive redistribution of Lamp1 compartments occur upon infection. Another possible explanation is that IgA1 protease may reach Lamp1 in late endosomes via the endocytic route and cleave its substrate at this site. Endocytosed substrates reach endosomes within 15 minutes. Moreover, IgA1 protease can hydrolyze Lamp1 at pH 6.5, the pH of late endosomes (Lin *et al.*, 1997). *In vivo*, prolonged colonization by low-invasive *Neisseria* strains should provide additional

time for Lamp1 cleavage. GC can infect a site for several days before symptoms appear (Berger *et al.*, 1979; Hook and Holmes, 1985; Kristensen and Scheibl, 1984) and carriers can harbor culturable *Neisseria* for lengthy periods (Britigan *et al.*, 1985; Hunter Hansdsfield, 1990). Such extended contact between bacteria and host could present additional opportunity for Lamp1 cleavage.

The exocytic event releases the contents of lysosomes onto the cell surface. However, these lysosomal acid hydrolases are unlikely to cause harm to the apical membrane. As these enzymes have a low pH optimum, their activity should be much reduced at the higher pH of the cell surface.

The role of IgA1 protease-mediated Lamp1 turnover in *Neisseria* infections *in vivo* is unclear at present. This hydrolytic process leads to additional alterations in the lysosomes of infected cells (Ayala *et al.*, 1998). Moreover, *iga* mutants are deficient in intracellular replication and transcellular trafficking (Hopper *et al.*, 2000a; Lin *et al.*, 1997). A diminution in the effectiveness of a key cellular defense mechanism may promote *Neisseria* intracellular survival. Although limited human infection studies demonstrate that the IgA1 protease is unnecessary for the initiation of an infection in the male urethra, these experiments do not address clinically important issues such as maintenance of an infection (in men or women) or prolonged carriage of bacteria. IgA1 protease cleavage of Lamp1 may function in these and/or other aspects of a *Neisseria* infection. The pilus-induced Ca^{2+} flux is reported to result in the appearance of novel, undefined receptor(s) for *Neisseria* (Kallstrom *et al.*, 1998). It can be additionally speculated that this receptor(s) is derived from Lamp1 compartments. Further studies are

required to determine the extent of IgA1 protease/Lamp1 interactions *in vivo* and the biological relevance of these interactions.

Clarification of B Vasquez's role in this manuscript:

I was very much a part of the formulation and discussion that led to this hypothesis and the execution of this work. I designed and performed the microscopy experiments, including the development of the calcium imaging assay. I also assisted Dr. Ayala with developing assays to measure surface Lamp1 and lysosome exocytosis. We worked together very closely on this project and feel this manuscript integrates well with this thesis.

MANUSCRIPT 3:

Neisseria outer membrane proteins direct the trafficking of Lamp1 exocytic compartments

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ABSTRACT

The *Neisseria gonorrhoeae* porin and pili induce a series of calcium fluctuations in the target host cell. The former causes an influx of extracellular calcium, while the latter releases calcium from intracellular stores. These calcium transients trigger the exocytosis of hLamp1 compartments, bringing hLamp1 to the epithelial cell surface, where it is cleaved by the secreted *Neisseria* IgA1 protease.

We wished to determine whether the exocytic events in response to the *Neisseria*-induced calcium signals are specifically directed to the site of bacterial attachment or whether they are randomly distributed towards the plasma membrane. We observed that beads coated with bacterial membranes containing porin and pili triggered the redistribution of cellular hLamp1 to the site of contact between bead and plasma membrane. In the presence of extracellular calcium, beads coated with porin-containing preparations caused hLamp1 to concentrate in vesicles at the cell cortex. Fusion of these vesicles with the plasma membrane, however, required the presence of pili and release of calcium from intracellular stores. To visualize these exocytic events, we constructed an hLamp1-EGFP fusion and stably expressed it in the A431 human epithelial cell line. Live-cell microscopy of beads contacting these cells enabled dynamic visualization that supported our observations. Together, our results indicate that hLamp1 exocytosis occurs in a stepwise manner upon contact of *Neisseria* membranes with epithelial cells and is dependent on pilus-induced calcium transients. In concert with the known activity of the IgA1 protease, these results illustrate the ability of pathogenic *Neisseria* to manipulate hLamp1 trafficking to its advantage.

INTRODUCTION

Many pathogenic bacteria, including *Neisseria meningitidis* and *Neisseria gonorrhoeae*, must colonize, invade, and transcytose through an epithelial monolayer in order to cause disease. These pathogens have evolved mechanisms to avoid the innate cellular response which works to prevent them from making this voyage. While inside the cell, *Neisseriae* survive are seen to survive within a membrane-bound vacuole. Although the composition of the intracellular bacterial compartment is not well understood, it has been shown that it does not acquire lysosomal protein markers such as hLamp1, as would be the natural fate of endosomes (Hauck and Meyer, 1997; Lin *et al.*, 1997).

Recent work has revealed several details as to how pathogenic *Neisseriae* may avoid contact with degradative lysosomal compartments during their intracellular journey. The cleavage of hLamp1 by the bacterial IgA1 protease at its proline-rich lumenal hinge leads to accelerated degradation of hLamp1 and other lysosomal constituents (Ayala *et al.*, 1998; Lin *et al.*, 1997). *Iga*- mutants have an intracellular survival defect, suggesting that IgA1 protease cleavage of Lamp1 promotes avoidance of harmful lysosomes.

Lamp1 is a major lysosomal membrane protein of unknown function. It is present primarily in lysosomes and late endosomes, though a small fraction is present on the plasma membrane. Its IgA1-like hinge, the target of IgA1 protease, is intralumenal. One pathway by which the secreted IgA1 protease accesses the protease-sensitive Lamp1 hinge is at the surface of the cell, where the bacteria colonize. This is achieved when bacterial surface proteins come in contact with the epithelial cell, inducing the exocytosis

of Lamp1-rich late endosomes and lysosomes. Neisserial porin first causes an influx of calcium from outside the cell, which triggers the exocytosis of endosomes (Ayala, in press). A second, slower calcium release from intracellular stores occurs in response to pilus contact with the cell, resulting in lysosomal exocytosis (Ayala *et al.*, 2001).

The IgA1 protease secreted by colonizing bacteria then cleaves the newly exposed Lamp1 on the cell surface (Ayala *et al.*, 2001). A concurrent decrease in Lamp2, CD63 and lysosomal acid phosphatase, not directly via cleavage by the protease, suggests a generalized decrease in host cell lysosomes (Ayala *et al.*, 1998). This is one mechanism by which *Neisseriae* may avoid lysosomal degradation during their intracellular life.

We have reported, as described above, both an influx of extracellular calcium and a release of intracellular calcium which contribute to Lamp1 on the plasma membrane of colonized epithelial cells (Ayala *et al.*, 2001) (Ayala, in press). We wished to determine if these cellular mechanisms are a non-specific response to stimulation by foreign objects, or if they are directly driven by bacterial proteins in a bacteria-cell interaction. We also wished to investigate the directionality of lysosome exocytosis – whether it is a widely distributed process involving the entire plasma membrane or if it is specifically aimed at the site of *Neiseria* contact.

A green fluorescent protein- (EGFP) fusion to the cytosolic tail of hLamp1 was constructed in order to study the dynamics of this process. hLamp1-EGFP in a stably-transfected A431 epithelial cell line was shown to colocalize with endogenous lysosomal markers in fusion-competent vacuoles. Several experiments were then conducted "infecting" epithelial cells with protein-coated fluorescent beads. Both the fusion protein, as well as hLamp1 in non-transfected cells, localized beneath beads coated with a

crude preparation of *Neisseria* outer membrane proteins. The fusion of hLamp1 compartments was abolished when pili were not present on the beads, but these vesicles were still redistributed beneath or near the site of bead attachment with these outer membranes. Bead attachment in the absence of calcium or in the presence of the calcium chelator, BAPTA, abolished cortical relocalization of hLamp1 compartments altogether. Finally, by employing the stably- transfected cells, we were able to visualize directed exocytosis of hLamp1-EGFP compartments to the site of attachment in real-time. This data visually supports our conclusions that *Neisseria*-directed exocytosis occurs in a stepwise manner, specifically at the site of attachment. Taken together, we show that this event is calcium-dependent, that fusion with the plasma membrane requires the presence of pili, and that hLamp1 is consequently increased on the surface of the host cell where it may be cleaved by IgA protease.

MATERIALS AND METHODS

Bacterial strains

Crude membrane preparations were made from *Neisseria gonorrhoea* strain MS11A (Pili +, Opa-) or from MS11307, its isogenic *pilE-* knockout strain (Pili -) (Nassif *et al.*, 1993).

Cell cultures

A431 epithelioid carcinoma cells, obtained from S. Schmid, were maintained in Dulbecco modified Eagle's medium (DMEM; Cellgro) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL).

Construction of Lamp1-EGFP and stably-transfected cell line

A plasmid encoding the cDNA sequence of hLamp1 was obtained from M. Fukuda (pSV lamp-1 (Williams and Fukuda, 1990)). The 3' end of the Lamp1 coding sequence was PCR amplified using the following primers: 5'primer: TGCAACACGTTACAGCG; 3'primer: CGGGATCCTTACTTGTACAGCTCGTCCATGCCG. The latter eliminated the original stop codon for Lamp1 and replaced it with a BamHI site for subsequent cloning. Following restriction digests, this product and the rest of the Lamp1 coding sequence from pSVlamp-1 were cloned into a eukaryotic expression vector, into a site following a CMV promoter. The EGFP sequence was PCR amplified from pEGFP-C1 (Clontech) and cloned in frame with the Lamp1 sequence, encoding a linker as shown in Figure 1.

A431 cells were transfected using purified DNA (prepared using Wizard PureFection kit; Promega) and FuGene6 reagent (Roche) as directed. Stably transfected cells were selected in DMEM-10%FCS supplemented with 500mg/ml Geneticin (Gibco BRL).

Outer membrane-coated bead preparations

Crude outer membrane preparations (OMP) were made as described (Ayala *et al.*, 2001). Briefly, overnight cultures of MS11A (P+) or MS11307 (P-) were swabbed into 5ml of HBSS and vortexed for 2 min. These suspensions were then centrifuged at 14,000xg for 5 min to pellet any remaining whole bacteria and debris. The supernatents were collected and stored at -80C until use.

Carboxylate-modified blue fluorescent beads (Molecular Probes) were labeled with *Neisseria* outer membranes as per the manufacturer's directions. Briefly, 3mg of membrane preps or BSA were incubated with the beads in MES buffer (50mM MES, pH6.0). EDAC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; Molecular Probes) was added to activate the reactive groups on the beads, followed by a 1 hour incubation at room temperature. The reaction was quenched by adding glycine to give a final concentration of 100mM, followed by a 30min incubation at room temperature. Beads were then washed three times by centrifugation for 20 minutes at 4,000xg followed by resuspension in PBS. The final resuspension was done with the same volume of PBS as the volume of beads that we started with. The efficiency of labeling was determined by dot-blotting of the bead preparations, along

with the original membrane preparations,

Protein-conjugated beads were tested for the presence or absence of pilin, porin, and BSA in a dot-blot assay using anti-SM1 Ab (pilin specific), anti-3C8 Ab (porin specific), and an anti-BSA Ab; all at 1:2000 dilutions. (data not shown). Since the outer membrane preparations may have contained traces of residual IgA1 protease, beads were also tested for protease activity using a hIgA1 cleavage assay (as previously described (Lin *et al.*, 1997)). None of the bead preparations assayed demonstrated detectable protease activity (data not shown).

Detection of surface Lamp1

Cell-surface Lamp1 was quantified using an ELISA-like assay as follows: A431 cells were incubated with BSA, OMP-P+ or OMP-P- coated beads in normal culture medium for two hours at 37C, 5%CO2. Cultures were then chilled to halt endo- and exocytosis in an ice bath and by the addition of ice-cold medium. Cells were washed five times with cold DMEM containing 20mM Hepes and 1% BSA. Biotinylated mAb H4A3 (anti-Lamp1) (Pharmingen) was diluted in this medium and cells were probed with the antibody mixture for 1 hour on ice. After a second set of cold washes, cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Bound mAb was detected using streptavidin-HRP (Roche) in 1%BSA-PBS, and quantified using QuantaBlue (Pierce); fluorescence was measured on a SpectraMax fluorimeter (Molecular Devices).

Immunocytochemistry

For fixed preparations, tissue culture cells were grown on glass coverslips in 6-well plates (Falcon). Cells were fixed in 4% paraformaldehyde (EM Sciences) in PBS for 10-15 minutes at room temperature. Coverslips were blocked in 3% normal goat serum (Gibco BRL) in PBS containing 0.03% saponin (Sigma) for permeabilization. Antibodies were diluted to appropriate dilution in this solution, and exposed to the cells for up to 24 hours at 4°C. Coverslips were washed several times in PBS and stained with the appropriate secondary antibodies (Molecular Probes) for 1 hour at room temperature. After washing, coverslips were mounted on slides using Fluoromount-G (Southern Biotech), and visualized by fluorescence microscopy.

For live cell viewing, A431-LE1 cells were seated in Bioptechs DeltaT dishes. This enabled temperature control on the stage via the Bioptech heating system.

Microscopy

For the Texas Red Dextran loading experiment, images were acquired using a BioRad 1024 ES laser scanning confocal microscope (CLSM) system attached to a Nikon Eclipse TE300 inverted microscope. The Krypton/Argon laser (American Laser Co., Utah) generates excitation lines at 488, 568 and 647 nm.

All other images in this publication were acquired with the Applied Precision DeltaVision® image restoration system. This includes the API chassis with precision motorized SYZ stage, a Nikon TE200 inverted fluorescent microscope with standard filter sets, halogen illumination with API light homogenizer, a CH350L Camera (500 KHz, 12-bit, 2 Mp, KAF 1400 GL, 1317x1035, liquid cooled), DeltaVision software.

Deconvolution using the iterative constrained algorithm of Sedat and Agard and additional image processing was performed on an SGI Octane workstation.

All images were compiled and processed using Adobe Photoshop 6.0.

RESULTS

Construction of a hLamp1-EGFP fusion protein

In order to dynamically visualize compartments in the terminal endocytic pathway we engineered a DNA construct that translationally fuses EGFP to the carboxyl terminus of human Lamp1 (Fig. 1). The human epithelial cell line, A431, was transfected with this construct and two lines were obtained that stably produced the hLamp1-EGFP fusion protein. SDS-PAGE of cell lysates followed by immunoblotting with antibodies against Lamp1 or EGFP confirmed both components of the fusion protein and the molecular mass (data not shown). Control experiments (see below) indicated that the fusions expressed in both transfectant lines behaved similarly, and for this reason the experiments shown below were all done with one line, which was named A431-LE1.

Lamp1-EGFP fusion localizes with endogenous hLamp1 in functional lysosomes

To confirm that fusion of EGFP to the Lamp1 cytoplasmic tail did not cause mislocalization of the fusion protein, two approaches were used to determine the cellular distribution of LE1. First, we compared the subcellular distribution of Lamp1-EGFP with endogenouse hLamp1 by indirect immunofluorescence. Secondly, LE1 was shown to colocalize with two other late endosomal markers: Lamp2 and CD63. As shown in Figure 2, there were no regions of exclusivity between the Lamp1 signal and LE1 fluorescence assured us that the fusion protein is trafficked normally, intermixed with endogenous Lamp1. Total Lamp1 and Lamp2 both had the identical distributions as Lamp1-EGFP. In each case fluorescence was mainly observed on many vesicles

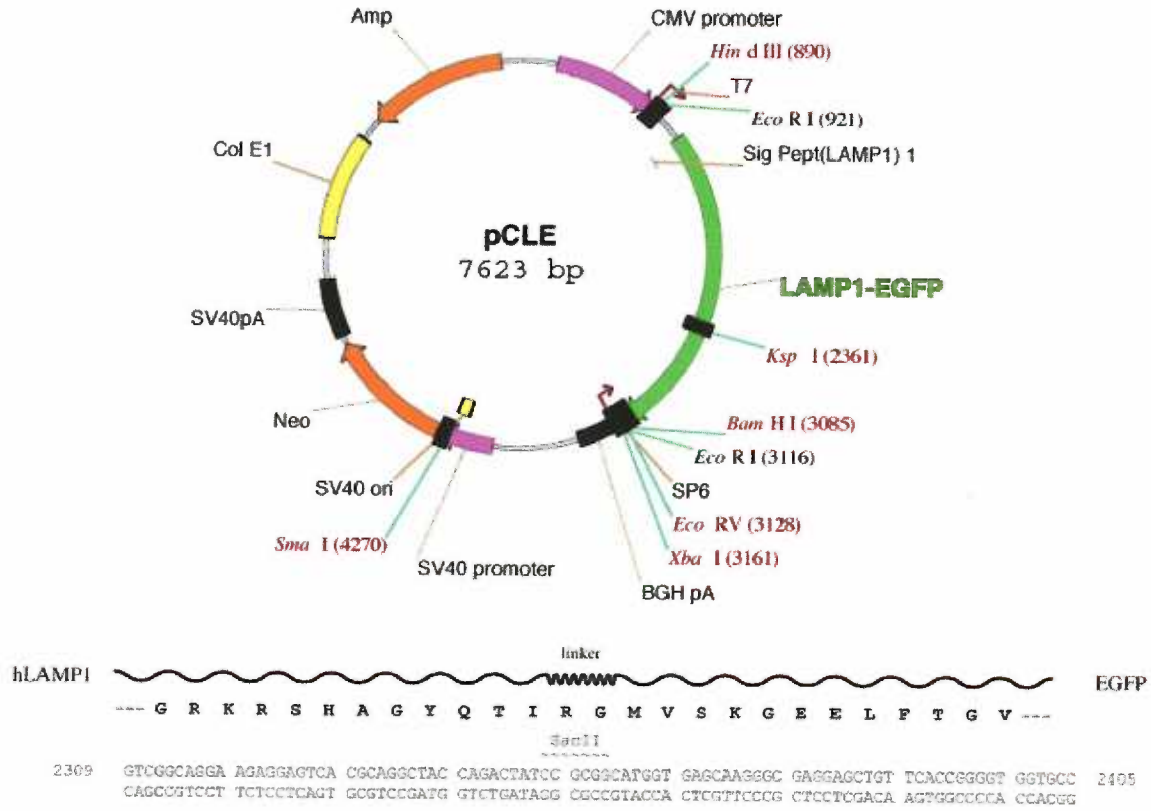


Figure 1. Map of the pCLE plasmid, which encodes the Lamp1-EGFP fusion protein.

throughout the cytoplasm, with a higher concentration of vesicles in the perinuclear region. CD63, a marker of late endosomes, significantly overlaps with the LE1 signal. In contrast, staining for transferrin receptor (TfR), a marker of the early endosome and recycling compartments, exhibited little or no overlap with Lamp1-EGFP fluorescence.

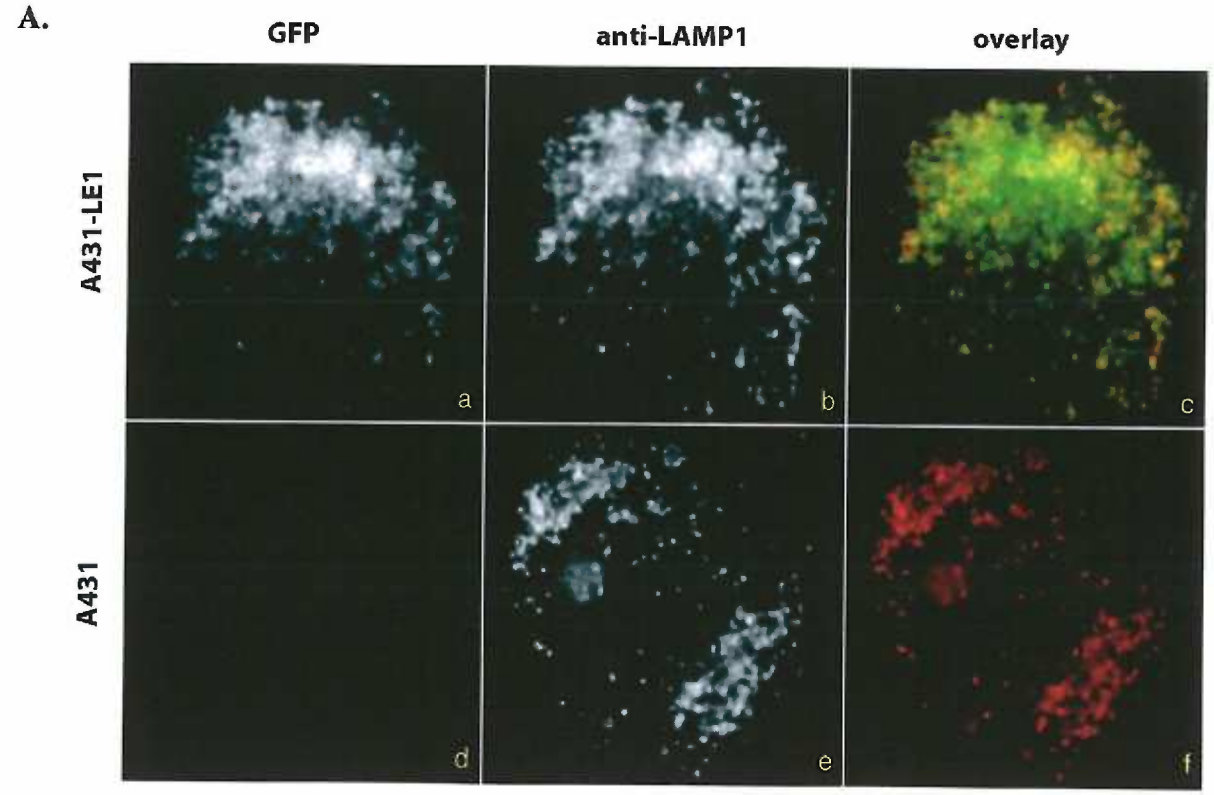
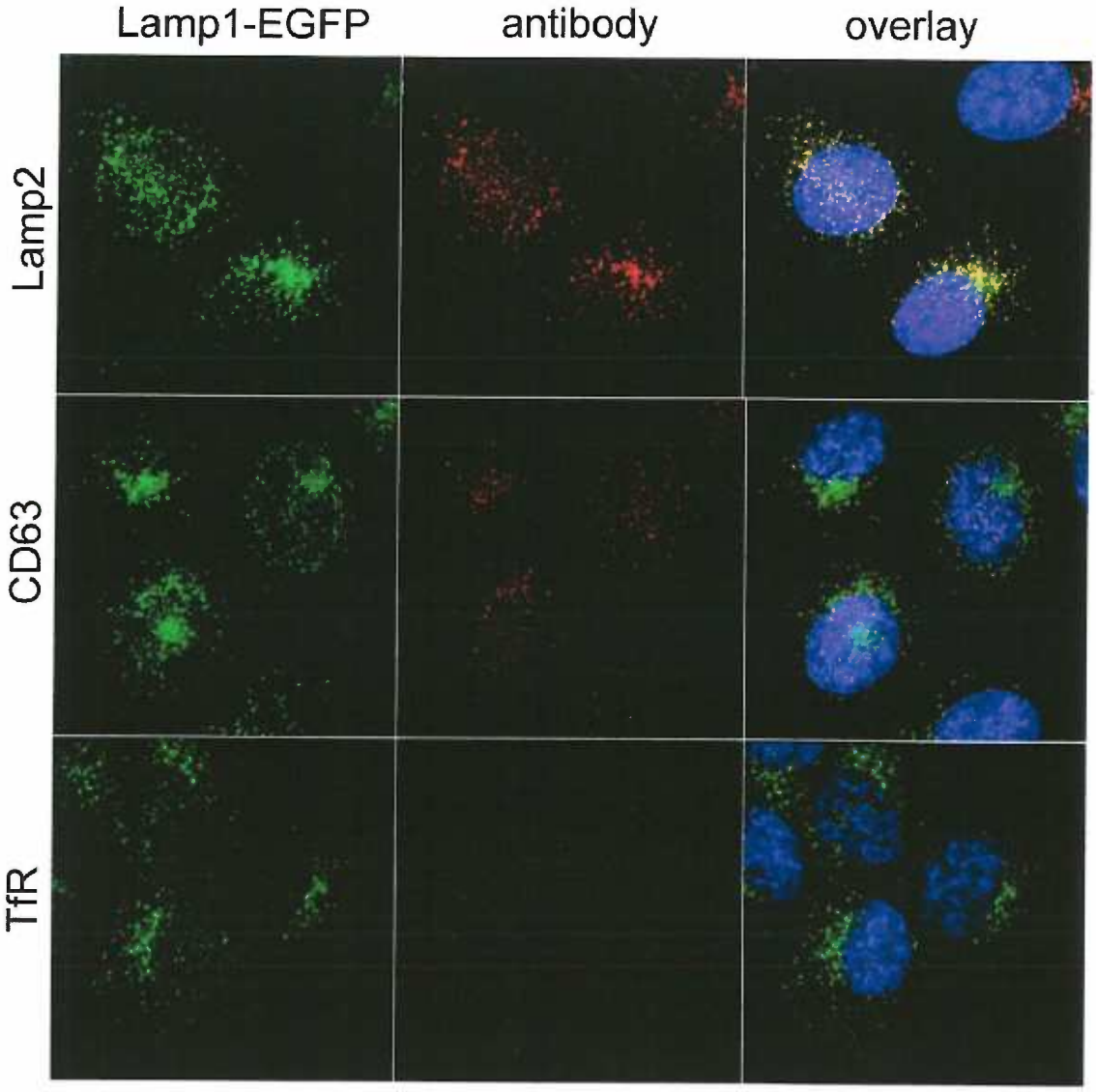


Figure 2 Lamp1-EGFP localizes with endogenous Lamp1 and other lysosomal markers, but not the early endosomal marker, TfR

A. A431 cells and A431 stably transfected with the Lamp1-EGFP (LE1) construct were fixed with 4% paraformaldehyde and stained by indirect immunofluorescence for Lamp1. Left panels: EGFP fluorescence. Center: a-Lamp1 staining. Right: overlay; overlapping fluorescence appears yellow.
a-c: Lamp1-EGFP stably transfected A431 cells
d-f: non-transfected A431 cells

B. Lamp1-EGFP cells were stained with markers to look for lysosomal (Lamp2), late endosomal (CD63) or early endosomal (TfR) localization of the GFP fluorescence.

B.



To confirm fusability and function of the LE1-labelled compartments, A431-LE1 cells were loaded overnight with Texas Red dextran (TxRedDex), washed, and chased with TxRedDex-free media for two hours. This preferentially labels organelles of the late endocytic pathway. Confocal images showed that a large fraction of the TxRedDex was present in compartments illuminated by LE1 (Fig 3). This demonstrates that the A431-LE1 compartments retained the fusion competence of endogenous late endosomes and

lysosomes. Together, these experiments demonstrate that hLamp1-EGFP is localized to the same late endocytic and lysosomal organelles as native hLamp1.

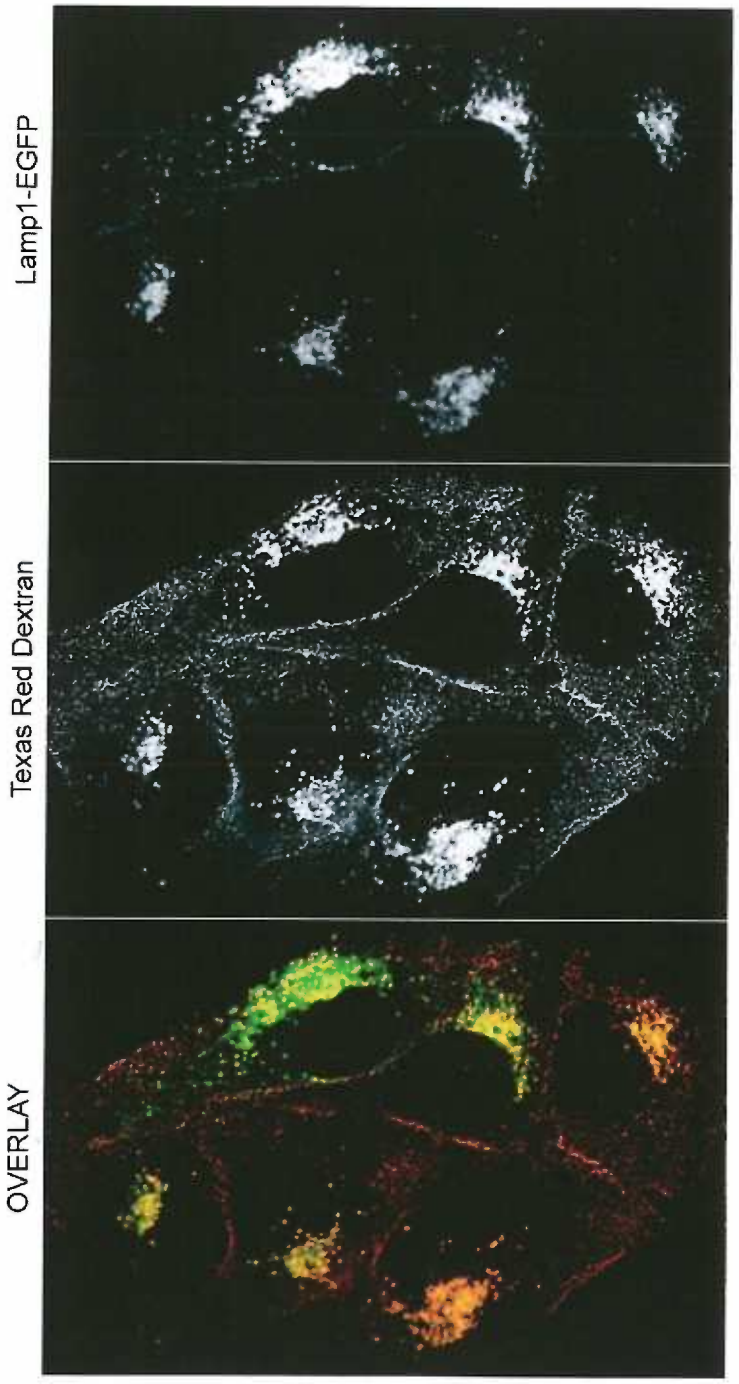


Figure 3. Lamp1-EGFP compartments can be loaded via endocytic uptake of a fluid phase tracer

A431 cells stably transfected with Lamp1-EGFP were incubated in the presence of Texas-Red conjucated dextran (Molecular Probes) for 22 hours.

top: EGFP fluorescence

middle: Texas Red dextran fluorescence

lower: overlay; overlapping fluorescence appears yellow

Lamp1-EGFP localization to the site of Neisseria-protein attachment

We wished to determine whether hLamp1-EGFP would be localized beneath the site of contact with *Neisseria* outer membrane proteins. For clear resolution of this contact, we employed fluorescent beads coated with crude outer membranes from wild-type, piliated *Neisseria gonorrhoeae*. At three hours post-inoculation with the beads, the green fluorescence in A431-LE1 cells was clearly localized beneath the beads (Fig. 4). At this time point, a few of the beads appeared completely endocytosed in a small number of cells; these were not imaged for analysis. Some of the blue fluorescent beads attached to the cells in clusters, resembling microcolonies of bacteria. The green fluorescence was non-homogeneous and brightest at the edges of most of the attached beads, eliminating the possibility that the detected light was bleed-through from the bright blue signal. These images demonstrate the localization of Lamp1-EGFP at the site of contact by neisserial outer membranes.

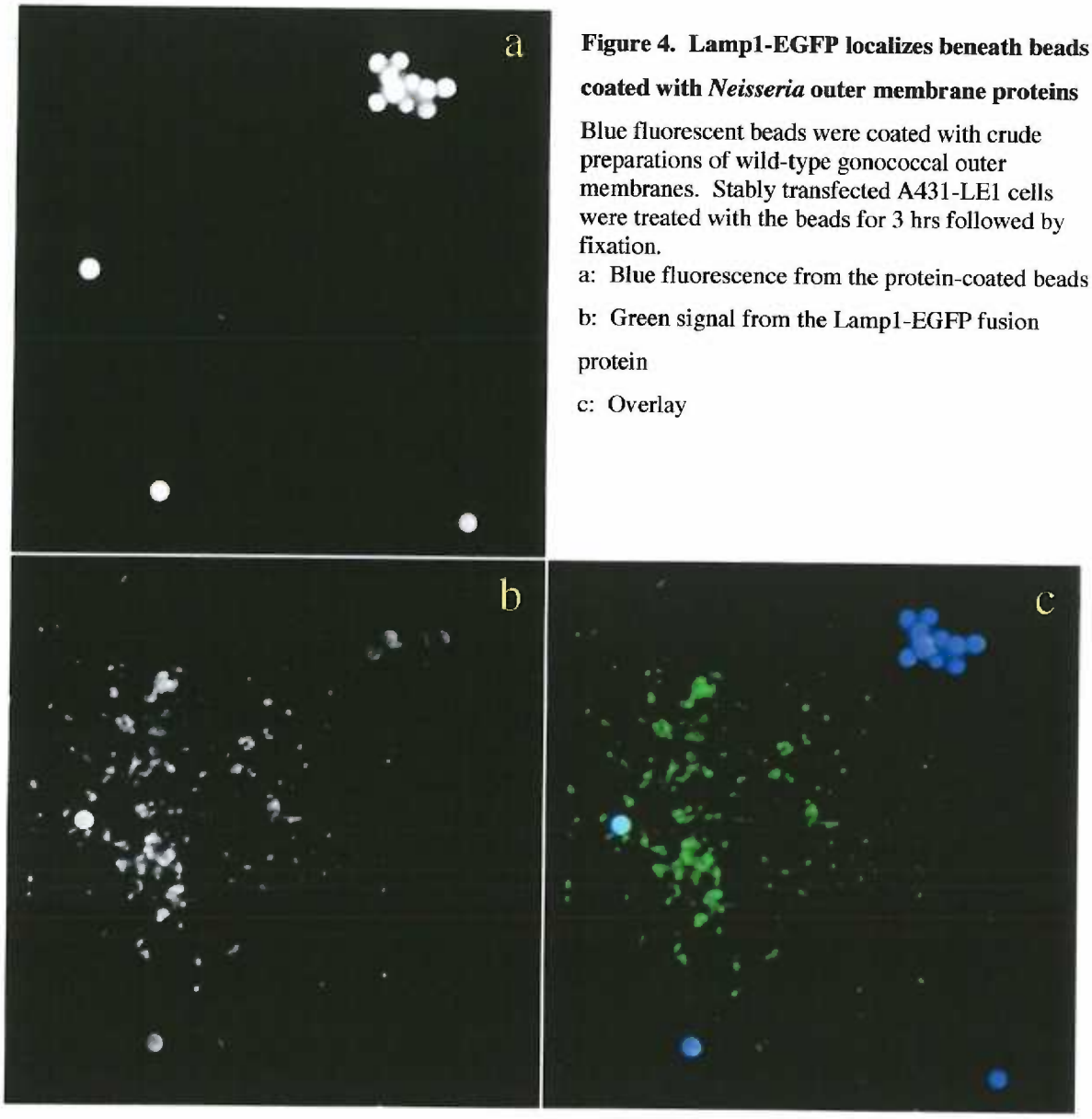


Figure 4. Lamp1-EGFP localizes beneath beads coated with *Neisseria* outer membrane proteins

Blue fluorescent beads were coated with crude preparations of wild-type gonococcal outer membranes. Stably transfected A431-LE1 cells were treated with the beads for 3 hrs followed by fixation.

a: Blue fluorescence from the protein-coated beads

b: Green signal from the Lamp1-EGFP fusion protein

c: Overlay

Lamp1 relocalization is pilus- and calcium-dependent

We previously demonstrated the calcium-dependence of lysosomal and endosomal exocytosis. We further wished to determine whether the targeting of Lamp1 is also dependent on a calcium signal. In addition, the previous results using porin and pili demonstrate both an extracellular influx of calcium as well as a separate intracellular

release, each having different effects (Ayala, in press, and (Ayala *et al.*, 2001)). Using similar preparations, we tested to see if directed lysosome exocytosis could also be factored into this model, utilizing one or both signals. For these experiments, we chose to focus on events occurring two hours after inoculation with the OMP-coated beads. At three hours, there is a small population of beads that have been completely endocytosed, and we wished to avoid confounding results due to a response to this invasion.

Our results are summarized in Figure 5. Immunofluorescence images were acquired after staining bead-stimulated A431 cells with a monoclonal antibody to hLamp1. The top row of images depict the effects on Lamp1 in the presence of extracellular calcium. Again, in cells exposed to beads coated with piliated outer membrane preparations (P+ beads), we observed a clear localization of Lamp1 at the site of bead attachment (Fig. 5A, arrows). This response did not occur, however, in the absence of extracellular calcium (middle), or after exposure to the calcium chelator, BAPTA (bottom).

Where non-piliated OMP-coated beads (P- beads) attached to cells, there was also a change in Lamp1 localization (Fig. 5B). Lamp1+ organelles appeared to be closely associated with the site of bead attachment. This phenomenon was observed in several samples. Again, the effect was abolished in the absence of calcium or the presence of BAPTA.

In addition, cells were treated with P+ or P- OMP beads to look for calcium-dependence of the LAMP1 exocytic reaction. To determine if an influx of extracellular calcium was important for the redistribution, cells were treated with the beads in calcium-free medium. In the absence of extracellular calcium, neither the aggregation of LAMP1

vesicles nor the exocytic reaction were observed (middle panels). BAPTA-AM, a chelator of both intracellular and extracellular calcium, also abolished all relocalization. These results together establish the effect as calcium-dependent. No change in Lamp1 localization was observed in cells stimulated with BSA-coated beads from unstimulated A431 cells, even with calcium present in the medium (Fig. 5C).

Figure 5. Lamp1 relocalization is pilus- and calcium dependent

Fluorescent beads were coated with either (**A**) P+: piliated or (**B**) P-: nonpiliated wild-type gonococcal outer membranes or (**C**)BSA: bovine serum albumin. A431 cells were exposed to these beads for 2 hours, then fixed. Indirect immunofluorescence was performed to look for Lamp1 redistribution to the sites of bead attachment. While P+ beads colocalized with exocytic Lamp1 on the plasma membrane, this was not observed with the P- beads. Instead, non-exocytosed Lamp1 vesicles appeared to aggregate at or near the site of P- bead attachment. (boxed) There was no redistribution of Lamp1 observed with BSA bead treatment.

Figure 5A

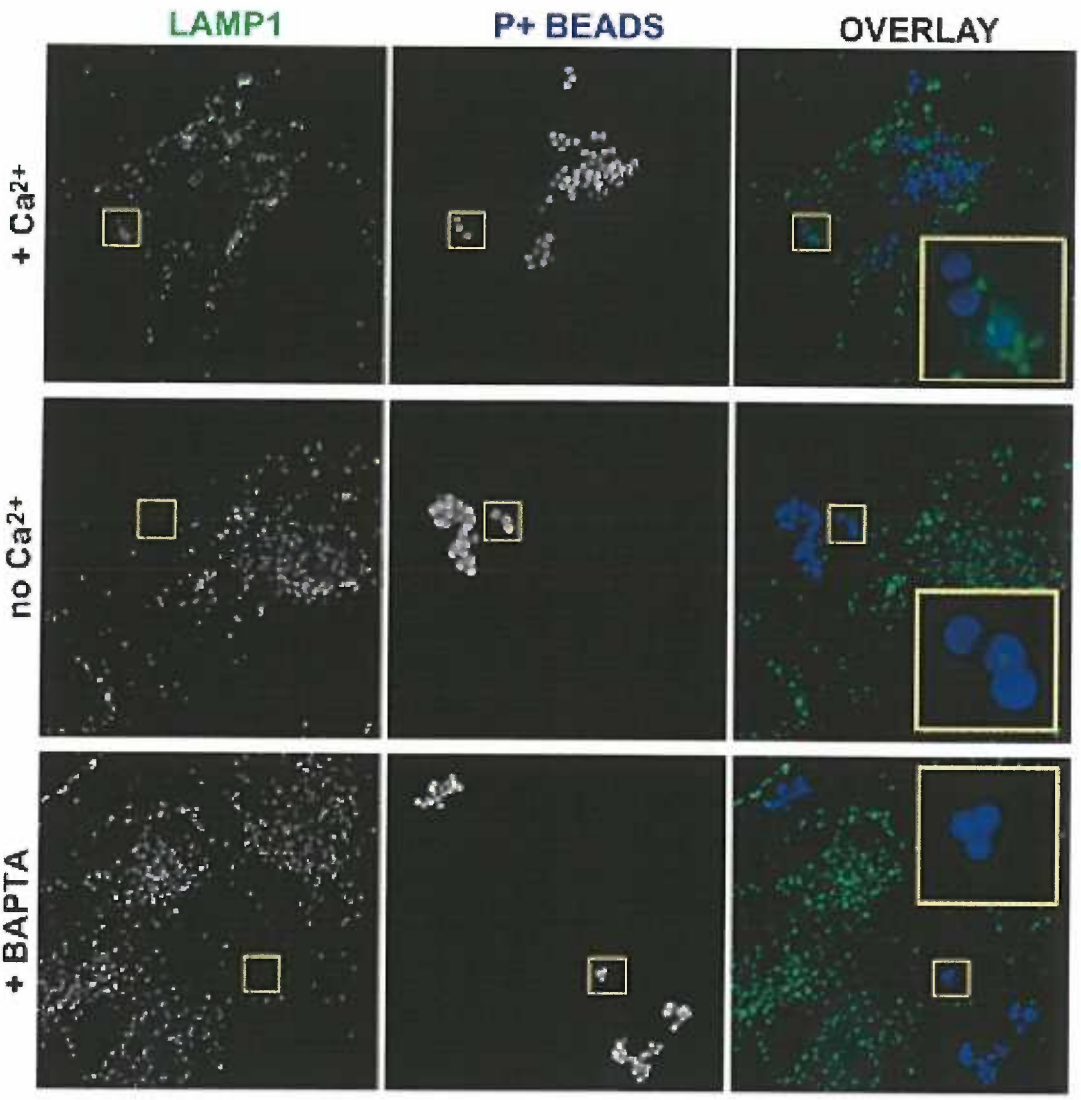


Figure 5b

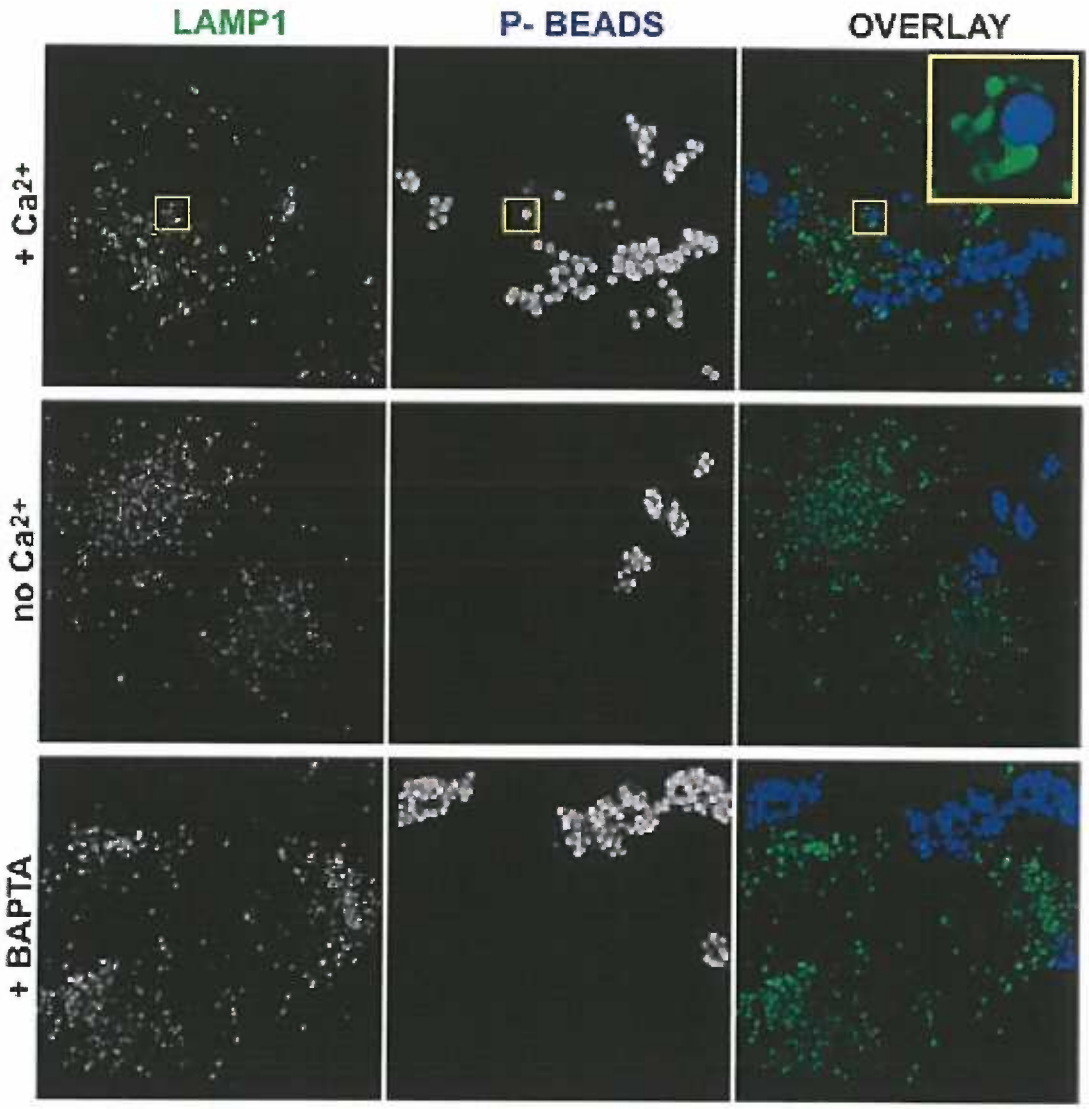
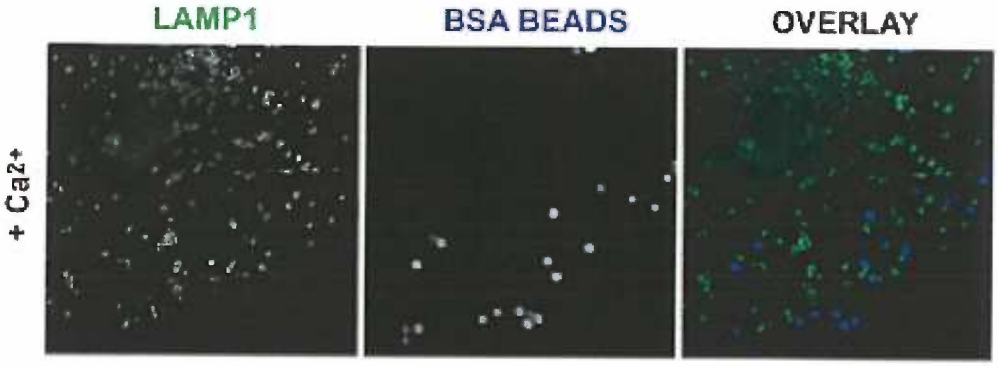


Figure 5c



Lamp1 exocytosis to the plasma membrane is pilus-dependent

We wished to confirm that P+ OMP-coated beads could induce the exocytosis of late endosomes and lysosomes, increasing surface Lamp1, as depicted in our images. It was also suggested by our image analysis suggests that Lamp1 does not increase on the cell surface in the absence of pili. Hence, using an ELISA-like assay previously described (Ayala *et al.*, 2001), we measured surface hLamp1 under the same conditions as the microscopy images above. Two hours after addition of outer membrane-coated beads, an increase in surface Lamp1 was detected in cells exposed to P+ beads only (Fig. 6). No increase was seen with either the P- beads or the BSA control over unstimulated cells. This confirms the necessity of pilus contact with cells for a significant increase in plasma membrane Lamp1 beyond a time point of 10 minutes. In addition, this result also shows the dependence of Lamp1 vesicular fusion with the plasma membrane on a pilus-induced signal.

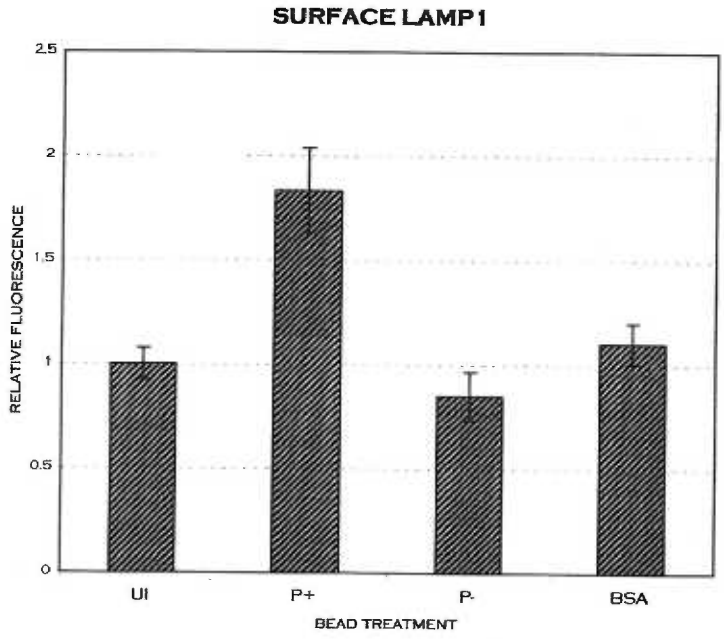


Figure 6. Lamp1 exocytosis to the plasma membrane is pilus-dependent

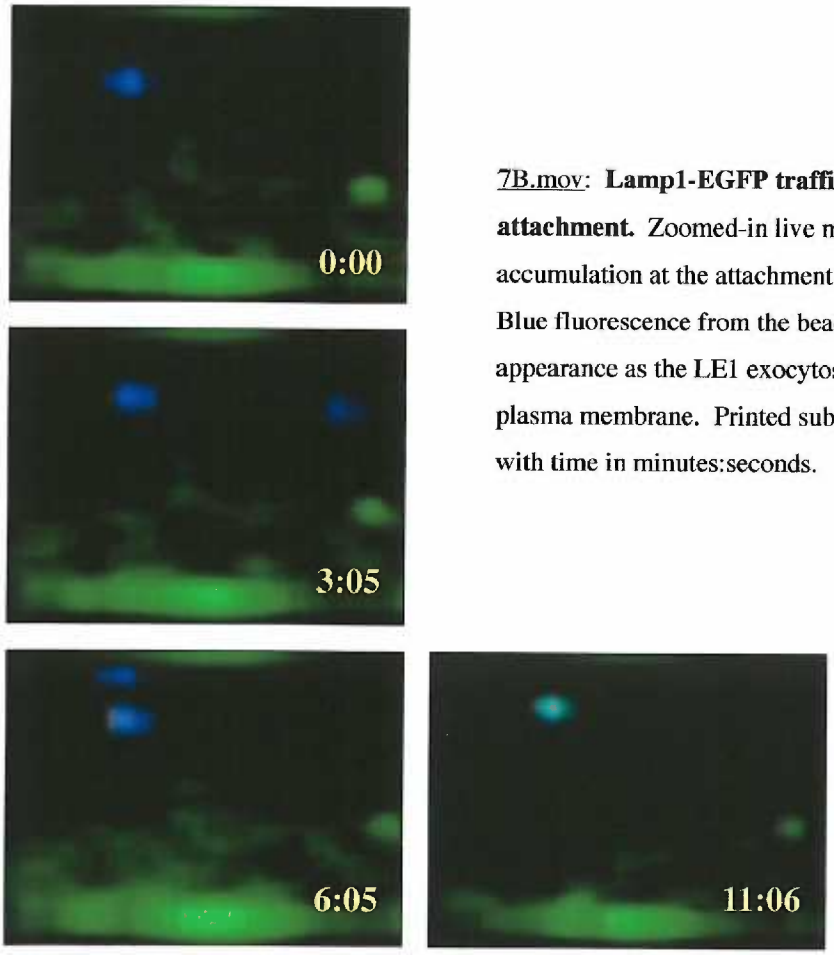
A431 cells were exposed to P+, P-, or BSA-coated beads as in previous experiments for 2 hours. Cells were then fixed, and surface Lamp1 was detected using a modified ELISA assay. Data shown is representative of trends observed in three experiments.

Dynamic visualization of directed lysosome exocytosis

Finally, we wished to demonstrate the directed exocytosis of Lamp1 in real-time using the stably transfected A431-LE1 cells. These cells show rapid movement of many fluorescent organelles throughout the cell cytoplasm, with a concentration of vesicles in the perinuclear region, as expected (Fig. 7A) High-magnification images were virtually impossible to capture when beads were freshly added to the cells due to constant movement by the beads and a low frequency of rapid attachment. To circumvent this problem, the A431-LE1 cells were chilled on ice to halt endocytosis and exocytosis, and kept at this temperature while incubated with the beads for 30-60 minutes before image acquisition. This procedure allowed the beads to attach to the cells and permitted the selection of pertinent fields. Samples were then warmed to 34°C, allowing lysosomal movement to occur. Over time, a strong increase in green fluorescence was observed beneath P+ OMP coated beads (Fig. 7B) indicating an accumulation of Lamp1-EGFP on the membrane at this site. This did not occur with the bound P- beads. Instead, small LE1 vesicles were seen changing their overall direction of movement to direct towards the attachment site. This result is consistent with the surface Lamp1 measurements in Figure 6. Thus, in contrast to the exocytosis of LE1-contining compartments to the site of P+ OMP attachment, P- OMPs appear to direct trafficking of these compartments to the site of binding *without* the occurrence of fusion to the plasma membrane.

Figure 7. Videos (please see accompanying CD): Lamp1-EGFP dynamics and exocytosis to a P+ bead

7A.mov: **Movement of Lamp1-EGFP vesicles in stably-transfected cells.** Live microscopy of A431 epithelial cells stably transfected with the Lamp1-EGFP construct. Images were captured every 6 seconds. Printed subset from movie are frames approximately 30 seconds apart.



7B.mov: **Lamp1-EGFP traffics to the site of P+ bead attachment.** Zoomed-in live microscopy of Lamp1-EGFP accumulation at the attachment site of a piliated bead. Blue fluorescence from the bead gains a green-white appearance as the LE1 exocytoses to its location on the plasma membrane. Printed subset of frames are labeled with time in minutes:seconds.

Taken together, these observations suggest a two-step model of Lamp1 exocytosis (Figure 8) – The first step involves relocalization of a subset of Lamp1 vesicles at or near the site of bacterial attachment. This is best demonstrated in the cells exposed to P-beads in the presence of extracellular calcium. It disappears when the extracellular calcium is depleted. Secondly, a pilus-induced exocytic event leads to fusion of these vesicles with the plasma membrane. The second process also appears dependent on the presence of extracellular calcium. Both steps may also require a release from intracellular calcium stores.

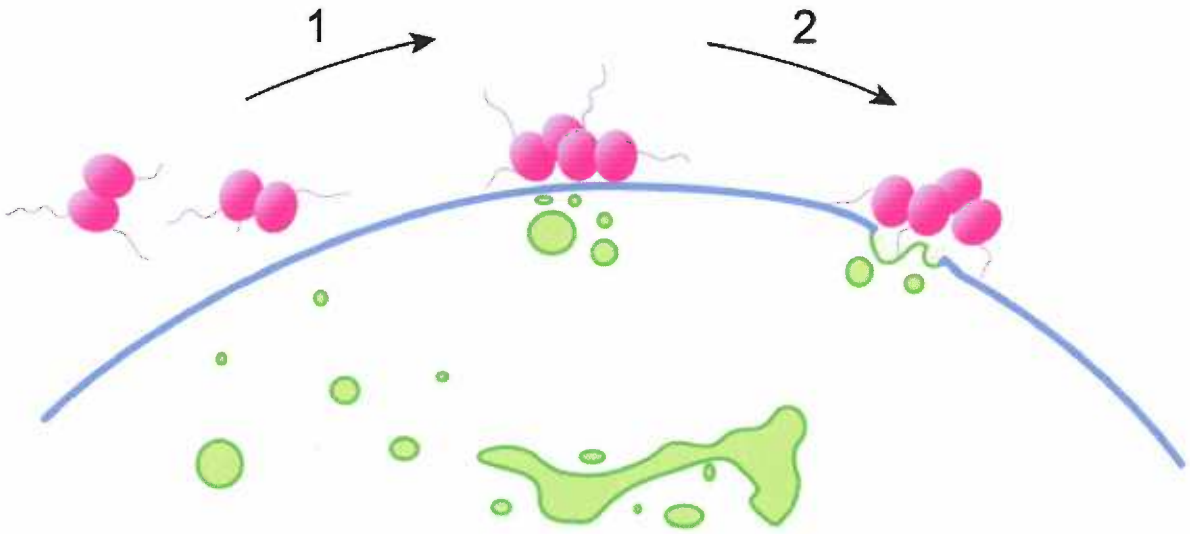


Figure 8. Two-step model for directed exocytosis of Lamp1 by contact with *Neisseriae*.

1. Lamp1 compartments are recruited beneath the plasma membrane at site of bacterial contact.
2. Fusion of Lamp1 compartments occurs secondary to a intracellular calcium signal. This results in an increase of surface Lamp1 at the site of bacterial attachment.

DISCUSSION

Results of the last decade are beginning to enlighten our ideas of how Neisseria is able to subvert killing within host epithelial cells during transcytosis across the mucosa. One key finding was that human Lamp1, with an IgA1-like proline-rich lumenal hinge, could be cleaved by IgA1 protease, and that this cleavage resulted in a decrease of total cellular lysosomes. The question remained as to how the secreted IgA protease reached Lamp1 as a substrate. Several models are conceivable including that verified by Ayala, et al (2000) showing that Lamp1 is exocytosed to the plasma membrane, where IgA protease secreted by adhered bacteria works to cleave it. This pilus- and calcium-dependent mechanism results in release of lysosomal hydrolases at the surface as well as the increase of lysosomal proteins to the surface.

If the bacteria simply wish to evade the degradative hydrolases and highly-glycosylated proteins which line the inside of lysosomes, they would direct exocytosis of the compartments elsewhere, or have them distribute randomly amongst the plasma membrane to dilute their effects. Our results suggest the opposite, however, indicating that lysosomal nutrients, enzymes and membrane-associated proteins might, in fact, be of some benefit towards establishing infection. Indeed, known bacterial receptors lie within these cellular treasure chests that enable these bacteria to exploit cellular traversal mechanisms (Kornfeld and Mellman, 1989; von Figura and Hasilik, 1986).

The recruitment of Lamp1 to the plasma membrane by a pathogen is not unique to *Neisseriae*. Elegant work has described how *Trypanosomes* direct the fusion of Lamp1 compartments to the site of contact by the parasite. Lamp1 is present on the membrane which surrounds the invader as it enters the cell. This process is activated in response to

a soluble trypanosomal protein binding to an unknown cellular receptor, followed by an intracellular calcium flux. It has also been considered that contact by the invading parasite may also induce a cellular repair mechanism, which occurs in response to an influx of extracellular calcium. Thus, the response of directing lysosomes to the site of entry is a perturbation of the cell's natural response to repair itself and/or recruit these organelles to the site of receptor stimulation.

The same mechanism may contribute to the directed exocytosis by *Neisseria*. It is known that porins from the bacterial outer membrane can insert into the plasma membrane of host cells, which may, like the trypanosomal contact, create enough of a break in the plasma membrane for the cell to respond as if it is injured. Recent work from our lab has shown that porin, itself, can induce a calcium flux which results in the exocytosis of endosomes, including some Lamp1-containing late endosomes (Ayala, in press). In addition, our results using P- outer membrane-coated beads, which contain porin, show that Lamp1 is not actually exocytosed to the surface. This changes with the presence of pili, which are less likely to cause membrane injury, than to signal through a receptor. CD46 has been identified as a putative receptor for pili (Kallstrom *et al.*, 1997) and induces a phosphorylation cascade with contact by pili (Kallstrom *et al.*, 1998; Lee *et al.*, 2002), but a connection between these events and the intracellular calcium release which is necessary for the exocytic fusion to occur, have not yet been detailed.

The stepwise process of lysosome exocytosis appears to occur as a concentration step facilitated by a localized influx of extracellular calcium, followed by a fusion step in response to a receptor-induced release of intracellular calcium. Fusion of vesicular compartments has been shown to be dependent on SNARE docking and is a stepwise

process in itself. Calcium is a known signaling molecule responsible for SNARE activation, facilitating membrane fusion. Whether or not this is the actual mechanism responsible for our observations is beyond the scope of this investigation.

It must be noted that these experiments, as well as several others looking at the exocytic phenomenon, use isolated membranes of bacteria and not live bacteria themselves. For our purposes, this enabled us to focus on localized events, alter experimental temperatures and avoid the confusion of soluble blebs, commonly released by *Neisseria* during infection. *In vivo*, these small lifeless organelles may create their own localized exocytic events at surface sites away from the site of bacterial attachment, perhaps preparing the cell surface for arrival of whole organisms.

There is supportive evidence where Lamp1 has been observed directly beneath colonizing *iga*- mutants. At that level of resolution, however, it is not clear if the Lamp1 has exocytosed or if there is simply an aggregation of apical vesicles. We have observed the localization in fixed A431-LE1 cells of green fluorescence beneath the site of bacterial attachment (Vasquez, unpublished results), but not to the extent of the results in this study. This suggests that either live bacteria actively suppress this response, or blebs released from the whole *Neisseriae* diffuse the exocytosis amongst the cell surface.

The Lamp1-EGFP fusion protein is shown here to be quite a useful tool for looking at lysosomal dynamics. This is the first known report of a successful GFP fusion to human Lamp1. It offers several advantages over other lysosomal markers. Many of the dyes used to track these organelles require activation in a low pH environment; many cell types don't actually achieve low-enough pH in their lysosomes to fluoresce. Fluid or bulk tracers used to follow the endocytic pathway may be excluded in studying the

uptake of pathogens. They may also interfere with binding to surface receptors, which is why lumenal or extracellular fusions of GFP to pertinent proteins also have their flaws. As the GFP is on the opposite side of the membrane from bacterial interaction, it is non-toxic to the infection process. In general, GFP fusions are very convenient and do not require washing or activation by a substrate in order to visualize the molecule of interest. In addition to a protein tracking molecule, the EGFP is useful as an epitope tag to aid in the isolation of Lamp1 which is overproduced in these stable transfectants.

In summary, our results show that *Neisseria* outer membrane components can direct the exocytosis of Lamp1 compartments to the site of attachment on the plasma membrane. This is achieved through a calcium-dependent mechanism, and appears to take place in two stages. The first stage relies on the presence of extracellular calcium and involves the relocalization of Lamp1 in the cell without its appearance on the cell membrane. The second, pilus-dependent stage, brings Lamp1 to be exposed on the surface via fusion of lysosomal compartments at the site of neisserial outer membrane contact. These results offer intriguing insights into Lamp1 trafficking and manipulation of the host cell by pathogenic *Neisseriae*.

SUMMARY AND CONCLUSIONS

IgA1 protease plays a significant role in the pathogenesis of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. This work explored several unknowns, including how protease expression is regulated within *Neisseria*, how it accesses the substrate Lamp1, and the significance and specificity of the two *iga* alleles. The finding of iron regulation of IgA1 protease is one of several newer discoveries of environmental regulation of virulence factors. Beyond isolated gene regulation, this finding has implications both for understanding disease pathogenesis as well as for treatment or prophylaxis development.

Our lab had previously shown the importance of the cleavage of Lamp1 by IgA1 protease. In this work, we expanded this model by studying the cell biology of infection. We were able to look at how the host epithelial cell responds to contact with bacterial factors, and how this influences the protease access to the lysosomal substrate. In the process, we identified key cell signaling pathways which *Neisseria* exploits during infection.

In Appendix A, we began an exploration of the similarities and differences of the two *iga* alleles. This work includes studies of both the genetic and functional specifics for each of the two protease types. In the process, we developed a new assay by which to assess IgA protease type in clinical isolates, and evaluated cleavage specificity within the Lamp1 hinge. This study further shows a clinical correlation which demonstrates significance of *iga* allele for disease phenotype.

This work lends further support to our understanding of IgA1 protease as a virulence factor which contributes to invasiveness of infections. Each of these studies focused on some aspect of bacteria-host interaction. It is hoped that the findings in this thesis will provide a basis for further understand of *Neisseria* pathogenicity and disease.

Appendix A

The IgA1 Protease types in Pathogenic *Neisseriae*: Cleavage of Lamp1 and influence on disease invasiveness

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ABSTRACT

All pathogenic *Neisseriae* secrete an IgA1 protease that is required for their virulence and survival within the mucosal environment of humans. Each strain produces one of two protease types, either Type 1 or Type 2 that differ, by definition, in the site they cleave within the human IgA1 hinge. Type 1 producing strains have anecdotally been correlated with more invasive disease manifestations in infections with both *N. meningitidis* and *N. gonorrheoae* (Mulks and Knapp, 1987). A molecular mechanism for this correlation, however, has yet to be shown. We wished to develop an assay by which clinical isolates could be "typed" and test for the correlation of protease type with invasiveness of disease. Firstly, we chose to sequence the Type 1 *iga* gene in order to provide a comparative understanding of its similarities and differences to the well-characterized Type 2 protease. Based upon our sequence comparison, we designed primers which allowed for protease "typing" of individual isolates. In this work, we confirm a significant correlation between *iga* genotype and invasiveness of gonococcal disease.

Besides hIgA1, the protease has been shown to have several other molecular substrates. The only one so far demonstrated in culture infections (as opposed to *in vitro* cleavage) is human lysosomal-associated membrane protein 1 (Lamp1). Cleavage of Lamp1 has been shown to enhance bacterial intracellular survival and transcytosis through cell monolayers(Hopper *et al.*, 2000a; Lin *et al.*, 1997). Although both IgA1 protease types are known to cleave hLamp1 at its IgA-like hinge, the specificity of this reaction is unknown. Type 1 protease cleaves a proline-serine (PS) bond within the IgA1

hinge; Type 2's specificity is for a proline-threonine (PT) site. The Lamp1 hinge contains five PS sites within its hinge, but only a single PT.

We wished to explore the possibility that bacteria which produce Type 1 IgA1 protease could cleave Lamp1 more efficiently, survive better intracellularly, and be more invasive in disease than an Type 2 producing strain, based on the potential for more cleavage sites in the Lamp1 hinge. We constructed mutagenized sequences within the cleavage site and established stably-transfected cells we could infect with *Neisseriae*. Strains which produce either Type1 protease or Type 2, and their isogenic *iga*- deletion mutants were then assayed for their abilities to invade these cell lines. In the process, we hoped to further show the significance of Lamp1 cleavage on the pathogenic progression of *Neisseriae*.

INTRODUCTION

IgA1 protease is a key factor in virulence of *Neisseria meningitidis* and *Neisseria gonorrhea*. All pathogenic strains of these bacteria express the gene, and disease severity has been linked with activity levels of the protease. Besides human IgA1, other substrates have recently been identified including synaptobrevin(Binscheck *et al.*, 1995), human chorionic gonadotropin (hCG)(Senior *et al.*, 2001), the TNF receptor II on the surface of monocytes (Beck and Meyer, 2000)and the major Lysosomal-associated membrane protein, Lamp1(Lin *et al.*, 1997). Cleavage of Lamp1 by IgA1 protease has been shown to increase gonococcal intracellular survival and speed transcytosis versus an *iga*- knockout strain(Lin *et al.*, 1997).

Both gonococci and meningococci strains carry one of two alleles of the *iga* gene(Mulks and Shoberg, 1994). These were designated Type 1 or Type 2 based on their site of proteolytic cleavage within the hIgA1 proline-rich hinge(Simpson *et al.*, 1988). Unlike several other virulence genes in these bacteria, this locus is not known to undergo variation antigenically or by phase. Instead each strain carries one or the other specific type. Although at least one Type 2 *iga* gene has been thouroughly sequenced and characterized(Pohlner *et al.*, 1987a), there is no *Neisseria iga* gene identified as Type 1 in the database. We therefore wished to sequence a known Type 1 gene for identification and comparison of the two alleles at the genetic level.

IgA1 protease was one the first bacterial autotransporters to be identified and characterized(Pohlner *et al.*, 1987a). An amphipathic β sheet at the C-terminus inserts into the outermembrane in oligomers to form a 2μm pore, through which the rest of the protease transports. The preprotein then undergoes autoproteolytic cleavage to produce 4

poplypeptides. (The N-terminal signal sequence, which targets translation to the periplasm, is a 5th domain.) Along with the pore-forming domain and the mature protease, there is an α-protein and a short γ-peptide. The γ-peptide has no known function; it is not clear if both protease types release this domain. The α-protein may act as a transcription factor in the host eukaryotic cell; it contains a functional eukaryotic nuclear localization signal and domains consistent with a leucine zipper for binding DNA(Pohlner *et al.*, 1995). Having this information on the basis of a Type 2 *iga* gene, we were able to conduct a comparison of the two alleles for this study.

Given the recent findings for the actions of IgA1 protease in pathogenesis beyond cleavage of IgA1, we wished to further investigate if there are separate roles for each type of IgA1 protease that may give specific strains more virulent properties. In particular, we wished to investigate the specificity for the protease types within the Lamp1 proline-rich hinge sequence, hypothesized to be the site of cleavage by IgA1 protease. If the sequence specificity for cleavage is like that for the hinge in IgA1, Type 2 protease cleaves a proline-threonine (PT) bond whereas Type 1 cleaves a proline-serine (PS) bond. Within the Lamp1 hinge, there are 5 PS bonds, but only a single PT; we therefore hypothesized that the Type 1 protease may have greater access and thus efficacy at cleaving Lamp1 than the second type.

Given that IgA1 protease activity correlates with invasiveness of disease(Vitovski *et al.*, 1999; Vitovski *et al.*, 2002), we further believed that Type 1 producing strains would tend to cause more invasive diseases. We therefore wished to investigate whether or not there is a correlation between IgA1 protease type and disease phenotype. Specifically, for gonorrhea, we compared isolates causing urethritis (non-invasive),

pelvic inflammatory disease (PID) (ascending infection), and disseminated gonoccocal infection (DGI) (invasive). Based on comparison of known *iga* sequences and correlation with IgA1 cleavage patterns, we developed a simple PCR assay by which we could rapidly type gonococcal isolates. Using this assay, we blindly tested a panel of gonococcal isolates and discovered that indeed, protease allele appears to correlate with disease phenotype.

Taken together, our results further demonstrate the significance of IgA1 protease cleavage of Lamp1 in the pathogenesis of Neisseriae. Not only does the presence and level of protease expression appear to be important, but the allele of each strain appears to play different roles in disease as well. We have developed a simple means by which this allele may be identified from gonococcal isolates. Finally, we show that there is clinical evidence which suggests that type of IgA1 protease offers invasive properties to it strain carrier.

MATERIALS AND METHODS

Bacterial strains

Neisseria gonorrhoeae strains MS11A, MS11-500, MC8013.6, and MC500 have all been previously described (Ayala *et al.*, 2001; Hopper *et al.*, 2000a). Clinical strains were obtained from a bank of isolates from patients at the Boston University School of Medicine.

Cell culture

A431 epitheliod carcinoma cells were a kind gift from S. Schmid. Both these cells, as well as their transgenic derivatives, were maintained in Dulbecco's Modified Eagle Medium (DMEM; Cellgro) supplemented with 10% FCS (Gibco BRL).

Type 1 *iga* sequence determination

pGIGA100 contains the Type 1 *iga* gene from N. gonorrhoeae 32819 (AHU-), derived from pFY23 (Fishman *et al.*, 1985), cloned into a pUC18 backbone. Sequencing was performed beginning from both ends of the cloning region and proceeded in both directions for overlapping control of errors. DNA sequence determination was performed by the OHSU-MMI Research Core Facility (http://www.ohsu.edu/core) on a model 377 Applied Biosystems Inc. automated fluorescence sequencer. Base calling is performed on a Macintosh computer with the ABI base calling program. Cycle sequencing is performed with AmpliTaq FS DNA Polymerase using "big-dye" labeled terminators, both from Applied Biosystems Inc.

Primers were sequentially designed approximately 250 bp apart according to sequence determination. Oligonucleotides were synthesized on an Applied Biosystems 394 synthesizer using standard (ß-cyanoethyl-phosphoramidite) chemistry. Following deprotection, the oligonucleotides were desalted by Nap-10 (Sephadex[a]) chromatography, eluted in `milliQ' water, and used without further purification.
Sequence analysis and alignment was performed using both MacVector software and Vector NTI Suite v.5.0 for Macintosh.

PCR analysis

Primers were designed flanking the major variable region of iga. "igaIN4203" = GCGGAGCAAGTGAAGCG, and "igaOUT4984" = CGCGGCTGCGCGGC. These products typically yielded two sets of products, differing by approximately 100-200 bp in size, from Neisseria genomes with an intact *iga* gene. Bacteria from single colonies were resuspended from culture in 100µl water using a sterile toothpick; 1µl of this suspension was used as template in each 20µl PCR reaction mixture. PCR was conducted in a Perkin Elmer Gene Amp 9600 PCR System using Taq polymerase (Gibco BRL). Products were separated by agarose gel electrophoresis and stained with EtBr, and visualized on a Gel Doc 1000 (BioRad).

hIgA1 cleavage

Neisseria were cultured in GCB liquid medium for six hours at 37°C, 5%CO_2. IgA1 protease-containing supernatants were then collected following centrifugation. Either samples from these supernatants or purified IgA1 protease (Type 2, Roche) were

incubated with purified human IgA1 (from colostrum, Calbiochem) at 37°C for 2-3 hours. Samples were analyzed by SDS-PAGE stained with Coomassie Blue. Images of these gels were obtained by a BioRad Gel Doc 1000, and densitometry was performed using NIH Image, v.1.62.

Lamp1 hinge mutagenesis and transfection

The cDNA encoding Lamp1 had previously been cloned into a eukaryotic expression vector under the CMV promoter (Vasquez, manuscript in preparation). This plasmid was subject to site-directed mutagenesis by the ssDNA *in vitro* synthesis (BioRad) using the following primers: To create an "all PT" hinge, "CAAGACAGGCCTACCCCAACCACGGCGCCCCCTGCGCCACCCACCCCCACTCCCACTCC CGTGCCCAAGAGCCCCACTGTGGACAAGTACAACGTG" was used to create five S to T mutations. For the "all PS" hinge, only one T to S mutation was required, and the primer "CAAGACAGGCCTTCCCCAAGTACTGCGCCCCCTGCGCCACCC" was used for mutagenesis.

Transfections of the resulting LWT, LPS, and LPT plasmids was carried out using Fugene 6 transfection reagent (Roche) as directed. Cells were selected for stable transfection with 0.5 mg/ml geneticin (Sigma) in DMEM with 10% FCS.

Adhesion and invasion assays

Stably-transfected cell lines, LWT, LPS, and LPT,were grown to 50% confluency in 24-well tissue-culture plates (Falcon). MS11A and MS11-500 were resuspended in this medium and cells were infected at an MOI of 5. MC8013.6 or MC500 were

resuspended and applied to cells at an MOI of 1. Cultures were incubated for 12hrs at 37°C, 5% CO_2. Media was changed to 50mg/ml gentimycin in DMEM with 10% FCS in half of the wells, and plates were incubated for an additional hour. All wells were then washed with warm GCB five times. Cultures were then lysed in 0.5ml GCB containing 0.5% saponin (Sigma) for 15 minutes at 37%C. Lysates were collected and serial dilutions were made prior to plating on GCB agar plates containing CNV (Beckton Dickinson).

Colonies were counted and scored. Calculations for figure 4 were made as follows: Each cell type and condition was collected in quadruplicate for each assay. These numbers were averaged between gentimycin-protected (GP) or cell-associated (CA) colony forming units. GP/CA gave the "invasion index" for each bacterial type on each of the three cell types. Results from two experiments for each bacterial type were averaged, then grouped by IgA1 protease type (e.g. MC8013.6 = Type 1 vs MC500 *iga*-; MS11A = Type 2 vs MS11-500 *iga*-). Relative invasion indices were calculated by dividing the invasion index of each *iga*- strain by that of its corresponding wild type strain.

RESULTS

Comparison of the Type 1 and Type 2 *iga* sequences.

In the current gene databases, both for genomes and for fragments, IgA1 proteases are not described as being either of Type 1 or Type 2. We obtained possession of the plasmid pGIGA100 (Mulks) with a known Type 1 IgA1 protease encoded. We sequenced the entire ORF and flanking sequences and compared it to other known *iga* sequences. Figure 1 depicts many of the similarities and differences between them and their polypeptide products.

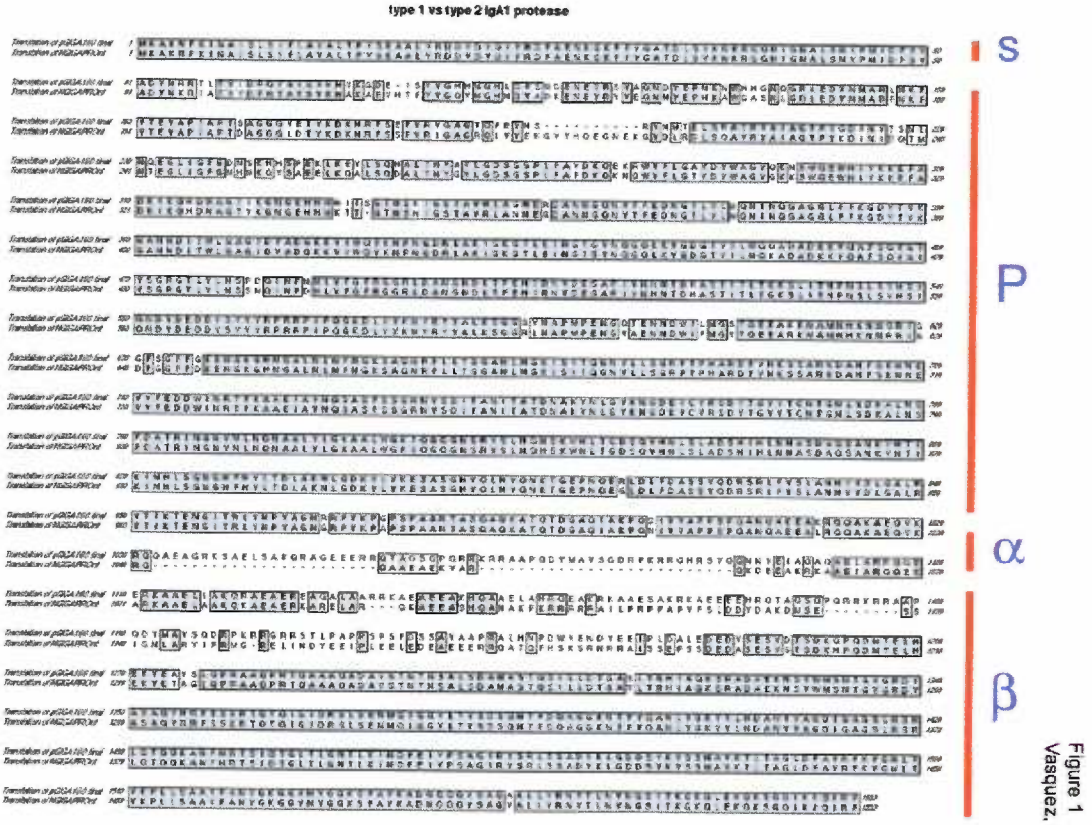


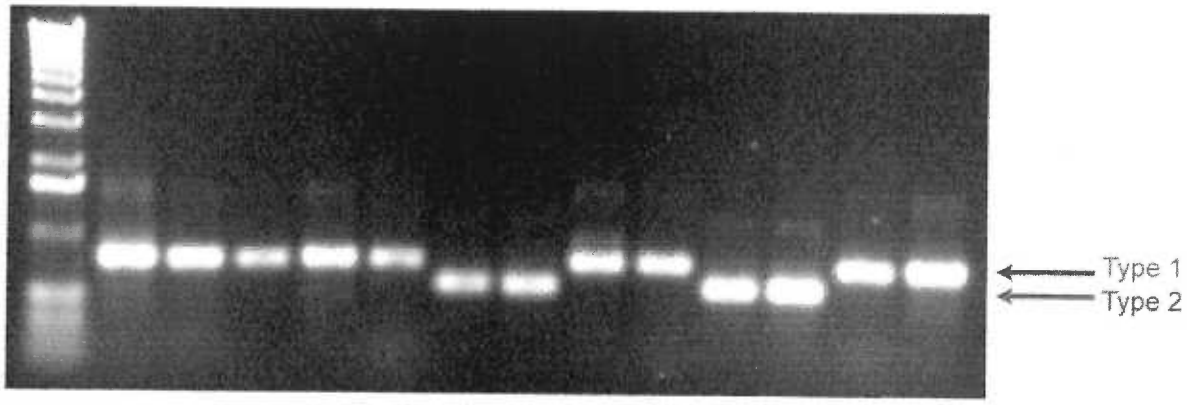
Figure 1
Vasquez, et al.

Type 1 and Type 2 *iga* genes are highly conserved; Overall, they are > 96% identical at the amino acid level. The majority of differences were within the α, and γ domains. In comparing several other *iga* sequences from genomes and fragments in

GenBank, there appears to be two distinct gene types – one group containing a larger variable region (VR) than the others (data not shown), differing in size by approximately 60 amino acids. The coding sequence for both types of mature protease have notably few differences; they are >97% identical at the amino acid level.

PCR analysis of iga and correlation to protease allele.

We therefore wished to determine whether this primary genotype difference correlated with phenotype of the protease allele. The sequences characterized above were used as templates to design a PCR screening technique for rapid determination of *iga* VR length. Essentially, conserved sequence flanking the variable regions within sequences was used as a template for PCR primers. Colony PCR was then performed and products were subject to agarose gel electrophoresis (Fig. 2a). Each isolate was then scored as either "long" (> 700 bp) or "short" (< 650bp), and results were compared with known protease typing by cleavage.



All "long" PCR products correlated with the Type 1 protease phenotype, and all "short" products were produced from Type 2 strains. This perfect correlation enabled us

to establish the colony PCR assay as a quick method of typing *iga* alleles for *Neisseria* isolates.

IgA1 protease types of clinical isolates

We performed the above PCR assay on several isolate of gonococci. These were obtained from a bank of isolate strains from patients with either urethritis/cervicitis, pelvic inflammatory disease (PID) or disseminated gonococcal infections (DGI). For the purposes of assessing invasiveness, we considered urethritis or cervicitis a mildly invasive disease, since the bacteria are required to pass through an epithelium to induce symptoms. PID is an ascending disease, where the bacteria ascend the female genital tract without invading and cause inflammation of the pelvic cavity upon exiting from the fallopian tubes. DGI strains were considered strongly invasive, as they pass through multiple cellular barriers before invading into the bloodstream.

Gonococcal clinical isolates

disease	total	Type 1	Type 2
Urethritis/cervicitis	27	19	8
PID	21	7	14
DGI	20	16	4

Table 1. Correlation of IgA1 Protease type with invasiveness of disease. A number of isolates from each disease type were typed using the PCR assay, with results as shown. More invasive diseases – urethritis, cervicitis and DGI – are associated with a higher incidence of Type 1 IgA1 protease-producing strains.

Results are shown in Table 1. The Type 1 allele of *iga* correlated significantly with those strains we considered invasive (urethritis/cervicitis or DGI), whereas PID strains were predominantly Type 2 producers. This supported the hypothesis that disease

invasiveness would correlate with *iga* allele; we investigated further to try to understand the etiology of this correlation.

Lamp1 hinge modification and cleavage by IgA1 protease types

To test the specificity of each protease type within the Lamp1 hinge, we constructed Lamp1 hinge mutants and overexpressed them in transfected cells. We considered all of the five proline-serine (PS) bonds and the single proline-threonine (PT) bond as potential proteolytic targets. An all-PT and all-PS hinge mutants were developed by site-directed mutagenesis, and these, as well as the wild-type Lamp1 gene, were cloned into a eukaryotic expression vector, with their expression driven by the CMV promoter (Fig 3a). Using this pIRES vector (Clontech), cells which are resistant to selection by the drug geneticin transcribe the transgenic gene.

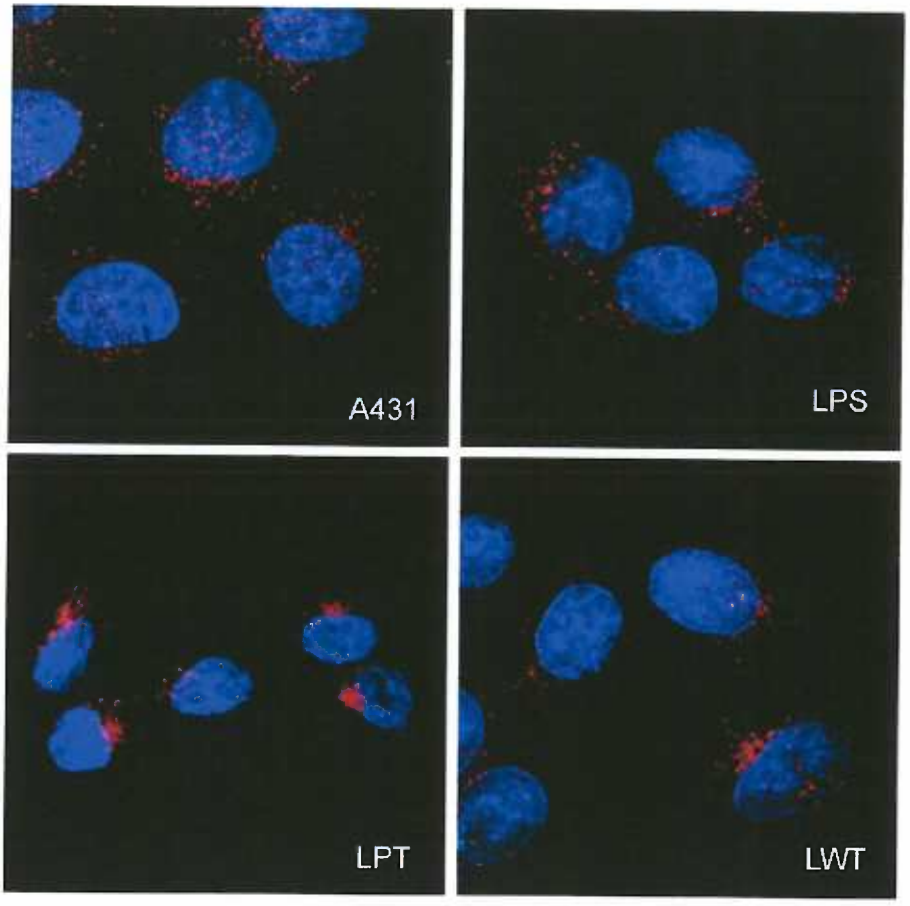
These stably-transfected cell lines were subject to multiple controls to assure that the localization of Lamp1 or the mutant was not drastically different from the norm. Immunocytochemistry was performed to look at Lamp1 distribution (Figure 3b), and colocalization studies were performed to assure overlap with late endosomal/lysosomal markers (Lamp2, CD63) and not with endosomes (transferrin receptor) (data not shown). Western blot verified the correct size of the transgenic Lamp1, which runs together with endogenous Lamp1, and compared relative expression between cell lines (Figure 3c). All cell lines chosen for further assay were selected based upon these criteria as indistinguishable from untransfected A431 cells as possible. They were named "LWT"

with the wild-type gene in trans, "LPS" for trans-Lamp1 with an all-PS hinge, or "LPT" for trans-Lamp1 with an all-PT hinge.

a

LWT ...RPSPTTAPPAPPSPSPSPVPKSPSVDKYNVSGT...
LPS ...RPSPSTAPPAPPSPSPSPVPKSPSVDKYNVSGT...
LPT ...RPSPTTAPPAPPTPTPTPVPKSPTVDKYNVSGT...

b



c

Isogenic analysis of protease types in mutant Lamp1 transfectants

To investigate the cleavage specificity of each IgA1 protease type, as well as to test the contribution of Lamp1 cleavage to intracellular survival, we conducted invasion assays on each of the transgenic cell types. *Neisseriae* which produce IgA1 protease type 1 (MC strain 8013.6) or type 2 (GC strain MS11A) were compared versus their corresponding *iga*- deletion mutant (MC500 or MS11-500, respectively). These experiments were all conducted in each of the three transgenic cell types in parallel.

Results are summarized in Figure 4. To obtain comparable invasion indicies, the number of fraction of intracellular bacteria was divided by the number of cell-associated colony forming units after 12 hours. These numbers were made relative by dividing the invasion index of the *iga*- mutant by its corresponding protease-producing parent. Thus the relative index expressed is the relative ablilty of the bacterium to invade the cell without producing IgA1 protease.

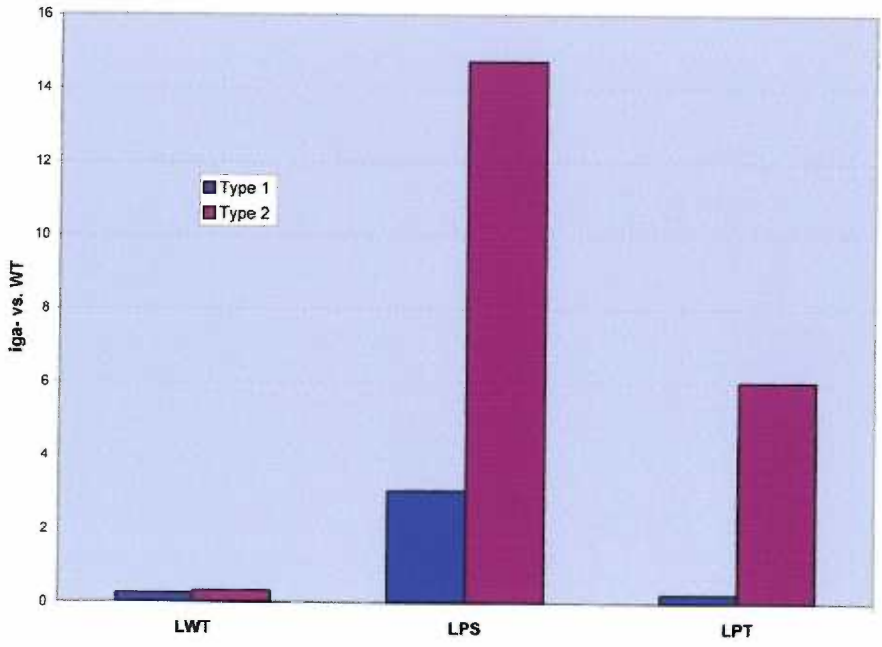


Figure 4. IgA1 protease type and impact on invasion of Lamp1-hinge transfectants. Invasion assays were performed using WT Type 1 or 2 IgA1 type or *iga*- gonococci. Bars represent fold-increase in invasion between WT and *iga*- bacteria. LWT, LPS, LPT transfeted cell lines as described in text.

Both Type 1 and Type 2 *iga Neisseriae* are affected in the LWT cell line, as expected. This confirms the fact that both protease types have the ability to cleave the wild-type Lamp1 hinge. In the LPS cell line, however, both bacterial strains appeared to invade intracellularly better than strains that produced protease. This suggests that other components of the protease (e.g. the autotransporter pore) may actually compromise its ability to survive in late endosomes or lysosomes, and that neither protease type is able to cleave an all-PS Lamp1 hinge. Lastly, Type 1 protease appears to cleave an all-PT Lamp1 hinge, while Type 2 does not. This interpretation arises from the fact that wild-type MC8013.6 survive considerably better than their *iga*- mutant (MC500) where as the same cannot be said for the gonococccal Type 2 *iga* strains in the LPT cell line.

DISCUSSION

To date, no analysis has been made which correlates IgA1 protease type with disease, although recent studies have looked at levels of protease expression. We have sequenced the first full-length *iga* gene of known Type 1, and compared it with the well-characterized sequence of the Type 2 allele. On the basis of this comparison, a simple PCR assay was developed by which clinical isolates may be rapidly assessed for the *iga* allele which they express. This assay was further used to analyze a panel of clinical gonococcal isolates, and showed significant correlation between *iga* type and invasiveness of disease. Type 1 producing strains were more likely to cause invasive manifestations such as urethritis, cervicitis or DGI, whereas PID (ascending) strains were predominantly of Type 2.

The IgA1 protease has been shown to have multiple functions in *Neisseria* interaction with the host. Besides the cleavage of hIgA1, the mature protease also cleaves human chorionic gonoadotropin (Type 1 only), the TNF receptor II on monocytes, and synaptobrevin *in vitro*. The significance for each of these interactions in pathogenesis is unknown.

We have performed several studies to look at the significance of IgA1 protease interaction with the major lysosomal protein, Lamp1. Although this protein has unknown function in host cells, it has been shown that its cleavage by IgA1 protease accelerates its degradation and the concurrent reduction of other lysosomal markers. This suggests that Lamp1 cleavage leads to a reduction in total lysosomes within host cells. This, then, is a proposed mechanism for how *Neisseriae* are able to avoid lysosomal degradation and survive within host epithelial cells.

The results described in this manuscript further advance our understanding of the interaction of IgA1 protease with host Lamp1. When we mutagenized the proline-rich hinge of Lamp1, which is the target of IgA1 protease, we showed that the production of protease no longer benefited the bacterium for survival. In fact, an *iga*- mutant survived better than its wild-type parent intracellularly in cells with "uncleavable" Lamp1. This is most likely due to other processes modulated by IgA protease that are yet unknown. For instance, the transporter subunit of IgA1 protease remains in the outer membrane of the bacterium as an oligomeric structure, forming a 2nm pore. Besides the secretion of the mature protease, the function of this pore is unknown.

Our results advance the understanding of the role IgA1 protease plays in *Neisseria* virulence. We have now characterized the Type 1 *iga* allele and its basic relationship to the Type 2 gene. In addition, there is evidence that Type 1 predisposes a gonococcal strain to cause more invasive disease. Finally, we further characterize the specificity of each protease type for cleavage of the Lamp1 hinge. These results further our understanding of *Neisseria* pathogenesis and the bacteria's interaction with the host.

REFERENCES

Akasaki, K., Michihara, A., Mibuka, K., Fujiwara, Y., and Tsuji, H. (1995) Biosynthetic transport of a major lysosomal membrane glycoprotein, lamp-1: convergence of biosynthetic and endocytic pathways occurs at three distinctive points. *Exp Cell Res* **220**: 464-473.

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

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

Beck, S.C., and Meyer, T.F. (2000) IgA1 protease from Neisseria gonorrhoeae inhibits TNFalpha-mediated apoptosis of human monocytic cells. *FEBS Lett* **472**: 287-292.

Berger, R.E., Alexander, E.R., Harnisch, J.P., Paulsen, C.A., Monda, G.D., Ansell, J., and Holmes, K.K. (1979) Etiology, manifestation, and therapy of acute epididymitis: prospective study of 50 cases. *J Urology* **121**: 750-754.

Berish, S.A., Subbarao, S., Chen, C.Y., Trees, D.L., and Morse, S.A. (1993) Identification and cloning of a fur homolog from Neisseria gonorrhoeae. *Infect Immun* **61**: 4599-4606.

Binscheck, T., Bartels, F., Bergel, H., Bigalke, H., Yamasaki, S., Hayashi, T., Niemann, H., and Pohlner, J. (1995) IgA protease from Neisseria gonorrhoeae inhibits

exocytosis in bovine chromaffin cells like tetanus toxin. *J Biol Chem* **270**: 1770-1774.

Blake, M., Holmes, K.K., and Swanson, J. (1979) Studies on gonococcus infection. *J Infect Dis* **139**: 89-92.

Bonnah, R.A., Lee, S.W., Vasquez, B.L., Enns, C.A., and So, M. (2000) Alteration of epithelial cell transferrin-iron homeostasis by Neisseria meningitidis and Neisseria gonorrhoeae. *Cell Microbiol* **2**: 207-218.

Britigan, B.E., Cohen, M.S., and Sparling, P.F. (1985) Gonococcal infection: a model of molecular pathogenesis. *N Engl J Med* **312**: 1683.

Carbonetti, N., Simnad, V., Elkins, C., and Sparling, P.F. (1990) Construction of isogenic gonococci with variable porin structure: effects on susceptibility to human serum and antibiotics. *Mol Micro* **4**: 1009-1018.

Carlsson, S.R., Roth, J., Piller, F., and Fukuda, M. (1988) Isolation and characterization of human lysosomal membrane glycoproteins, h-lamp1 and h-lamp-2. *J. Biol. Chem.* **263**: 18911-18919.

Carlsson, S.R., and Fukuda, M. (1992) The lysosomal membrane glycoprotein lamp-1 is transported to lysosomes by two alternative pathways. *Arch Biochem Biophys* **296**: 630-639.

Chai, S., Welch, T.J., and Crosa, J.H. (1998) Characterization of the interaction between Fur and the iron transport promoter of the virulence plasmid in Vibrio anguillarum. *J Biol Chem* **273**: 33841-33847.

Chen, J.W., Murphy, T.L., Willingham, M.C., Pastan, I., and August, J.T. (1985) Identification of two lysosomal membrane glycoproteins. *J Cell Biol* **101**: 85-95.

Coorssen, J.R., Schmitt, H., and Almers, W. (1996) Ca2+ triggers massive exocytosis in Chinese hamster ovary cells. *Embo J* **15**: 3787-3791.

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

Davletov, B.A., Meunier, F.A., Ashton, A.C., Matsushita, H., Hirst, W.D., Lelianova, V.G., Wilkin, G.P., Dolly, J.O., and Ushkaryov, Y.A. (1998) Vesicle exocytosis stimulated by α-latrotoxin is mediated by latrophin and requires both external and stored Ca^{2+}. *Embo J* **17**: 3909-3920.

Dietrich, G., Kurz, S., Hubner, C., Aepinus, C., Theiss, S., Guckenberger, M., Panzner, U., Weber, J., and Frosch, M. (2003) Transcriptome analysis of Neisseria meningitidis during infection. *J Bacteriol* **185**: 155-164.

Dubrac, S., and Touati, D. (2000) Fur positive regulation of iron superoxide dismutase in Escherichia coli: functional analysis of the sodB promoter. *J Bacteriol* **182**: 3802-3808.

Dunn, L.A., and Holz, W. (1983) Chatecholamine secretion from digitonin-treated adrenal medullary chromaffin cells. *J Biol Chem* **258**: 4989-4993.

Edwards, J.L., Brown, E.J., Ault, K.A., and Apicella, M.A. (2001) The role of complement receptor 3 (CR3) in Neisseria gonorrhoeae infection of human cervical epithelia. *Cell Microbiol* **3**: 611-622.

Elkins, C., Barkley, K.B., Carbonetti, N.H., Coimbre, A.J., and Sparling, P.F. (1994) Immunobiology of purified recombinant outer membrane porin protein I of *Neisseria gonorrhoeae*. *Mol Micro* **14**: 1059-1075.

Fishman, Y., Bricker, J., Gilbert, J.V., Plaut, A.G., and Wright, A. (1985) Cloning of the Type 1 Immunoglobulin A1 Protease from Neisseria gonorrhoeae and Secretion of the Enzyme from Escherichia coli. In *The Pathogenic Neisseriae*. Schoolnik, G.K. (ed). Washington, D. C.: American Society for Microbiology, pp. 164-168.

Frangione, B., and Wolfenstein-Todel, C. (1972) Partial duplication in the "hinge" region of IgA1 myeloma proteins. *PNAS* **69**: 3673-3676.

Genco, C.A., and Desai, P.J. (1996) Iron acquisition in the pathogenic Neisseria. *Trends Microbiol* **4**: 179-184.

Griffiths, G., Matteoni, R., Back, R., and Hoflack, B. (1990) Characterization of the cation-independent mannose 6-phosphate receptor-enriched prelysosomal compartment in NRK cells. *J Cell Sci* **95 (Pt 3)**: 441-461.

Halter, R., Pohlner, J., and Meyer, T.F. (1984) IgA protease of Neisseria gonorrhoeae: isolation and characterization of the gene and its extracellular product. *Embo J* **3**: 1595-1601.

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

Hedges, S.R., Mayo, M.S., Kallman, L., Mestecky, J., Hook, E.W., 3rd, and Russell, M.W. (1998) Evaluation of immunoglobulin A1 (IgA1) protease and IgA1 protease-inhibitory activity in human female genital infection with Neisseria gonorrhoeae. *Infect Immun* **66**: 5826-5832.

Holtzman, E. (1989) *Lysosomes*. New York, NY: Plenum Press.

Hook, E.W., 3rd, and Holmes, K.K. (1985) Gonococcal infections. *Ann Internal Med* **102**: 229-243.

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

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

Hunter Hansdsfield, H. (1990) *Principles and practice of infectious diseases*. New York: Churchill Livingstone.

Johannsen, D.B., Johnston, D.M., Koymen, H.O., Cohen, M.S., and Cannon, J.G. (1999) A Neisseria gonorrhoeae immunoglobulin A1 protease mutant is infectious in the human challenge model of urethral infection. *Infect Immun* **67**: 3009-3013.

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

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

Kasai, H.T., Kishimoto, T.T., Liu, Y., Miyashita, P., Podini, F., Grohovaz, F., and Meldolesi, J. (1999) Multiple and diverse forms of regulated exocytosis in wild type and defective PC12 cells. *PNAS* **96**: 945-949.

Kornfeld, S., and Mellman, I. (1989) The biogenesis of lysosomes. *Annu Rev Cell Biol* **5**: 483-525.

Kornfeld, S.J., and Plaut, A.G. (1981) Secretory immunity and the bacterial IgA proteases. *Rev Infect Dis* **3**: 521-534.

Kristensen, J.K., and Scheibl, J.H. (1984) Etiology of acute epididymitis presenting in a veneral disease clinic. *Sex. Transm. Dis.* **11**: 32-33.

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

Lightfoot, R.W., Jr., and Gotschlich, E.C. (1974) Gonococcal disease. *Am J Med* **56**: 327-356.

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

Lorenzen, D.R., Dux, F., Wolk, U., Tsirpouchtsidis, A., Haas, G., and Meyer, T.F. (1999) Immunoglobulin A1 protease, an exoenzyme of pathogenic Neisseriae, is a potent inducer of proinflammatory cytokines. *J Exp Med* **190**: 1049-1058.

Madara, J.L., Stafford, J., Dharmsathaphorn, K., and Carlson, S. (1987) Structural analysis of a human intestinal epithelial cell line. *Gastroenterology* **92**: 1133-1145.

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

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

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

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

Mulks, M.H., and Knapp, J.S. (1987) Immunoglobulin A1 protease types of Neisseria gonorrhoeae and their relationship to auxotype and serovar. *Infect Immun* **55**: 931-936.

Mulks, M.H., and Shoberg, R.J. (1994) Bacterial immunoglobulin A1 proteases. *Methods Enzymol* **235**: 543-554.

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

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

Parge, H.E., Bernstein, S.L., Deal, C.D., McRee, D.E., Christensen, D., Capozza, M.A., Kays, B.W., Fieser, T.M., Draper, D., So, M., and et al. (1990) Biochemical purification and crystallographic characterization of the fiber-forming protein pilin from Neisseria gonorrhoeae. *J Biol Chem* **265**: 2278-2285.

Plaut, A.G., Gilbert, J.V., Artenstein, M.S., and Capra, J.D. (1975) Neisseria gonorrhoeae and Neisseria meningitidis: extracellular enzyme cleaves human immunoglobulin A. *Science* **190**: 1103-1105.

Pohlner, J., Halter, K., Beyreuther, K., and Meyer, T.F. (1987a) Gene structure and extracellular secretion of *Neisseria gonohroeae* IgA protease. *Nature* **325**: 458-460.

Pohlner, J., Halter, R., Beyreuther, K., and Meyer, T.F. (1987b) Gene structure and extracellular secretion of Neisseria gonorrhoeae IgA protease. *Nature* **325**: 458-462.

Pohlner, J., Langenberg, U., Wolk, U., Beck, S.C., and Meyer, T.F. (1995) Uptake and nuclear transport of Neisseria IgA1 protease-associated alpha-proteins in human cells. *Mol Microbiol* **17**: 1073-1083.

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

Qiu, J., Brackee, G.P., and Plaut, A.G. (1996) Analysis of the specificity of bacterial immunoglobulin A (IgA) proteases by a comparative study of ape serum IgAs as substrates. *Infect Immun* **64**: 933-937.

Reinholdt, J., and Kilian, M. (1997) Comparative analysis of immunoglobulin A1 protease activity among bacteria representing different genera, species, and strains. *Infect Immun* **65**: 4452-4459.

Rodland, K.D., Muldoon, L.L., Lenormand, P., and Magun, B.E. (1990) Modulation of RNA expression by intracellular calcium. *J Biol Chem* **265**: 11000-11007.

Rodriguez, A., Rioult, M.G., Ora, A., and Andrews, N.W. (1995) A trypanosome-soluble factor induces IP_3 formation, intracellular calcium mobilization and microfilament rearrangement in host cells. *J Cell Biol* **129**: 1263-1273.

Rodriguez, A., Samoff, E., Rioult, M.G., Chung, A., and Andrews, N.W. (1996) Host cell invasion by trypanosomes requires lysosome and microtubule/kinesis-mediated transport. *J Cell Biol* **134**: 349-362.

Rodriguez, A., Webster, P., Ortego, J., and Andrews, N.W. (1997) Lysosomes behave as Ca2+-regulated exocytic vesicles in fibroblasts and epithelial cells. *J Cell Biol* **137**: 93-104.

Rodriguez, A., Martinez, I., Chung, A., Berlots, C.H., and Andrews, N.W. (1999) cAMP regulates Ca^{2+}-dependent exocytosis of lysosomes and lysosome-mediated cell invasion by trypanosomes. *J Biol Chem* **274**: 16754-16759.

Rosenstein, N.E., Perkins, B.A., Stephens, D.S., Popovic, T., and Hughes, J.M. (2001) Medical progress: Meningococcal disease. *N Engl J Med* **344**: 1378-1388.

Rudel, T., Schmid, A., Benz, R., Kolb, H.A., Lang, F., and Meyer, T.F. (1996) Modulation of Neisseria porin (PorB) by cytosolic ATP/GTP of target cells: parallels between pathogen accommodation and mitochondrial endosymbiosis. *Cell* **85**: 391-402.

Ruden, A.K., Backman, M., Bygdeman, S., Jonsson, A., Ringertz, O., and Sandstrom, E. (1986) Analysis of serovar distribution as a tool in epidemiological studies in gonorrhoea. *Acta Derm Venereol* **66**: 325-333.

Ruden, A.K., Jonsson, A., Lidbrink, P., Allebeck, P., and Bygdeman, S.M. (1993) Endemic versus non-endemic gonorrhoea in Stockholm: results of contact tracing. *Int J STD AIDS* **4**: 284-292.

Scheenen, W.J., Wollheim, C.B., Pozzan, T., and Fasolato, C. (1998) Ca2+ depletion from granules inhibits exocytosis. A study with insulin-secreting cells. *J Biol Chem* **273**: 19002-19008.

Schryvers, A.B., and Stojiljkovic, I. (1999) Iron acquisition systems in the pathogenic Neisseria. *Mol Microbiol* **32**: 1117-1123.

Sebastian, S., Agarwal, S., Murphy, J.R., and Genco, C.A. (2002) The gonococcal fur regulon: identification of additional genes involved in major catabolic, recombination, and secretory pathways. *J Bacteriol* **184**: 3965-3974.

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

Senior, B.W., Stewart, W.W., Galloway, C., and Kerr, M.A. (2001) Cleavage of the hormone human chorionic gonadotropin, by the Type 1 IgA1 protease of Neisseria gonorrhoeae, and its implications. *J Infect Dis* **184**: 922-925.

Serkin, C.D., and Seifert, H.S. (2000) Iron availability regulates DNA recombination in Neisseria gonorrhoeae. *Mol Microbiol* **37**: 1075-1086.

Shoberg, R.J., and Mulks, M.H. (1991) Proteolysis of bacterial membrane proteins by Neisseria gonorrhoeae type 2 immunoglobulin A1 protease. *Infect Immun* **59**: 2535-2541.

Simpson, D.A., Hausinger, R.P., and Mulks, M.H. (1988) Purification, characterization, and comparison of the immunoglobulin A1 proteases of Neisseria gonorrhoeae. *J Bacteriol* **170**: 1866-1873.

Stojiljkovic, I., Baumler, A.J., and Hantke, K. (1994) Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated Escherichia coli genes by a fur titration assay. *J Mol Biol* **236**: 531-545.

Tam, M.R., Buchanan, T.M., Sandstrom, E.G., Holmes, K.K., Knapp, J.S., Siadak, A.W., and Nowinski, R.C. (1982) Serological classification of *Neisseria gonorrhoeae* with monoclonal antibodies. *Infect. Immun.* **36**: 1042-1053.

Thomas, C.E., and Sparling, P.F. (1996) Isolation and analysis of a fur mutant of Neisseria gonorrhoeae. *J Bacteriol* **178**: 4224-4232.

Tsien, R.Y. (1998) The green fluorescent protein. *Annu Rev Biochem* **67**: 509-544.

Tsirpouchtsidis, A., Hurwitz, R., Brinkmann, V., Meyer, T.F., and Haas, G. (2002) Neisserial immunoglobulin A1 protease induces specific T-cell responses in humans. *Infect Immun* **70**: 335-344.

Turner, C.F., Rogers, S.M., Miller, H.G., Miller, W.C., Gribble, J.N., Leone, P.A., Cooley, P.C., Quinn, T.C., and Zenilman, J.M. (2002) Untreated Gonococcal and Chlamydial Infection in a Probability Sample of Adults. *Jama* **287**: 726-733.

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

Veiga, E., Sugawara, E., Nikaido, H., de Lorenzo, V., and Fernandez, L.A. (2002) Export of autotransported proteins proceeds through an oligomeric ring shaped by C-terminal domains. *Embo J* **21**: 2122-2131.

Viitala, J., Carlsson, S.R., Siebert, P.D., and Fukuda, M. (1988) Molecular cloning of cDNAs encoding lamp A, a human lysosomal membrane glycoprotein with apparent Mr approximately equal to 120,000. *Proc Natl Acad Sci U S A* **85**: 3743-3747.

Vitovski, S., Read, R.C., and Sayers, J.R. (1999) Invasive isolates of Neisseria meningitidis possess enhanced immunoglobulin A1 protease activity compared to colonizing strains. *Faseb J* **13**: 331-337.

Vitovski, S., Dunkin, K.T., Howard, A.J., and Sayers, J.R. (2002) Nontypeable Haemophilus influenzae in Carriage and Disease: A Difference in IgA1 Protease Activity Levels. *Jama* **287**: 1699-1705.

von Figura, K., and Hasilik, A. (1986) Lysosomal enzymes and their receptors. *Annu Rev Biochem* **55**: 167-193.

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

Warren, R.A., Green, F.A., Stenberg, P.E., and Enns, C.A. (1998) Distinct saturable pathways for the endocytosis of different tyrosine motifs. *J Biol Chem* **273**: 17056-17063.

Weel, J.F., and van Putten, J.P. (1991) Fate of the major outer membrane protein P.IA in early and late events of gonococcal infection of epithelial cells. *Res Microbiol* **142**: 985-993.

Weir, S.S., Feldblum, P.J., Roddy, R.E., and Zekeng, L. (1994) Gonorrhea as a risk factor for HIV acquisition [see comments]. *Aids* **8**: 1605-1608.

Williams, M.A., and Fukuda, M. (1990) Accumulation of membrane glycoproteins in lysosomes requires a tyrosine residue at a particular position in the cytoplasmic tail. *J Cell Biol* **111**: 955-966.

Xu, T., Binz, T., Niemann, H., and Neher, E. (1998) Multiple kinetics components of exocytosis distinguished by neurotoxin sensitivity. *Nature Neurosci* **1**: 192-200.