PG0553: A ROLE IN POR SECRETION AND THE PROCESSING OF GINGIPAINS IN
PORPHYROMONAS GINGIVALIS STRAIN W83

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2013
To Jewel and Anabell, for their constant comfort, reassurances and fuzziness
ACKNOWLEDGMENTS

I would like to thank my graduate committee for their support and guidance, especially my mentor for helping me get this far. I would like to thank my lab mates for always having my back. I would like to thank my friends and family for their emotional support and encouragement. I would like to thank my boyfriend for being awesome. I would like to thank my cats for keeping my lap warm through many late nights sitting in front of my computer, and I would like to thank Swamphead Brewery for making my time in Gainesville filled with good beer.
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A-LPS The lipid A moiety of LPS: highly conserved in gram negative bacteria, anchors LPS to the cell wall

APOE Apolipoprotein E: lipoprotein that transports lipids in cardiovascular tissues and mediates cholesterol metabolism. The ApoE-/- mouse is prone to atherosclerosis and is used as model for studying CVD. Porphyromonas gingivalis strain W83; an oral pathogenic bacterium that is a major contributor to periodontal disease.

CPG70 Metalloprotease associated with gingipain processing in Pg (PG0232)

CTD C-terminal Domain: a conserved, 80 amino acid long C-terminal tail found in a family of proteins associated with the Por secretion system

CVD Cardiovascular Disease: A class of diseases that affect cardiovascular tissues such as the heart and arteries.

HCAEC Human coronary artery endothelial cells; primary human cells obtained from patient tissue and cultured under laboratory conditions. These cells are the primary in vitro model used in this dissertation to explore the relationship between Pg and human cardiovascular disease.

HUVEC Human Umbilical Vein Endothelial Cells: primary human cells obtained from patient tissue and cultured under laboratory conditions.

KGP Lys- gingipain

LPS Lipopolysaccharide: major component of the bacterial cell wall in gram-negative bacteria

PORSS Por secretion system: a novel secretion system responsible for secretion of major extracellular proteinases in Pg.

PD Periodontal Disease: A class of inflammatory diseases that affect oral tissues and the bone that supports the teeth.

RA Rheumatoid Arthritis: A systemic, inflammatory disorder that principally affects synovial joints.

RGP A/RGP B Arg- gingipain
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<td>Human osteosarcoma cells; immortalized human cells used as a model system for studying the activation of autophagy in host cells by <em>Pg</em>.</td>
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<td>TLR2</td>
<td>Toll-like Receptor 2: Immune cell receptor that recognizes conserved, pathogen-associated molecular patterns</td>
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<td>W83Δ0553</td>
<td>Mutant strain of <em>Pg</em> W83 in which the gene <em>PG0553</em> has been excised and replaced with the <em>ermF ermAM</em> antibiotic resistance cassette; this is the experimental strain being characterized in this dissertation.</td>
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Porphyromonas gingivalis (Pg) is a periodontal pathogen that has been detected in atherosclerotic plaque and has been shown to invade and persist within human coronary artery endothelial cells (HCAEC) in vitro. Microarray analysis was used to identify genes differentially regulated by Pg during invasion of HCAEC. One such gene, PG0553, was found to be upregulated at multiple time points during invasion. PG0553 belongs to a family of proteins containing a C-terminal domain sequence (CTD) associated with por-mediated secretion (1) and is currently thought to play an important role in the processing of other CTD proteins. The por secretion pathway is still poorly understood, but it is essential for the secretion of gingipains in Pg. The gingipains are CTD family proteins that are important both for nutrition of the bacterium and its interactions with host systems. A disruption in gingipain processing or secretion would have significant consequences for Pg virulence and survival.

A deletion mutant was constructed in Pg strain W83 by allelic replacement of gene PG0553 with an ermF/ermAM antibiotic resistance cassette. This W83Δ0553 mutant exhibits altered protease activity in the soluble intracellular fraction, including a reduction in measurable gingipain activity. In addition, knocking out PG0553 was
sufficient to significantly impact adherence to host cells, host cell invasion and activation of host autophagy. The mutant phenotype is consistent with PG0553 interacting with key effectors of gingipain processing, potentially in conjunction with the por secretion system.
CHAPTER 1
PORPHYROMONAS GINGIVALIS AND HUMAN DISEASE

Introduction

Systemic disease progression in humans is an increasingly complicated picture. The more that is understood about host-pathogen interactions and the long term effects of infection, the less likely a direct ‘cause and effect’ relationship becomes. For example, a localized infection can trigger chronic inflammation, contributing to disease pathology in unexpected ways. Several pathogens, such as Chlamydia pneumoniae (2), Helicobacter pylori (3), and Cytomegalovirus (4) are associated with a systemic inflammatory response. New evidence is continually surfacing that links a systemic inflammatory response, resulting in cumulative damage, to the development of “chronic” diseases such as diabetes, rheumatoid arthritis and cardiovascular disease.

Periodontitis (PD) is a chronic inflammatory disease that is generally considered to be the second most common infection in the world. Epidemiological studies support a connection between PD and several systemic diseases, including cardiovascular disease (5) and diabetes mellitus (6, 7). The severity of PD also correlates with increased risk for coronary heart disease (8) and poor glycemic control in some patients (9). Severe instances of PD are strongly associated with colonization by specific oral bacteria, including Treponema denticola, Tannerella forsythia and Porphyromonas gingivalis (10, 11).

Pg primarily colonizes the subgingival crevice; where its ability to adhere tightly to surfaces and invade host cells allows it to persist even as the array of proteases produced by Pg damage the surrounding tissues. Oral microbes and their byproducts have been demonstrated to gain systemic access to the circulatory system through
dental procedures (12), toothbrushing (12) and the simple act of chewing (13). The transient bacteremia associated with periodontitis can induce endotoxemia with a severity that directly correlates to the degree of periodontal disease (13). Because of their ability to gain systemic access to the circulatory system, oral pathogens have the potential to directly influence subclinical mediators of systemic disease. Mounting evidence from a vast array of clinical and laboratory research indicates that some of the same virulence factors that make *Pg* a potent agent of periodontal disease may contribute to the progression of various other human diseases as well.

**Virulence Mechanisms of *Pg***

The human oral microbiome is incredibly diverse, with recent estimates suggesting as many as 25,000 different phylotypes reside in the mouth (14). The accumulation of oral plaque biofilm, the primary etiology of periodontal disease, is a complex process that involves several species of bacteria in a symbiotic relationship. Aerobic bacteria such as *Streptococci* stabilize oxygen levels, making it possible for anaerobes to colonize the maturing biofilm. *Pg* is a late-comer in this process, and is known to trigger the transition to an inflammatory disease state even at low levels (15). Other periodontopathogens associated with disease biofilm have been shown to interact synergistically with *Pg*, amplifying tissue damage and the inflammatory response. For example, in a murine lesion model, mixed infections consisting of *Pg* and *Fusobacterium nucleatum* (16) and *Pg* and *Bacteroides forsythus* (17) resulted in significantly higher rates of tissue damage than monoinfections in the subcutaneous lesions. This synergistic effect was dependent on gingipain activity in both studies; one of the many virulence mechanisms employed by *Pg* to maintain a disease state that may also facilitate systemic spread.
*Pg* expresses a wide range of virulence factors that facilitate adherence, host tissue destruction, evasion of host immunity and cellular invasion. As a non-motile organism fighting against the laminar flow of sub-gingival fluid in the oral cavity, *Pg* must strongly adhere to the epithelial surface of the mouth. This is accomplished through the production of fimbriae, adhesive proteins strands that adhere to both host cells and other bacteria (18), as well as hemagglutinins which facilitate direct adherence to host cells (19). Research also suggests that *Pg* uses hemagglutinins to adhere to erythrocytes as a means of circulating through the bloodstream (20), providing access to a wide range of tissues.

*Pg* is asaccharolytic and relies on amino acids as its primary source of carbon. Gingipains are necessary for housekeeping functions such as the uptake of amino acids and maturation of fimbriae, however these cysteine proteinases also target host proteins such as collagen, fibrin/fibrinogen, human immunoglobulin and cell surface receptors (21, 22). An array of proteases, including the gingipains, breaks down environmental proteins and facilitates the acquisition of iron in the form of heme (23). Iron is an absolute requirement for anaerobic respiration in *Pg*, making iron acquisition critical to survival in the host (24). *Pg* has been shown to lyse erythrocytes through gingipain activity (25), an efficient method for acquiring the heme *Pg* requires for growth. However, the release of cytoplasmic components into the bloodstream is highly inflammatory and contributes to tissue destruction and vascular disruption (26).

*Pg* has evolved mechanisms to take advantage of the increased accessibility to essential nutrients in the subgingival crevice provided by the host inflammatory response while evading clearance by immune mechanisms (27). *Pg* expresses
proteases that degrade immunoglobulins and complement, deregulating the immune response while generating digestible peptides. In addition, *Pg* capsular polysaccharide, or K-antigen, has been shown to inhibit the production of inflammatory cytokines to varying degrees, depending on the components of the capsule (28). A distinct anionic polysaccharide molecule produced by *Pg*, characterized as a type of LPS, has also been shown to inhibit complement deposition (29, 30). These evasive strategies are highly effective in non-oral environments and cell types as well.

Cellular invasion is another mechanism by which bacteria can evade host immunologic defenses. *Pg* has been shown to be capable of invading several human cell types *in vitro*, including umbilical vein endothelial cells (HUVEC) (31), macrophages (32), trophoblasts (33) and coronary artery endothelial cells (HCAEC) (34). Through interactions between the fimbriae and surface integrins (35), *Pg* has been shown to be able to induce cytoskeletal rearrangement and internalization in epithelial cells (36). While the process through which *Pg* invades HCAECs is still being characterized, *Pg* has been shown to both activate autophagy (37) and inhibit apoptosis (38) within the HCAECs, greatly disrupting the life cycle of the host cells and creating a specialized niche for its replication. This cycle of inflammation and immune evasion is a significant mechanism by which a localized *Pg* infection could have systemic effects, potentially contributing to a disease state.

**Pg and Periodontal Disease**

The bacteria of the oral cavity present a constant challenge to the mucosa. If left unchecked, the accumulation of pathogenic organisms and bacterial metabolic waste products leads to leukocyte infiltration, tissue damage and loss of bone supporting the teeth. The majority of periodontal diseases consist of plaque-induced inflammatory
lesions in the gingival tissue, with *Porphyromonas* species being one of the major constituents of the plaque biofilm. *Pg* is strongly associated with adult periodontitis, with elevated levels of the organism found in periodontal lesions and low levels in healthy sites. The interactions between *Pg* and the oral mucosa and the relationship between *Pg* and periodontal disease progression have been thoroughly studied and reviewed elsewhere (39-41); however, a chronic *Pg* infection in the oral cavity has potential systemic repercussions as well. A chronic, localized infection is associated with both lymphocyte migration (42) and the secretion of inflammatory cytokines by the host (43), which may lead to tissue damage. This inflammation is exacerbated by the proteolytic activity of the gingipains, which break down the fibrin and fibrinogen that would normally promote blood coagulation and wound healing at the damaged site (41). The oral cavity thus provides a route for *Pg*, and the virulence factors *Pg* produces, to be transmitted hematogenously via oral mucosal lesions.

**Pg and Cardiovascular Disease**

Several epidemiological studies have demonstrated an association between periodontal disease and cardiovascular disease, with periodontitis patients found to have a 25% increased risk of coronary heart disease compared to those with minimal periodontal disease (8). The connection between the severity of periodontal disease and risk for coronary heart disease was further established by an independent cross-sectional study involving over 5,500 patients (5). Dental health status has also been linked with the risk for myocardial infarction (44, 45) and coronary atheromatosis (46). Significant controversy exists in regards to whether or not this association is causative and how it should impact patient care (47). Several important risk factors are associated with both PD and CVD, including: smoking, obesity, age, ethnicity and diabetes mellitus.
Some studies suggest that this overlap generates a confounding effect that may partially account for the observed association between PD and CVD (50, 51). The potential for these variables to confound an observational study cannot always be completely eliminated; however several studies involving never-smokers found that the association between PD and CVD was independent of smoking history (52-54). Because smoking is a powerful predictor of both PD and CVD, these studies strongly support the conclusion that the association between these diseases is independent of this potential confounding effect.

CVD is a complex disease with multiple contributing factors that develops over a lifetime, making conclusive etiological assessment difficult in short-term observational studies. While improvements in endothelial cell function and reductions in inflammatory markers have been reported by several clinical studies in response to periodontal intervention (55-57), it has not been established that periodontal therapy reduces the risk of CVD. Patients have also demonstrated a significant increase in circulating inflammatory mediators, including TNF- and IL-6, immediately following periodontal procedures (58, 59). The risk for endothelial deterioration (57) and vascular events (60) also increases significantly following invasive dental procedures. While not conclusive, these studies strongly suggest that factors associated with the oral cavity contribute to cardiovascular events.

Despite the lack of an etiological proof, an accumulation of experimental evidence suggests that some periodontopathogenic bacterial species may be involved in cardiovascular disease progression. Genomic DNA from periodontal pathogens such as \textit{Pg} and \textit{Aggregatibacter actinomycetemcomitans} (Aa) has been detected by Q-PCR
and immunofluorescent microscopy in atheromas dissected from vascular tissues as well as atherosclerotic plaques (61). Confirming the viability of \textit{Pg} associated with atherosclerotic tissue has proven problematic. \textit{Pg} has been shown experimentally to persist within various cell types and tissues, both \textit{in vivo} and \textit{in vitro}, only to prove unculturable or recoverable at surprisingly low levels following exposure to the host environment (62, 63). One study found that plaque isolates testing positive for \textit{Pg} by Q-PCR were unculturable, but was able to confirm the presence of viable \textit{Pg} by demonstrating that the bacteria were transmissible from plaque tissue to uninfected host cells (64). The recovery of viable \textit{Pg} from human atherosclerotic lesions makes a compelling argument for a link between cardiovascular disease and periodontal pathogens, but additional research is needed to verify these results. Thus, though not conclusively proven, \textit{Pg} is strongly associated with diseased tissue and may also be a contributor to the development of atherosclerosis.

Atherosclerosis, the underlying cause of many forms of cardiovascular disease, has often advanced to dangerous levels by the time a patient is diagnosed. Vascular injury has been shown to accumulate from childhood (65), and remains undiagnosed in two out of three cases of cardiac death. The difficulties associated with diagnosis make identification of risk factors and prevention, especially early in life, crucial tools in the battle against heart disease. Epidemiological studies have led to the development of an infectious theory of atherosclerosis. This hypothesis suggests that a localized infection can trigger a chronic inflammatory response, contributing to atherosclerosis through the accumulation of subclinical mediators of cardiovascular disease.
It has been established that systemic inflammation is predictive of cardiovascular events (66, 67). Inflammatory markers are often used as tools to assess potential risk and vascular health in patients (68, 69). For example, increased levels of C-reactive protein in patient serum are an indicator of future risk of myocardial infarction and stroke (70). This predictive relationship strongly supports the potential for a chronic inflammatory response to contribute to atherosclerosis. The oral cavity is a significant source of inflammation, with clinical studies supporting a direct link between specific periodontal pathogens, such as \( Pg \) and \( Treponema denticola \), and markers of subclinical atherosclerosis in humans (71).

Another hypothesis addressing the connection between chronic infections and atherosclerosis suggests that cross-reactivity between bacterial and host antigens is to blame for systemic inflammation. For example, the common bacterial chaperonin, GroEL, is strongly homologous to the human heat shock protein 60 (hHSP60). Anti-GroEL and anti-\( Pg \) antibodies from atherosclerosis patients have been shown to cross-react with hHSP60 (72), which is expressed by coronary artery endothelial cells. Arterial tissues could therefore be susceptible to inflammatory damage as part of the immune response against \( Pg \). This theory is supported by the detection of GroEL, hHSP60 and periodontopathogens in patient carotid endarterectomy samples; 52% of which were found to contain \( Pg \) specifically (73). The anti-hHSP60 antibody response was also significantly reduced in patients at risk for cardiovascular disease when periodontal health was improved (74). The molecular mimicry hypothesis is currently unable to conclude that periodontopathogens specifically are the source of the initial immune response, but rather suggests that cross-reactivity to bacterial antigens maintains
inflammation in arterial tissues in a manner that correlates with the presence of periodontopathogenic species such as \textit{Pg}.

The development of atherosclerotic plaque is primarily associated with the accumulation of foam cells in the vasculature, and foam cell formation is significantly enhanced in \textit{Pg}-infected macrophages (75, 76). \textit{Pg} may be activating foam cell formation and persisting, either within atherosclerotic plaque or potentially within the foam cells that comprise plaque tissue. More research is necessary to convincingly demonstrate the persistence and viability of internalized \textit{Pg} and to determine the effects of persistence on cardiovascular disease development.

Animal studies demonstrating the contribution of \textit{Pg} to the progression of CVD have been performed using various animal models and modes of infection. The ability of \textit{Pg} to induce or accelerate cardiovascular events is well-documented in the ApoE\textsuperscript{-/-} mouse, a common model for studying atherosclerosis. In this model system, apolipoprotein E is disrupted, leading to the spontaneous formation of atherosclerotic lesions. Intravenous injection with \textit{Pg} dramatically accelerated atherosclerosis in ApoE\textsuperscript{-/-} mice (77) and induced myocarditis in C57BL-6 mice (78), demonstrating the potential of \textit{Pg} or \textit{Pg}-associated components to contribute to cardiovascular events. Atherosclerotic lesion development in the ApoE null mouse has also been correlated with anti-GroEL antibody levels, strongly supporting the molecular mimicry hypothesis of atherosclerosis (79). Inoculation with heat-killed \textit{Pg} is also protective against plaque accumulation in ApoE\textsuperscript{-/-} mice (80), convincingly demonstrating a direct relationship between \textit{Pg} and a specific symptom of atherosclerosis.
Periodontitis induced by oral inoculation with \textit{Pg} in the ApoE\textsuperscript{−/−} mouse resulted in detectable \textit{Pg} DNA localized to the aortic tissue of infected mice, demonstrating that an oral infection can be transmitted systemically in the ApoE\textsuperscript{−/−} mouse model (81). Accelerated early atherosclerosis was also observed as a result of \textit{Pg} oral inoculation in ApoE null mice (81), while lipid accumulation in the aorta was found to be significantly increased in rabbits experiencing \textit{Pg}-induced periodontitis (82). An infection in the oral cavity can thus affect distant sites in a manner that potentially contributes to atherosclerotic development, showing a specific relationship between oral inoculation with \textit{Pg} and manifestations of CVD. A potential mechanism for this relationship has also been described in a TLR2-deficient ApoE\textsuperscript{−/−} mouse model. Oral infection with \textit{Pg} increased levels of inflammatory mediators detected in serum, an effect that was significantly reduced in the absence of TLR2 (83). Thus innate immune recognition of \textit{Pg} via TLR2 may be a major contributing factor to the heightened inflammatory state associated with PD and the development of CVD.

Endothelial cell function is considered to be a metric of arterial health and is often measured by detecting biomarkers of inflammation, coagulation and endothelial cell activation. Using these metrics, an association was established between oral health and improved endothelial function (57). Altered endothelial function or disregulation can promote inflammation and hypertension, both significant risk factors for the development of CVD. Exposure to gingipains from \textit{Pg} is known to negatively impact endothelial cell adhesion, leading to cell detachment and apoptosis (84). Thus, gingipain activity could contribute to the formation of arterial lesions and the activation of the inflammatory response through this mechanism.
*Pg* is also known to invade and persist within HCAECs *in vitro* (34), dramatically altering cellular functions in the process. As part of the invasion process, *Pg* activates autophagy (37) in human endothelial cells and trafficks to autophagosomes (85), forming a protected niche for replication while significantly disregulating the host cell. *Pg* has also been shown to inhibit apoptosis (38) by up-regulating Bcl-2 in infected gingival epithelial cells, despite causing significant DNA damage to the host (86). Additional research is required to determine the extent to which invasion by *Pg* negatively impacts endothelial cell function and how that dysfunction may predispose patients to hypertension and CVD.

In humans, there is significant evidence demonstrating a relationship between *Pg* infection and specific cardiovascular events. During stages of bacteremia *Pg* induces platelet aggregation (87) and hypercoagulation (88), significantly increasing the likelihood of blood clot formation and further complications such as a myocardial infarction or heart attack. Taken together with the epidemiological studies and *in vivo* animal data, the available evidence strongly supports a direct connection between infection with *Pg* and the development of atherosclerosis in humans. However, more research is necessary to confirm this hypothesis in humans.

**Pg and Adverse Pregnancy Outcomes**

Bacteria infecting placental tissues can cause cellular damage and disrupt the unique immune-privileged status of the fetus, leading to an inflammatory condition known as chorioamnionitis. This condition is associated with pre-term delivery and may cause bacteremia in the mother and infection in the newborn. A connection between periodontal disease and pre-term delivery and low birth weight has not yet been firmly established due to conflicting epidemiological studies. Clinical studies examining the
role of oral care (89) and humoral immunity (90) against periodontal pathogens have found no associations based on treatment beginning in the second trimester. However, these studies do not account for the potential of *Pg* and other periodontal pathogens to have an impact during the earlier stages of pregnancy.

Bacterial infection of the genital tract is present in 40-50% of pre-term births (91). The systemic bacteremia associated with periodontal disease provides *Pg* with access to placental tissues, and it has been demonstrated that *Pg* is capable of invading and persisting within HUVECs *in vitro* (31). The ability of *Pg* to invade placental tissues and contribute to chorioamnionitis development has been demonstrated in a pregnant rat model, with *Pg* detected at varying levels depending on which strain of *Pg* was used (92). The presence of *Pg* antigens in preterm placental tissues taken from chorioamnionitis patients has also been confirmed by immunocytochemistry (93), reflecting the experimental results from the rat model.

Pre-eclampsia is a hypertensive disorder that can develop during pregnancy. It is characterized by hypertension and proteinuria during the second trimester and is potentially life-threatening to the mother. Though the condition has no single causative agent, recent evidence suggests an association with periodontal disease. A recent epidemiological study found a significant association between chronic periodontitis and pre-eclampsia in pregnant women (94) and periodontal pathogens have been detected in the placenta of women experiencing hypertension during pregnancy (95). DNA from several periodontal pathogens, including *Pg*, was also detectable by Q-PCR in placental samples from pre-eclampsia patients at significantly higher levels than healthy controls (96). During pre-eclampsia, acute atherotic lesions form on the placenta that are very
similar pathologically to atherosclerotic lesions. The causative agents of pre-eclampsia are still unknown, but there are several parallels to the association between periodontitis and cardiovascular disease. *Pg* is not currently considered a risk factor for pre-eclampsia, but the available data warrant further investigation.

Intrauterine growth restriction (IUGR) is a significant reduction in fetal growth that can be caused by pre-eclampsia as well as other placenta-related complications. This condition sharply increases the risk to the developing fetus as well as complications during delivery. Epidemiological studies demonstrate a connection between humoral immunity to *Pg* in early pregnancy and IUGR as well as pre-term birth, with patients experiencing complications related to IUGR showing significantly lower levels of *Pg* antibodies in serum (97). In a pregnant mouse model, the development of growth-restricted fetuses was associated with the spread of *Pg* to non-oral tissues and elevated levels of Il-10 and TNF-alpha in the mother (98). These results suggest that exposure to *Pg* during pregnancy may contribute significantly to complications such as IUGR, while an effective immune response against *Pg* may be protective.

**Pg and Rheumatoid Arthritis**

Rheumatoid arthritis (RA) is a systemic inflammatory disorder that primarily targets synovial joints. The specific cause of RA is currently unknown, but it is considered a systemic autoimmune disease. It can take years for an abnormal immune response to fully develop, making it difficult to identify other hallmarks and auto-antigens.

Currently, anti-citrullinated protein, or ACP, is one of the most promising auto-antigens in the study and diagnosis of RA (99). Citrullination is the post-translational
modification of arginine into citrulline, as mediated by peptidylarginine deiminases (PADs). This conversion increases the hydrophobicity of the target protein, potentially leading to significant alterations in protein folding. Several proteins, including fillagrin and many histones, are citrullinated normally. However, proteins such as fibrin, fibrinogen and vimentin can become citrullinated during tissue inflammation and cell death, potentially leading to the development of auto-antibodies against these key mediators of joint and tissue integrity. Interestingly, \textit{Pg} is the only human pathogen known to produce PADs that are capable of citrullinating proteins (100). Cross-reactivity has also been observed between antibodies against human citrullinated \(\alpha\)-enolase and citrullinated \textit{P. gingivalis} enolase (101). The potential for infection with \textit{Pg} to impact the levels of self-reactive antibodies against citrullinated proteins has not yet been investigated; however it represents a potential mechanism for \textit{Pg} to contribute directly to RA development.

There is significant evidence supporting a link between periodontitis and rheumatoid arthritis. Patients with RA are twice as likely to have periodontal disease (102), and both conditions are characterized by chronic inflammation and bone and tissue damage. \textit{Pg} is directly associated with periodontal disease and patients with RA are significantly more likely to test positive for specific antibodies against \textit{Pg} in serum (103). DNA from periodontal pathogens, including \textit{Pg}, has also been detected in the synovial fluid of RA patients (104). RA disease progression is complex and is likely due to numerous contributing factors, such as genetic predisposition and abnormal immune cell interactions, but the potential exists for \textit{Pg} to influence RA development. More
research is needed to understand how various influences, including infection with \textit{Pg}, can lead to the development of RA.

\textbf{Pg and Diabetes Mellitus}

Diabetes mellitus is a complex group of metabolic diseases characterized by hyperglycemia. There are three major classifications of diabetes, which describe the different contexts in which hyperglycemia can develop. Type 1 diabetes develops as a result of auto-immune targeting of pancreatic islet cells, resulting in a lack of insulin production. In type 2 diabetes fat and muscle cells develop insulin resistance, resulting in relative insulin deficiency. Gestational diabetes is specific to pregnant women. In some pregnancies, the body of the pregnant woman does not secrete enough insulin to compensate for the additional requirements of pregnancy, resulting in hyperglycemia. Diabetes mellitus is diagnosed when hyperglycemia becomes chronic, regardless of the instigating factors. Because hyperglycemia is the common factor between these vastly different disease classifications, the relationship between diabetes and \textit{Pg} will be presented here in the context of hyperglycemia.

Chronic hyperglycemia is recognized as a risk factor for periodontitis. This connection is well-established and has been thoroughly reviewed (6, 7). Hyperglycemic patients experience a 2-3 fold higher incidence rate for periodontal disease and are known to experience severe periodontitis, accompanied by acute alveolar bone loss. The most likely mechanism for this association is reduced regulation of the immune response in hyperglycemic patients (105). In chronic hyperglycemia, immune cells demonstrate a hyperinflammatory phenotype that is predisposed to chronic inflammation. This amplifies the systemic inflammation associated with PD, impeding bone formation and tissue repair. Cytokines such as IL-1 and TNF-alpha that are
released in association with periodontitis-induced bacteremia have also been shown to exacerbate insulin resistance, with improved oral care generating improved glycemic control in some patients (9).

The relationship between hyperglycemia and *Pg* specifically is not well-characterized. *Pg* is detectable at higher levels than other oral pathogens in hyperglycemic patients suffering from periodontitis (106, 107), but there is no significant difference observed in the oral microbiota of hyperglycemic patients compared to normoglycemic patients (108). These results suggest that the increased levels of *Pg* are associated with PD rather than chronic hyperglycemia. However, *Pg* is known to secrete inflammatory factors, such as LPS and TNF-alpha, which may contribute to the immune disregulation seen in hyperglycemic patients. When inoculated with *Pg*, NOD mice (a standard model for studying diabetes) showed reduced levels of inflammatory cytokines as well as better glycemic control (109) when treated with an anti-TNF-alpha antibody. Hyperglycemic mouse macrophages stimulated with *Pg* LPS were also characterized as immunocompromised (110), but this abnormal differentiation was found to be dependent on glycemic levels rather than *Pg* LPS specifically. While these studies certainly demonstrate the importance of immune regulation, they do not specifically associate *Pg* with a hyperglycemic outcome.

One epidemiological study did find that the persistence of certain strains of *Pg*, specifically those expressing Type II fimbriae, affected glycemic levels in patients (111). Patients without Type II-fimbriated strains showed improved glycemic control. However, current research suggests that the most significant role for *Pg* in the development or
exacerbation of chronic hyperglycemia is as a primary etiological agent of periodontal disease.

**Discussion**

The systemic inflammation and bacteremia associated with periodontal disease clearly has the potential to impact non-oral tissues in many ways, including the hematogenous spread of periodontopathogens. *Pg*, a major etiological agent of periodontal disease, contributes to the systemic impact of PD. *Pg*-related virulence factors and interactions with a wide range of cells and tissue-types demonstrate that *Pg* may be a specific contributor to systemic disease progression (Table 1-1) as well.

However, in the case of *Pg*, not all strains are created equal. Significant variations in capsular (112) and fimbrial (113) serotypes have been observed, and the *Pg* strains that have been characterized experimentally vary considerably in their virulence characteristics (Table 1-2). The most invasive strains, for example, tend to express K¹ capsular polysaccharide in conjunction with Type 2 or Type 4 fimbriae. The potential exists for different strains and serotypes to affect disease progression in different ways, as observed with type-II fimbriated strains of *Pg* and glycemic control (111) and the varying degrees to which capsular polysaccharides are able to modulate host inflammatory responses (102). Colonization of placental tissue in the rat pregnancy model was also observed to be strain-dependent, with strain A7436 detected in 100% of inoculated animals and strain W83 detected in only 33% (92). This strongly suggests that strain A7436 is more likely than strain W83 to play a role in diseases associated with placental tissues, such as pre-eclampsia.

The potential for a strain-dependent relationship between *Pg* and disease progression may represent a significant factor in experimental analysis. This is
especially relevant in human disease studies, which often specifically determine the presence or absence (but not the specific strain) of *Pg* in human tissues associated with disease. Methods for distinguishing between *Pg* genotypes (114-116) are available and could be employed to gain a clearer picture of the path from oral infection to systemic disease. Future studies examining the role of *Pg* in human disease would be well-served to address strain-specific virulence factors. If a pattern is established between specific strains of *Pg* and disease progression in humans, a simple oral swab and genotyping procedure could contribute significantly to establishing a patient's risk based on the strain of *Pg* present in the oral cavity.

The development of systemic disease is a complex process, often with no single instigating factor. Infection with *Pg* may be a condition that predisposes patients to diseases like rheumatoid arthritis and cardiovascular disease. Identifying the presence of *Pg*, *Pg*-specific virulence factors and *Pg*-specific antibodies could be useful, in combination with other known risk factors, for determining overall patient risk for developing a particular systemic disease. There are currently no cures for RA, CVD or diabetes, making prevention and risk awareness crucial aspects of treatment of these diseases. Improving our understanding of the relationship between *Pg* infection, with recognition of the potential significance of strain, and systemic disease could make that process more effective and is worthy of further investigation. Much work remains to be done to conclusively prove these disease relationships and translate them into patient care, but it is clear from the present data that *Pg* expresses a number of virulence factors that have the potential to impact human systemic disease progression.
<table>
<thead>
<tr>
<th>Systemic Disease</th>
<th>Epidemiology</th>
<th>Animal (\textit{in vivo})</th>
<th>Human (\textit{in vitro})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular Disease</td>
<td>Strong link between PD and CVD</td>
<td>Inoculation induces/accelerates cardiovascular events; heat-killed cells protective against plaque formation</td>
<td>(P_g) bacteremia (\rightarrow) Platelet aggregation; Induces foam cell formation; isolated from atherosclerotic plaque</td>
</tr>
<tr>
<td>Chorioamnionitis</td>
<td>Conflicting associations with PD, established connection with bacterial infection</td>
<td>Contributes to disease development in rats</td>
<td>Invades HUVECs; antigens to (P_g) detected in preterm placental tissues</td>
</tr>
<tr>
<td>Pre-eclampsia</td>
<td>Connection between chronic PD and pre-eclampsia</td>
<td></td>
<td>(P_g) DNA detected in atherotic placental lesions</td>
</tr>
<tr>
<td>Rheumatoid Arthritis</td>
<td>RA patients more likely to have PD; test positive for (P_g) antibodies</td>
<td></td>
<td>(P_g) DNA detected in synovial fluid – produces PADs</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Strong, but not causative, link between PD and diabetes</td>
<td>Anti-TNF-alpha antibodies improved glycemic control/(P_g) clearance; development of immunocompromised macrophages in mice</td>
<td>Persistence of type II fimbriated strains affects glycemic control in patients</td>
</tr>
<tr>
<td></td>
<td><em>Pg W83</em></td>
<td><em>Pg 33277</em></td>
<td><em>Pg W50</em></td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------------------</td>
<td>--------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Capsular Polysaccharide (117)</td>
<td>K&lt;sup&gt;+&lt;/sup&gt; serotype</td>
<td>K&lt;sup&gt;-&lt;/sup&gt; serotype</td>
<td>K&lt;sup&gt;+&lt;/sup&gt; serotype</td>
</tr>
<tr>
<td>Fimbrial Serotype (118, 119)</td>
<td>Type 4 (Fimbrial deficient)</td>
<td>Type 1</td>
<td>Type 4 (Fimbrial deficient)</td>
</tr>
<tr>
<td>Evades phagocytosis and opsonization (120, 121)</td>
<td>Highly resistant, digests C3</td>
<td>Less resistant</td>
<td>Highly resistant</td>
</tr>
<tr>
<td>Tissue Invasiveness in Mouse Model (122, 123)</td>
<td>Invasive, septicemia and death</td>
<td>Non-invasive, localized abscess</td>
<td>Invasive, septicemia and death</td>
</tr>
</tbody>
</table>
CHAPTER 2
PROTEASE SECRETION IN PORPHYROMONAS GINGIVALIS

Protein Secretion in Gram-Negative Bacteria

The ability of gram-negative bacteria to translocate proteins across the inner and outer membranes is critical for virulence and pathogenicity. Toxins, proteases and other virulence factors must be secreted in order to alter the host environment. This process generally favors the pathogen and promotes survival and virulence. Accordingly, significant resources are dedicated to the formation of translocase machinery that facilitates protein transport and secretion. Six conserved secretion systems have currently been identified and characterized in gram-negative bacteria (types I-VI).

These fall into two main categories: one-step systems (types I, III, IV, and VI) and two-step systems (types II and V). In a one-step system, the transport complex spans both the inner and outer membranes, allowing effectors to be secreted directly from the bacterial cytoplasm into the extracellular milieu or the cytoplasm of host cells. A two-step system works in conjunction with either the Sec or Tat pathways, which transport proteins that contain specific signal peptides across the inner membrane and into the periplasm. Once in the periplasm, a second transport step occurs across the outer membrane through the specific secretion machinery.

Protease Secretion in Pg

The proteins that form these translocase nanomachines are highly conserved among species. Interestingly, while Pg is known to secrete an array of proteases and other virulence factors, the Pg genome does not contain homologues to any of the currently characterized secretion systems in gram-negative bacteria. However a novel two-step secretion system, known as the por secretion system (PorSS), has recently
been identified in *Pg*. The PorSS complex is composed of approximately 12 proteins: PorK, PorL, PorM, PorN, PorO, PorP, PorQ, PorT, PorU, PorV (PG27, LptO), PorW and Sov (124). While a majority of this gene cluster is unique, *Flavobacterium johnsoniae* contains six gliding motility proteins that are orthologous to components of PorSS (124). PorT in particular also has orthologs in several *Bacteroidetes* species, including *Tannellera forsythia* and *Prevotella intermedia* (125). However the PorSS complex, and its function as a secretion system, is novel and unique to *Pg*.

The mechanism of gingipain secretion in *Pg* is a matter of great interest to researchers because of the major role gingipains play in virulence. Gingipain peptides are known to be secreted into the periplasm via the Sec pathway (126), but the method of secretion across the outer membrane was unknown until 2010 when the por secretion system was first described (124). The PorSS complex consists of 12 proteins, but their functions are only partly understood. PorT, PorV (PG27), and Sov are necessary for PorSS-associated secretion to occur, with the periplasmic accumulation of gingipain peptides and reduction in extracellular gingipain activity observed if any of them are disrupted (127-129).

PorT is an integral transmembrane protein localized to the outer membrane (130), but its specific role in PorSS is unknown. PG27 is predicted to form a β-barrel with its C-terminus localized to the periplasm (131), strongly suggesting a role in transport. This is supported by an association between PG27 and the deacylation of LPS in *Pg*, linking PG27 with both the secretion and cell-surface attachment of A-LPS and CTD proteins (132). Sov has been characterized as an outer membrane protein with its C-terminal region exposed to the extracellular milieu and is thought to play a role
in regulating PorSS-associated activity in response to environmental signals (133). PorSS gene expression is regulated by PorX and PorY, a response regulator and a histidine kinase respectively that are thought to form a signal transduction system that is regulated by Sov (124, 125).

Even less is known about PorSS components PorK, PorL, PorM, and PorN. Fractionation and immunoblotting of Pg cell lysates indicate that PorL and PorM are in a complex associated with the inner membrane while PorK and PorN are in a complex associated with the outer membrane, but the function of these protein complexes remains unknown (125). However, once a peptide is secreted though PorSS, it is known that a conserved C-terminal domain (CTD) is cleaved by the signal peptidase PG26 on the surface of the outer membrane; a process that may be mediated by interactions with PG27 (134). This cleavage step is necessary for secreted peptides to be released from PorSS and attached to A-LPS by a glycosylation reaction (135).

Through analysis of the Pg proteome, the gingipains were found to share a common C-terminal domain with numerous outer membrane proteins (1). This CTD was found to be necessary for the secretion and attachment of the gingipains to the bacterial cell surface (1, 136) and is thought to function as a signal peptide for por-mediated secretion. Site-directed mutagenesis of various CTD residues in RgpB resulted in accumulation of RgpB peptides in the periplasm, loss of surface attachment and accumulation within the secretion system (136). CTD proteins and A-LPS both accumulate in the periplasm of PorSS-deficient mutants in Pg, indicating that secretion and attachment of the CTD protein to the A-LPS anchor are coordinated (134).
In addition to the gingipains, multiple outer membrane proteins and secreted effectors were identified as CTD family proteins that rely on PorSS for transport (137). Thirty-four CTD family proteins are currently recognized, 18 of which are outer membrane proteins (summarized in Table 2-1). The secretion and glycosylation of HBP35, a hemin-binding outer membrane protein that contributes to heme utilization in *Pg*, was characterized and found to be dependent on the por secretion system (135). CPG70, a carboxypeptidase associated with gingipain processing and virulence in *Pg* (138, 139), has also been characterized as a CTD family protein that is secreted through PorSS (1). Multiple hemagglutinins and adhesins, including, a major *Pg* virulence factor, HagA (140), are also secreted in a PorSS-dependent manner. Disrupting this system has the potential to significantly impact virulence in *Pg*.

Our current understanding of the por secretion system, summarized in Figure 2-1, is limited. PorSS is a two-step secretion system that relies on the Sec pathway to transport gingipains and other CTD family peptides into the periplasm. The conserved CTD functions as a signal peptide, associating the gingipain polypeptides with PorSS and facilitating transport across the outer membrane. At the bacterial cell surface, the CTD is cleaved and the gingipains are either secreted into the extracellular milieu or attached to A-LPS by a glycosylation reaction on the bacterial cell surface.

CTD family proteins, including the gingipains, are often heavily processed prior to secretion. For example, the molecular weight of CPG70 ranges from 70-92kD in culture extracts. Very little is known about what factors mediate this processing or how it is regulated. Recently the Progulske-Fox lab has investigated the CTD family protein PG0553, a putative endopeptidase that has been shown experimentally to impact the
processing and function of CTD family proteins PG0027, CPG70, Kgp, RgpA and RgpB. Additional research is necessary to fully characterize this novel secretion system, which is critical for gingipain secretion and cell-surface attachment in \textit{Pg}, and to further elucidate the role of PG0553 in por-mediated secretion and the processing of CTD family proteins. This was the focus of the studies described in this dissertation.
<table>
<thead>
<tr>
<th>TIGR Annotation</th>
<th>Proposed Protein Function</th>
<th>CTD Sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG0026</td>
<td>C-terminal signal peptidase associated with PorSS</td>
<td>MASGHWVKIRDASGVYRTLDEQLRANGFSDPSKV VGVFGYGGVLPEDLSRITDDLPPV</td>
<td>(1, 134, 141)</td>
</tr>
<tr>
<td>PG0111</td>
<td>Capsular polysaccharide biosynthesis, putative von Willebrand factor type A domain protein, putative</td>
<td>MLNNASYRLKYWLRKAVWKMPAYRKTYREAHV SVSEQEHLFWEELKYVIVHVYPYRYDV</td>
<td>(1, 117)</td>
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<tr>
<td>PG0182</td>
<td></td>
<td>MMKRYTIILAVFLLFCTVTFQIKARPYERFADVEK PWWIQKHSMSKLVPANKGNIQAE</td>
<td>(1, 141)</td>
</tr>
<tr>
<td>PG0232</td>
<td>Zinc carboxypeptidase (CPG70)</td>
<td>M KKKNFLLLGIFVALLTFGSMQAQKDYFnFDE RGEAYFSFKEVFRAVLQELALI MS</td>
<td>(1, 139)</td>
</tr>
<tr>
<td>PG0290</td>
<td>Hypothetical protein</td>
<td>MAVGSGNANRQMNMLYVFIMAMMNLSVEV</td>
<td>(1)</td>
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<tr>
<td>PG0350</td>
<td>Internalin-related protein</td>
<td>MKRKLPSLVSFFGSVHPASAQKVPAVPVDG JERIMELSEADVCTIEAEDGAN</td>
<td>(1, 141)</td>
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<tr>
<td>PG0411</td>
<td>Hemagglutinin, putative</td>
<td>MAIMMKSVIFRAFLTILLSWAINTPATAQEQSMNAS CLAAPAQDPTILYESFENGVPN</td>
<td>(1, 141)</td>
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<tr>
<td>PG0495</td>
<td>Hypothetical protein</td>
<td>MKRIMKKKLFLAASLAVLCAWIAAQCTMAPYNFH A DPQQKHRIVEKFSSSYNSYEG</td>
<td>(1)</td>
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<td>PG0506</td>
<td>Arg-gingipain, RgpB</td>
<td>MKKKNFSRIVSIVASSLGLGMAFQAQERGRNPQVR LLSAEQMSKQFRMNDLQFTGVQ</td>
<td>(1, 142)</td>
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<tr>
<td>PG0553</td>
<td>Endopeptidase, putative</td>
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<td>(1, 136)</td>
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<tr>
<td>PG0611</td>
<td>Lipoprotein, putative</td>
<td>MKTNIKRMKTIIFCLLALFGCSWAQERVDEKVFSAGT SIFRGELEKVKAPLMMYGDRE VW</td>
<td>(1)</td>
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<td>PG0614</td>
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<td>(1, 135)</td>
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<tr>
<td>PG0616</td>
<td>Heme-binding outer membrane protein (HBP35)</td>
<td>MKRLLSAIALLSMALVNQAEQLKSADMKGSFKKNV VLEVFETAEWGCYGCGPKERIAK</td>
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<tr>
<td>PG0626</td>
<td>Hypothetical protein</td>
<td>MCHLQINVMSMRKLFLFVSLGLCFLGSLQETLVTFTGEG PGFDPCWTSVTSDAITTSQPOP</td>
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<td>PG0769</td>
<td>Fibronectin type III domain protein</td>
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<tr>
<td>PG1326</td>
<td>Hemagglutinin, putative</td>
<td>MCENTLAAKTEEFAPVSDLRAEAYGSTVFHLWTPP YDNPMILPSESFGAPIWKTD</td>
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<tr>
<td>PG1374</td>
<td>Immunoreactive 47 kDa antigen PG97</td>
<td>MKLSSKKILAIALLTLMGHAVQAOFVPTTNGRMSVT TTKAVKIELVHSEIEKKGW</td>
<td>(1, 100)</td>
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<tr>
<td>PG1424</td>
<td>Peptidylarginine deaminase (PAD)</td>
<td>MKKLQAKALALALGLFQLPAIAQTQMQADRTNGOFAT EEMQRFAQETNPAPGVRKIAE</td>
<td>(1, 143)</td>
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<tr>
<td>PG1427</td>
<td>Thiol protease/hemagglutinin PrtT precursor, putative</td>
<td>MKKSFLAVMFLGAMQGHSAPVTKERALSLARLALR QVSLRMGQTAVSDKISIDYVVR</td>
<td>(1, 141)</td>
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<tr>
<td>TIGR Annotation</td>
<td>Proposed Protein Function</td>
<td>CTD Sequence</td>
<td>References</td>
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<td>PG1795</td>
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<td>PG1837</td>
<td>Hemagglutinin protein HagA</td>
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<td>PG1844</td>
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<td>(1)</td>
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<td>MKLNK...</td>
<td>(1, 145)</td>
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<td>PG2100</td>
<td>Immunoreactive 63 kDa antigen PG102</td>
<td>MPRIMKL...</td>
<td>(1)</td>
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<tr>
<td>PG2102</td>
<td>Immunoreactive 61 kDa antigen PG91</td>
<td>MKT...</td>
<td>(1)</td>
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<td>PG2172</td>
<td>Hypothetical protein</td>
<td>MNKKT...</td>
<td>(1, 141)</td>
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<td>PG2198</td>
<td>Hemagglutinin protein, truncation</td>
<td>MKONYFKRVC...</td>
<td>(1)</td>
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<tr>
<td>PG2216</td>
<td>Hypothetical protein</td>
<td>MWFVN...</td>
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Figure 2-1. The Por secretion system (PorSS) in *Pg*. PorSS consists of a number of membrane or periplasmic proteins including PorK, PorL, PorM, PorN, PorO, PorP, PorQ, PorT, PorU, PorV (PG27, LptO), PorW and Sov. The precise functions of many of these proteins are still unknown, and some have not been included in this model. 1) Gingipains and other proteins are secreted across the IM into the periplasmic space via the Sec pathway (127, 135). 2) A conserved C-terminal domain (CTD) is associated with the por secretion system and functions as a signal peptide, directing effector proteins to PorSS for secretion (1, 135, 136). This process is poorly understood. 3) PG26, a surface-associated signal peptidase, cleaves the C-terminal domains of PorSS secretion products in the final step of por secretion. PG26 is likely associated with PorSS via PG27 (134). 4) Following cleavage of the CTD, the gingipains are either secreted or attached to the OM by a glycosylation reaction (135, 146, 147).
CHAPTER 3
PG0553 IS ASSOCIATED WITH PROTEASE ACTIVITY AND CTD FAMILY PROTEINS IN PORPHYROMONAS GINGIVALIS

Introduction

*Porphyromonas gingivalis* (*Pg*), an oral pathogen, is one of the primary etiological agents of periodontal disease. The systemic inflammation and bacteremia associated with periodontal disease (12, 13) facilitates the systemic spread of oral pathogens to cardiovascular tissues, potentially contributing to the development of cardiovascular diseases such as atherosclerosis. Epidemiological studies have demonstrated an association between periodontal disease and the development of cardiovascular disease (5, 8), prompting researchers to investigate the impact of periodontal pathogens on human arterial tissues and endothelial cells. Endothelial cell dysfunction is thought to be an initiating factor in atherosclerosis development (148) and endothelial cells are known to be activated during ischemic stroke and carotid atherosclerosis (149). *Pg* is able to invade human coronary artery endothelial cells (HCAEC) as well as several additional host cell types (31, 34, 150). The impact of *Pg* on the endothelial cell layer and other subclinical mediators of cardiovascular disease are currently being investigated.

In 2005, Rodrigues et al. (151) performed microarray experiments to determine which genes in *Pg* were differentially regulated over time in the context of HCAEC invasion (151). One such gene, *PG0553*, was found to be significantly up-regulated at 5 minutes and 60 minutes during the course of invasion. Annotated as an extracellular protease in the JCVI database, *PG0553* was identified as a potential virulence factor during the early stages of host cell invasion.
*Pg* is asaccharolytic, relying on peptides rather than carbohydrates as a source of carbon, and employs an array of proteases that serve both metabolic and virulence-related functions. The gingipains, trypsin-like cysteine proteases, are critical for both virulence and survival in the host environment. Targeting host proteins such as collagen, fibrin/fibrinogen, human immunoglobulin and cell surface receptors, the gingipains facilitate the acquisition of amino acids and heme while evading the host immune response (21, 22, 27). Degradation of host proteins and disregulation of the host immune response by *Pg* is directly linked to disease pathogenesis (41, 152).

The gingipains are secreted across the outer membrane and either released into the extracellular milieu or attached to lipid A moieties on the bacterial surface via glycosylation in the form of a protease-adhesin complex (126, 146), but not much is known about the mechanism of gingipain secretion. Interestingly, the *Pg* genome does not contain homologues to any of the currently characterized protease secretion systems (types I-VI). A novel system, known as the por secretion system (PorSS), is currently being characterized in *Pg* and is thought to be responsible for gingipain secretion.

A unique C-terminal domain (CTD), first identified in RgpB and found to be necessary for the post-translational processing of gingipains (136), functions as a recognition signal for PorSS secretion (135). This CTD is conserved in *Pg*, and has been used to identify a family of proteins associated with the PorSS pathway (1). The *PG0553* sequence contains this conserved CTD and is included in this unique family of proteins, as illustrated in Figure 3-1A. STRING, an online software program designed to predict protein-protein interactions (153), identified potential interaction partners for
PG0553 that included several other CTD family proteins. Most notably this list contained *PG0027* and *sov*, PorSS components that are necessary for gingipain secretion to occur ([127, 129, 133](#)), as well as gingipains Kgp and RgpA (Figure 3-1B). Based on CTD homology and predicted interactions with the PorSS pathway, PG0553 is potentially associated with this novel secretion system in *Pg* and may play an important role in the regulation and processing of secreted proteins, such as the gingipains, associated with PorSS.

**Results**

**Soluble Protease Activity is Reduced in the PG0553 Knockout**

PG0553 is annotated in the JCVI database as a putative extracellular protease, yet bioinformatic analysis of the peptide sequence through Blastp and the Conserved Domain Database detected no domains associated with protease activity. However, a conserved C-terminal domain (CTD) associated with protease secretion was identified in PG0553. In order to determine if *PG0553* was important for protease activity in W83, a commercially available protease assay capable of detecting all four proteolytic classes (serine, aspartic, cysteine and metalloproteinases) was used to measure general protease activity in *Pg* samples taken from both the wild type W83 and the mutant W83Δ0553. *Pg* produces and secretes a wide array of proteases that are processed internally, and then secreted across the outer membrane via PorSS. Secreted proteases are both released into the supernatant and anchored in the outer membrane. Accordingly, samples taken from growth supernatants, whole cells and cell lysates were analyzed for measurable protease activity.

These experiments generated diverse readings in the supernatant and whole cell samples. As indicated in Figure 3-2, this range of activity readings precluded the
possibility of demonstrating a significant difference when the results of several experiments were taken together. The PG0553 sequence contains no identifiable protease domains and is unlikely to contribute directly to protease activity. While no significant difference was observed in samples associated with secreted protease activity, protease activity in the Pg cell lysate samples was consistently and significantly reduced in the W83Δ0553 mutant. The localization of the reduction in protease activity to the soluble, intracellular fraction is consistent with PG0553 playing a role in protease processing prior to por secretion.

**Soluble Gingipains-specific Protease Activity is Reduced in the PG0553 Knockout**

The gingipains are responsible for at least 85% of the proteolytic activity observed in Pg (154), both secreted and cell surface-associated activity. The Lys-gingipain, Kgp, and Arg-gingipain, RgpA, were also predicted by the STRING database to be interaction partners for PG0553 (Figure 3-1B). To determine if the difference in overall intracellular protease activity observed in the soluble lysates was specifically related to a decrease in gingipains activity, a similar assay was performed using gingipains-specific substrates.

The results of the gingipain assay mirrored those of the general protease assay, as seen in Figure 3-3. No significant differences were observed between mutant and wild type in either the supernatant or whole cell fractions in their ability to process the Kgp substrate over time, while the reaction rate in the lysate samples was significantly reduced in W83Δ0553. Over time however, the wild type and mutant lysate samples reach the same maximum level of gingipain activity as measured by cleavage of the
Kgp substrate. However this reduction in the rate of cleavage by Kgp does not completely account for the reduced level of overall protease activity seen in Figure 3-2.

**Proteases and CTD Family Proteins were Affected in the PG0553 Knockout**

Zymograms were used to generate a clearer picture of the differences in protease activity between the W83 wild type and the W83Δ0553 mutant. Lysate samples were mildly denatured with SDS buffer and run on casein (Figure 3-4A) and gelatin (Figure 3-4B) zymogram gels, sorting the soluble protein fractions by molecular weight. Areas of clearing in the gels represent proteolytic processing of the embedded substrates. A distinct difference in clearing patterns between the wild type and W83Δ0553 mutant lysates was observed with both casein and gelatin substrates, as seen in Figure 3-4. However there was no band of proteolytic clearing observed at 102kD, the molecular weight of PG0553 itself, in either substrate tested.

Distinct bands in the wild type lysates that were characterized by markedly reduced or ablated clearing in the W83Δ0553 lysates (as indicated by the black arrows in Figure 3-4) were excised and submitted for identification by mass spectrometry. These results, summarized in Table 3-1 and Figure 3-5, strongly support the STRING predictions regarding PG0553 interaction partners (Figure 3-1B). The 70kD band in the gelatin zymogram, altered significantly in the W83Δ0553 lysate, was positively identified as an Arg-gingipain. However the sequence homology between the catalytic domain of RgpA and RgpB (41) makes any further distinction speculative. These results are consistent with both the STRING predictions and the altered gingipain activity observed in Figure 3-3A.
Surprisingly PG0027, also identified by STRING as a potential interaction partner for PG0553, was also detected in a band excised from the casein zymogram. While PG0027 (LptO, porV) is required for normal gingipain activity (127), it is a membrane protein thought to form a β-barrel that is anchored in both the inner and outer membranes (131). There is no protease activity associated with PG0027, which is instead thought to coordinate the secretion of CTD proteins and attachment to A-LPS (132). The molecular weight of the band containing PG0027 (MW 43.2 kD) was also far smaller than expected, running on the zymogram gel at approximately 13kD. Components of the Rag receptor complex, similarly lacking in proteolytic activity, were also detected in the 125kD band in the gelatin zymograms. These bands were absent in W83Δ0553 lysates, suggesting that PG0553 may facilitate an interaction between these membrane structures and other, currently unidentified, proteases.

Interestingly, the band excised at 75kD in the casein zymogram was identified as PG0232 (91.5 kD). PG0232 is a carboxypeptidase that was first identified by the Reynolds lab as a 69.8 kD protein (CPG70) secreted by Pg that worked in concert with the gingipains (139). When the Reynolds lab sequenced the CPG70 protein they had isolated, it was identified as a C- and N-terminally truncated form of PG0232. The C-terminus of PG0232 contains the CTD signal peptide associated with PorSS, which is thought to be cleaved by PG0026 as part of the por secretion process (1, 134, 135). The molecular weight of the band suggests that the N-terminal cleavage of PG0232, thought to be necessary to activate the carboxypeptidase domain, is occurring normally in the W83 lysates but is absent in the W83Δ0553 lysates. CPG70 processes C-terminal lysine residues in RgpA and Kgp domains, a critical step in the formation of the
gingipain protease-adhesin complex on the bacterial cell surface (138). This role can also be filled by Kgp, though likely with reduced efficiency (138, 139).

When CPG70 was first characterized through its association with the gingipains, it was also found to be essential for virulence in a mouse murine lesion model (139). Insertional inactivation of CPG70 did not block gingipain secretion, but rather resulted in the secretion of differentially processed gingipains characterized by altered isoelectric points and molecular weights (138). These subtle changes were sufficient to impact virulence in the murine lesion model. We have observed the absence of CPG70-associated protease activity via zymogram and a significant reduction in gingipain activity by both zymogram and enzymatic assays. Therefore, we hypothesize that PG0553 is involved with the recruitment or N-terminal processing of PG0232, which in turn processes and activates the gingipains. The absence of CPG70 activity in the W83Δ0553 mutant strain has the potential to significantly impact multiple aspects of virulence in Pg W83 by altering gingipains activity.

**Methods**

**Bioinformatic Analysis of PG0553**

Conserved domains in PG0553 were identified using the BLASTp database. The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families (155, 156).

STRING was used to identify potential interaction partners for PG0553. STRING is a database of known and predicted protein interactions. The interactions include both
direct and indirect associations and are derived from four sources: genomic context, high-throughput experiments, conserved coexpression and published information. STRING quantitatively integrates interaction data from these sources for a large number of organisms, and transfers information between these organisms where applicable (153, 157).

Mutant Construction

Gene \textit{PG0553} was insertionally inactivated in wild type \textit{Pg} strain W83 by allelic exchange. 500 bp fragments from the C- and N-termini of gene \textit{PG0553} were amplified by PCR and cloned into plasmid pPR-UF1, flanking an \textit{ermF/ermAM} antibiotic resistance cassette. The vector was linearized and electroporated into \textit{Pg} W83. Successful mutations were selected for by growth on erythromycin plates and confirmed by Southern blot (data not shown).

Bacterial Cell Growth and Sample Preparation

Cultures of \textit{P. gingivalis} W83 and W83\textit{Δ}0553 were grown anaerobically overnight for 15 hours in tryptic soy broth supplemented with 0.5% yeast extract, 0.05% L-cysteine hydrochloride, 5 µg/mL hemin and 1 µg/mL vitamin K1. Overnight cultures were diluted to an OD of 0.5 in pre-reduced, supplemented TSB and grown anaerobically until the OD had doubled to 1.0, approximately 3.5 hours, to homogenize cultures in the exponential phase of growth.

Cultures in exponential phase were centrifuged at 5000XG for 5 minutes. Supernatant samples were collected and sterile filtered through a 0.2 micron filter. Bacterial cell pellets were either washed twice in PBS to collect the whole cell fraction or lysed with BugBuster (EMD Millipore 70584) to collect the cytoplasmic fraction. Lysates
were also centrifuged at 10,000XG for 10 minutes to remove non-soluble proteins. All samples were kept on ice to prevent the degradation of proteases.

Protease Assay

The Sigma-Aldrich Protease Fluorescent Detection System (PF0100 Sigma) was used to assess secreted protease activity in *Pg* supernatant, membrane-associated protease activity in whole cells and soluble protease activity in cellular lysates. Serine, aspartic, cysteine and metalloproteinase activity was measured via digestion of FITC-casein. Samples were prepared as previously described and measurements were taken fluorometrically per manufacturer’s instructions.

Gingipain Assays

*Pg* strains W83 and W83Δ0553 were grown anaerobically as previously described. The supernatants are collected, filtered and mixed with the reaction buffer. The pellets are washed with PBS or lysed with BugBuster, then resuspended in the reaction buffer. The gingipain enzymatic substrate, GPR-pNA or GPK-pNA, was added to the reaction at a final concentration of 0.4 mM. Enzymatic activity was measured colorimetrically every 30 seconds over a 4 hour time course.

Zymograms

*Pg* lysate and supernatant samples were prepared as previously described and run on Novex zymogram gels from Invitrogen. Samples were prepared and run on 12% casein (EC6405BOX) and 10% gelatin (EC6175BOX) gels in tris-glycine SDS running buffer using the XCell SureLock Mini-Cell. Zymograms were renatured and developed using Novex Zymogram Renaturation and Developing Buffers per the manufacturer’s instructions. Gel bands of interest were excised from the zymograms and sent to the
University of Florida Interdisciplinary Center for Biotechnology Research for mass spectroscopy identification by LC-MS/MS.

**LC: MS/MS**

The enzymatically digested samples were injected onto a capillary trap (LC Packings PepMap) and desalted for 5 min with a flow rate 3 µl/min of 0.1% v/v acetic acid. The samples were loaded onto an LC Packing® C18 Pep Map nanoflow HPLC column. The elution gradient of the HPLC column started at 3% solvent A, 97% solvent B and finished at 60% solvent A, 40% solvent B for 30 min for protein identification.

Solvent A consisted of 0.1% v/v acetic acid, 3% v/v ACN, and 96.9% v/v H2O. Solvent B consisted of 0.1% v/v acetic acid, 96.9% v/v ACN, and 3% v/v H2O. LC-MS/MS analysis was carried out on a hybrid quadrupole-TOF mass spectrometer (QSTAR Elite, Applied Biosystems, Framingham, MA). The focusing potential and ion spray voltage was set to 225 V and 2400 V, respectively. The information-dependent acquisition (IDA) mode of operation was employed in which a survey scan from m/z 400–1800 was acquired followed by collision induced dissociation (CID) of the four most intense ions. Survey and MS/MS spectra for each IDA cycle were accumulated for 1 and 3 s, respectively.

**Protein Search Algorithm**

Tandem mass spectra were extracted by ABI Analyst version 2.0. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.2.2). Mascot was set up to search NCBI-Bacteria database assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 0.50 Da. Iodoacetamide derivative of Cys, deamidation of Asn and Gln, oxidation of Met, are specified in Mascot as variable modifications. Scaffold
(version Scaffold 3.6.5, Proteome Software Inc., Portland, OR) will be used to validate MS/MS based peptide and protein identifications. Peptide identifications are accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (158). Protein identifications are accepted if they can be established at greater than 99.0% probability and contain at least 2 identified unique peptides. Protein probabilities were assigned by the Protein Prophet algorithm (159).

**Discussion**

There is a distinct alteration in protease activity in the W83Δ0553 mutant, as indicated by different patterns of clearing in zymogram gels and measured directly by cleavage of FITC-casein and GPK-pNA. This data indicates that the activity of multiple proteases is altered in the absence of *PG0553* in a manner that is localized to the cytoplasm. Rather than directly affecting gingipain processing, PG0553 potentially plays a role in the processing or recruitment of several effector proteins in conjunction with the por secretion pathway. PG0553 is predicted to function as an intermediary in the processing of gingipains prior to por secretion through the N-terminal cleavage and activation of CPG70 (PG0232). Gingipain processing is a multi-step process that is only partially understood. As described in Figure 3-6, gingipain polypeptides are first secreted across the inner membrane into the periplasmic space via the sec pathway (127, 135). Not all CTD family proteins contain a sec signal sequence and are potentially translocated through an alternative mechanism. PG0553 and PG0232 are both members of the CTD family of proteins associated with PorSS. The absence of CPG70-associated protease activity and the overall reduction in protease activity in W83Δ0553 cell lysates is consistent with PG0553 directly processing or facilitating the
N-terminal cleavage of PG0232, activating the carboxypeptidase domain (CPG70). Carboxypeptidase CPG70 processes eight different C-terminal lysine residues of RgpA and Kgp polypeptides, enabling various adhesin and proteolytic domain conformations to form on the bacterial cell surface (138, 139).

The processed gingipains in the periplasmic space contain the conserved CTD sequence, which facilitates translocation across the outer membrane through PorSS (125, 137). PG0026, which is also thought to associate with PG0027, functions as a C-terminal signal peptidase on the cell surface (134). The CTD is cleaved, and the mature gingipain is either secreted or permanently attached to the outer membrane by glycosylation to A-LPS (135, 146, 147).

Curiously, the W83Δ0553 protease phenotype is restricted to the cell lysates. Though surprising, it is possible for PG0553 to play a significant role in protease processing and por secretion intracellularly without a significant impairment in overall secreted or cell surface-associated protease activity as measured in vitro. Pg produces a wide array of proteases, many of which overlap in terms of function. CPG70, for example, shares the role of C-terminal processing of lysine residues with Lys-gingipain Kgp (138). It is possible that Kgp is able to compensate somewhat for reduced functionality in CGP70, or that an alternative system exists for activating PG0232 that is simply less efficient than PG0553.

While the zymogram results clearly indicate that carboxypeptidase CPG70 is proteolytically active intracellularly, it is unknown whether CPG70 interacts with the gingipains intracellularly or following secretion through PorSS. The extent to which CPG70 processing impacts the overall activity of the gingipain protease domain is also
unknown. However, experiments performed by Chen et al. (139) using a CPG70 knockout strain have demonstrated that disrupting CPG70-mediated gingipain processing has a significant impact. Surface protein extracts from the CPG70 knockout strain contained proteins of altered mass and isoelectric characteristics (138), which was sufficient to disrupt virulence in the murine lesion model (139). The \textit{in vitro} assays used to assess protease activity in the experiments reported here are unable to detect differences in the structure of the gingipain protease-adhesin complex that may impact virulence without impacting overall protease activity.

PorSS is a novel secretion system that has yet to be thoroughly characterized. Exploring the role of PG0553 in this unique system suggests a complex story, with the activity of multiple proteases altered in the W83Δ0553 knockout. We hypothesize that these pleiotrophic effects are due to PG0553 playing a role in the post-translational modification and processing of CPG70, which in turn processes the gingipains and contributes significantly to virulence. The diverse phenotype observed in the W83Δ0553 mutant suggests that PG0553 may interact with other CTD proteins as well, but the mechanism and significance of this potential interaction remains unclear. Many new and interesting questions have been raised regarding how the various PorSS components work together to promote protease secretion and virulence in W83. Further experimentation is required to determine the biological relevance of this phenotype, as well as to further clarify the specific role of PG0553 in por secretion and gingipains processing.
Table 3-1. Identification and significance of proteins associated with differential protease activity observed in the W83Δ0553 mutant

<table>
<thead>
<tr>
<th>Band</th>
<th>Conf.</th>
<th>%Cov</th>
<th>Accession</th>
<th>Gene Name</th>
<th>Molecular Weight</th>
<th>Biological Relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>~75kD casein</td>
<td>100</td>
<td>3</td>
<td>GI:34540084 (PG0232, CPG70)</td>
<td>PG0232</td>
<td>91.4kD (whole), 77.3kD (N-terminally truncated), 69.8kD (N- and C-terminally truncated)</td>
<td>Associated with gingipains processing and maturation. Contains C-terminal domain associated with PorSS (1, 138, 139).</td>
</tr>
<tr>
<td>~15kD casein</td>
<td>100</td>
<td>10</td>
<td>GI:34539904 (IptO, porT)</td>
<td>PG0027</td>
<td>43.1kD</td>
<td>Essential for por secretion in Pg. Thought to form a β-barrel in the outer membrane. Contains C-terminal domain associated with PorSS (1, 127, 131, 132).</td>
</tr>
<tr>
<td>~125kD gelatin</td>
<td>99</td>
<td>4</td>
<td>GI:34540042 (RagA)</td>
<td>PG0185</td>
<td>112.2kD</td>
<td>Outer membrane protein. Forms a receptor-transporter complex with RagB on the surface of Pg. Functionally linked to TonB.</td>
</tr>
<tr>
<td>~125kD gelatin</td>
<td>100</td>
<td>7</td>
<td>GI:34540043 (RagB)</td>
<td>PG0186</td>
<td>56kD</td>
<td>Lipoprotein. Forms a receptor-transporter complex with RagA on the surface of Pg. Functionally linked to TonB.</td>
</tr>
<tr>
<td>~70kD gelatin</td>
<td>100</td>
<td>2</td>
<td>GI:34541613 (RgpA, HagE)</td>
<td>PG2024</td>
<td>185.5kD, 12+ proteins are derived from RgpA with varying MW (160)</td>
<td>Arg-gingipain. RgpA and RgpB share 93% sequence homology between their catalytic domains. Contains C-terminal domain associated with PorSS (1).</td>
</tr>
<tr>
<td>~70kD gelatin</td>
<td>100</td>
<td>5</td>
<td>GI:34540322 (RgpB, prtRII)</td>
<td>PG0506</td>
<td>80.8kD, 56kD (mature)</td>
<td>Arg-gingipain. RgpA and RgpB share 93% sequence homology between their catalytic domains. Contains C-terminal domain associated with PorSS (1).</td>
</tr>
</tbody>
</table>
a) Bands were excised from zymogram gels (Figure 3-4). Samples were processed for LC-MS/MS and analyzed using Mascot (Matrix Science, London, UK; version 2.2.2).
b) Scaffold (version Scaffold 3.6.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability.
c) Percent coverage refers to the number of matching amino acids (from peptides) divided by the total number of amino acids in the protein sequence, expressed as a percentage.
d) Accession numbers refer to the NCBI Database, (http://www.ncbi.nlm.nih.gov/). Mascot was set up to search NCBI-Bacteria database assuming the digestion enzyme trypsin.
e) Functional data was obtained from the NCBI Database or from indicated references.
Figure 3-1. Bioinformatics assessment of PG0553. A) PG0553 contains a conserved C-terminal por secretion tag associated with the secretion of proteases across the outer membrane in Pg. No other conserved domains were detected. Conserved domains were identified using NCBI blastp suite (155). Image generated using PROSITE MyDomain image creator (161). B) STRING analysis (153) determined that PG0553 is conserved across Pg strains and identified potential interaction partners. PG0769, Kgp and RgpA are also characterized by a C-terminal domain associated with PorSS (1), while sov and PG0027 mediate por secretion together with porT (127-129).
Figure 3-2. W83Δ0553 mutant is defective in overall protease activity. Protease activity in *P*. *fluorescens* W83 samples as measured by digestion of a FITC-conjugated casein substrate. W83 cell lysates, associated with the cytoplasmic and periplasmic fraction, showed a significant reduction in protease activity in the W83Δ0553 mutant (p<0.005). These results are the average of three experiments (n=3), with four replicates per experiment. Results were analyzed using student’s t-test. Error bars are standard deviation.
Figure 3-3. Measurement of Kgp activity in the W83Δ0553 mutant. Colorimetric measurement of Kgp activity over time indicates a significant decrease in gingipains activity in the W83Δ0553 mutant associated with the bacterial cell lysates (A). This decrease was not observed in the supernatant (B) or whole cell (C) fractions. These results are representative of two experiments, with five replicates in each experiment. Error bars are standard deviation.
Figure 3-4. Differential protease activity in W83Δ0553 mutant. Bacterial cell lysates were run on zymogram gels embedded with A) casein and B) gelatin substrates. Regions of clearing indicate protease activity associated with a specific molecular weight. The banding pattern, indicative of the complement of active proteases in the sample, varied significantly between the wild type and the W83Δ0553 mutant. Black arrows highlight distinct protease activity present in the wild type but reduced or ablated in the W83Δ0553 mutant. These bands were excised for identification by mass spectrometry. Letters correspond to band identifications in Figure 3-5.
Figure 3-5. Mass spectrometry identification of bands extracted from zymogram gels. A) Casein zymogram, ~75kD band, B) Casein zymogram, ~15kD band, C) Gelatin zymogram, ~125kD band and D) Gelatin zymogram, ~70kD band. Black lines indicate alignment of MS/MS sample peptides with positively identified proteins. A minimum of two unique peptides and 95% confidence was required to be considered a positive identification. Sample C) was positive for both RagA and RagB, which are known to form a complex on the bacterial cell surface. In sample D), a distinction between RgpA and RgpB cannot be made due to the alignment of the MS/MS peptides with the Arg-gingipain domain, which is 93% identical between RgpA and RgpB. MS/MS results were analyzed using Scaffold v3.
Figure 3-6. Proposed model of interactions between PG0553 and por secretion of gingipains. 1) Gingipains and other proteases are secreted across the IM into the periplasmic space via the Sec pathway (127, 135). 2) PG0553 and PG0232 are associated with the PorSS complex by their CTDs (1). This association is a potential mechanism for the interaction between PG0553 and PG0232 that may mediate cleavage of the N-terminal region of PG0232 to form active CGP70. 3) CPG70 processes RgpA and Kgp at eight potential C-terminal Lys sites. This function can also be fulfilled by Kgp. Cleavage of C-terminal Lys residues is thought to be necessary for the formation of the active protease-adhesin complex on the bacterial cell surface. (138, 139). 4) RgpA, Kgp and CPG70 are secreted across the OM into the extracellular space via PorSS (125, 137). This process is poorly understood. 5) PG26, a surface-associated signal peptidase, cleaves the C-terminal domains of the gingipains and CPG70 in the final step of por secretion. PG26 is likely associated with PorSS via PG27 (134). RgpA and Kgp can also be attached to A-LPS and attached to the OM via glycosylation following por secretion (135, 146, 147).
CHAPTER 4
THE DEFECT IN PROTEASE ACTIVITY ASSOCIATED WITH THE W83Δ0553 MUTANT SIGNIFICANTLY IMPACTS HOST CELL INTERACTIONS IN PORPHYROMONAS GINGIVALIS

Introduction

The integrity of the endothelium is critical for cardiovascular health. A layer of endothelial cells lines the vasculature, forming a semi-permeable barrier between circulating plasma and the blood vessel wall that is the major regulator of vascular homeostasis. Injury or cellular dysfunction within the endothelium plays a major role in the development of atherosclerosis and is one of the earliest, most recognizable factors available for diagnosing cardiovascular disease (162). Many of the known risk factors for cardiovascular disease, such as smoking and systemic inflammation, are also associated with endothelial cell dysfunction (163). By reducing the effectiveness of the epithelium and increasing the uptake of LDL into the blood vessel wall, damage to vascular epithelial cells may in fact be an early factor in the development of atherosclerosis (148).

*Porphyromonas gingivalis* (*Pg*) is a periodontal pathogen that has been associated with the development of cardiovascular disease (61, 64, 71, 73). The systemic inflammation and bacteremia associated with periodontal disease (12, 13, 57-60) facilitates the spread of oral pathogens and related effector proteins to cardiovascular tissues and the endothelium. The potential for oral pathogens to contribute to cardiovascular disease is significant, with epidemiological and experimental data supporting an association (5, 8, 61, 71, 73). *Pg* has been consistently detected in atheromatous plaque and has been shown to be viable and infectious, supporting a specific association between *Pg* and damaged vasculature (61-64).
One of the primary virulence factors of *Pg* are the gingipains, cysteine and lysine proteases that target host proteins such as collagen, fibrin/fibrinogen, human immunoglobulin and cell surface receptors (21, 22). In addition to the gingipains, *Pg* secretes an array of proteases which break down environmental and immune proteins and provides the bacterium a source of carbon and energy. *Pg* has also been shown to lyse erythrocytes through gingipain activity (25), an efficient method for acquiring iron in the form of heme. However, the release of cytoplasmic components into the bloodstream is highly inflammatory and contributes to tissue destruction and vascular disruption (26). *Pg* is also able to invade several host cell types, including human coronary artery endothelial cells (HCAEC) (31, 34, 150), potentially contributing to endothelial disregulation while evading the host immune response.

Invasion of host cells is initiated by interactions between bacterial adhesins, such as fimbriae and hemagglutinins, and integrins localized to lipid rafts in the host cell membrane (18). Fimbriae are proteinaceous appendages that are major adhesins in *Pg*; however W83 is a fimbriae-deficient strain and instead relies on surface-associated hemagglutinins like HagA and the gingipain protease-adhesin complex for attachment. The RgpA and Kgp polypeptides contain multiple adhesin domains in addition to the gingipain peptidase (160). In the absence of the gingipains, host cell adherence and invasion are significantly impaired. (164).

Upon internalization into HCAEC *in vitro*, *Pg* strain W83 is incorporated into the phagocytic pathway (Figure 4-1). The phagosome typically merges with the lysosome, resulting in bacterial cell death and degradation. However, *Pg* has been demonstrated to evade the phagocytic pathway by instead trafficking to the autophagic pathway (37,
85). Autophagy is a metabolic pathway that functions to break down unnecessary or malfunctioning cellular components to be recycled. A double-membrane vesicle called the autophagosome forms around components targeted for degradation and then merges with the lysosome, breaking down the contents of the vacuole. Autophagy also plays a role in innate immunity through the destruction of intracellular pathogens.

*Pg* is known to activate cellular autophagy in the host and localize to autophagosomes, however it is able to prevent the fusion of the lysosome with the autophagosome, and thus formation of the autophagolysosome is inhibited (37). These autophagic vacuoles thus function as a protected, nutrient-rich environment for *Pg* that may facilitate its persistence within the host. The detailed mechanisms of autophagy activation and lysosomal resistance in *Pg* are currently unknown. In the absence of the gingipains, however, *Pg* strain 33277 instead migrates to phagolysosomes, suggesting that the gingipains are necessary to avoid lysosomal destruction (165). The Progulske-Fox lab has also demonstrated that the gingipains are necessary for autophagy activation in SAOS-2 cells by *Pg* strain 381 (data unpublished). The disregulation of autophagy is strongly associated with human disease (166), and likely has a negative impact on the endothelium.

Thus, the gingipains are critical virulence factors in *Pg* that play a major role in nutrient acquisition, adhesion and persistence within autophagic vacuoles. We reported previously that PG0553, a putative virulence factor in *Pg* W83, was associated with the por secretion pathway and played a role in gingipain processing via CPG70. We predicted that gingipains would be secreted in an altered, “immature”, conformation, potentially having a significant impact on virulence and host cell interactions. This does
not eliminate the possibility that other surface-associated structures or adhesins are also affected in the W83Δ0553 mutant, but a potential mechanism for PG0553 to impact other surface proteins has not yet been identified. Here we examine the biological significance of the W83Δ0553 phenotype in HCAEC through in vitro cellular assays.

Results

The W83Δ0553 Mutant Demonstrated Growth and Biofilm Formation Comparable to W83

Growth of W83Δ0553 in supplemented tryptic soy broth was characterized alongside W83. Overnight cultures of Pg were diluted to an optical density of 0.1 in triplicate in pre-reduced tryptic soy broth. Optical density measurements were taken every two hours over the course of 48 hours of anaerobic growth. No significant differences were observed in growth rate.

A microtiter dish biofilm formation assay was used to assess biofilm formation in W83Δ0553. This assay allows for the formation of a biofilm on the wall and/or bottom of a microtiter dish. The extent of biofilm formation can be measured using the dye crystal violet. By measuring the optical density of the dye retained by the biofilms we were able to determine that W83Δ0553 forms biofilms of comparable density to W83. W83 is a weak biofilm former however, and the microtiter dish biofilm formation assay does not allow for the formation of mature biofilms. It is possible that in another system for measuring biofilm formation some differences could be observed.

Adherence and Invasion in HCAEC are Impaired in the PG0553 Knockout

Adherence and invasion assays were conducted in vitro in HCAEC to determine if the predicted conformational alterations in the gingipains had a significant biological impact. A 1.5-fold reduction in adherence was observed (Figure 4-2A), suggesting that
the ability of \textit{Pg} to interact with the host cell surface was impaired in the W83Δ0553 mutant. This is consistent with PG0553 having an impact on the function of surface-associated adhesins in \textit{Pg}, such as the gingipain-adhesin complex or HagA. While statistically significant and consistent, the difference is subtle. However, this model system does not reproduce the laminar flow of the bloodstream or the pressure of the host immune response. \textit{In vivo}, a 1.5 fold reduction in adherence may have a more dramatic impact.

As previously reported, \textit{PG0553} was found by microarray analysis to be significantly up-regulated after 5.0 minutes and after 1.0 hour of co-incubation with HCAECs. The invasion assay results reflected this, with W83Δ0553 showing a 3.4-fold reduction in recoverable cfu at 30 minutes and a 2.6-fold reduction at 1.0 hour, but no significant difference at 2.5 hours (Figure 4-2B). This is consistent with PG0553 playing a significant role in the early stages of host cell invasion. Persistence at 24 and 48 hours was also assessed in W83Δ0553, which was also found to be comparable to W83 (data not shown).

The 1.5-fold reduction in adherence does not account for the significantly higher impairment in invasion. The gingipains are important for adherence and cell entry (167), so if the W83Δ0553 mutant is expressing altered gingipain complexes or other surface-located adhesins on the bacterial cell surface, as predicted by our current model, these subtle differences may have a broad impact on virulence in \textit{Pg} strain W83.

\textbf{Activation of Autophagy and Trafficking to Autophagic Vacuoles in the PG0553 Knockout}

Strains of \textit{Pg} activate autophagy in HCAEC and traffick to the autophagosome, which forms a protected niche for replication (85). In experiments where autophagy is
inhibited in HCAEC by wortmannin, \( Pg \) strain 381 is instead transported to phagolysosomes and degraded (37). A strain 381 mutant lacking all three gingipain genes lost the ability to activate autophagy in SAOS-2 cells (Progulske-Fox lab, data unpublished). KDP136, a mutant in strain 33277, also lacking gingipains, trafficked to phagolysosomes and did not persist in HCAEC (165). It thus appears that if the ability of \( Pg \) to either activate autophagy or inhibit phagolysosome formation is impaired, \( Pg \) is more likely to be degraded. Significant evidence exists suggesting the importance of the gingipains in activation of autophagy and persistence within autophagic vacuoles in the host cell (37, 165). Measuring these variables in the host in conjunction with \( Pg \) infection is therefore a potential indicator of virulence and gingipain activity in \( Pg \).

The following experiments were performed in cell lines that were either stably transfected (in the case of SAOS-2) or transiently transduced via an adenovirus vector (in the case of HCAEC) to express GFP-tagged LC3, a marker of autophagic vacuoles. LC3 is ubiquitously expressed in the host cytosol. As the autophagic membrane forms, autophagy-associated proteins form a complex that recruits LC3 and incorporates it into the autophagosomal membrane. Thus, gfp-labeled vacuoles and puncta allow us to visualize ongoing autophagy. Though the role of LC3 in autophagy is not fully understood, it is considered a reliable marker for monitoring autophagic activity in host cells (168).

**The PG0553 Knockout Activates Autophagy to a Lesser Extent than W83 in SAOS-2 cells**

SAOS-2 (sarcoma osteogenic) cells are immortalized human osteosarcoma cells that have been well-characterized and are widely cultured. When grown to confluence, SAOS-2 cells deposit an extensive collagenous extracellular matrix around themselves.
(169). *Pg* cannot penetrate this extracellular matrix and consequently cannot invade SAOS-2 cells, but the presence of *Pg* in the surrounding culture media is sufficient to activate autophagy in these cells. The SAOS-2 model system can thus be used to assess autophagy activation independently from *Pg* invasion through the quantification of GFP-labeled autophagic vacuoles, or puncta, as observed in Figure 4-3. For these studies, a cell containing 5 or more puncta was considered to be undergoing autophagy.

Cells inoculated with *Pg* W83 and the W83Δ0553 mutant were undergoing autophagy at significantly higher rates than the fed controls. However, W83Δ0553 was significantly impaired when compared to the wild type in this model (Figure 4-3). The mechanism by which *Pg* activates autophagy in the host is currently unknown, though the SAOS-2 model system strongly establishes that a secreted factor is responsible. There is some evidence that the gingipains are involved, as a triple gingipain knockout in strain 381 lost the ability to activate autophagy in SAOS-2 cells (Progulske-Fox lab, data unpublished). This is consistent with our current model, which predicts that PG0553 indirectly impacts surface-associated gingipain functionality by processing CPG70 into an active form, which then is crucial for the processing of the RgpA and Kgp polypeptides. It is also possible that PG0553 plays a role in recruiting other, currently unknown, effector proteins that play a role in autophagy activation.

**The PG0553 Knockout is Impaired in Trafficking and Activates Autophagy to a Lesser Extent than W83 in HCAEC**

In HCAEC, *Pg* W83 activates autophagy and trafficks to the autophagosome. HCAEC transiently transduced with GFP-LC3 were used to observe both the activation of autophagy and trafficking through the autophagic pathway in HCAEC. This process was significantly altered in cells infected with W83Δ0553 (Figure 4-4). Quantification of
autophagic puncta and vacuoles within infected cells indicated a reduction in ongoing autophagy in HCAEC coincubated with W83Δ0553 (Figure 4-4C). Trafficking was also impacted, with fewer *Pg* colocalizing with autophagic vacuoles in the knockout strain (Figure 4-4D).

**Western Blot Analysis of GFP-LC3 Processing Confirm Activation of Autophagy in SAOS-2 and HCAEC Host Cells**

The GFP-LC3 conjugate has a molecular weight of 44kD. As autophagosomes mature and merge with lysosomes, the GFP tag is cleaved. By itself, the GFP tag has a molecular weight of 26 kD. The GFP cleavage product can be detected by western blot and is an indicator of ongoing autophagy. In a stable transfection, the ratio of cleaved GFP to GFP-LC3 can be calculated based on band density in the western blot to indicate differences in the rate of ongoing autophagy. However, the varying sensitivity of this technique can be a confounding factor in confirming subtly altered levels of autophagy in the host cell.

Western blots performed on inoculated SAOS-2 cell extracts confirmed that autophagy was occurring (Figure 4-5A). The GFP-LC3 expression is stable in the SAOS-2 cell line, but the ratio of GFP to GFP-LC3 indicated no difference in autophagic activity between the W83Δ0553 mutant and the wild type. This is inconsistent with the experiment described in Figure 4-4, where a decrease in the number of cells undergoing autophagy was observed in cells inoculated with the W83Δ0553 mutant. Autophagy was clearly being activated in both inoculation conditions. Western analysis may not be sufficiently quantitative in distinguishing subtle variations in ongoing autophagy.
GFP-LC3 is transiently expressed in HCAEC, meaning that to quantify the levels of ongoing autophagy the GFP and GFP-LC3 bands must be compared to a stable host protein. Tubulin is the standard most commonly used, but our lab has experienced consistent problems detecting tubulin in \emph{Pg}-infected HCAEC lysates. We suspect that the action of the gingipains and other secreted proteases in the lysates are degrading host tubulin, resulting in an extremely weak signal when the lysates are analyzed via western blot. We are currently investigating alternatives to allow for GFP band density analysis in HCAEC. Figure 4-5B confirms that autophagy is ongoing in the host HCAEC in a manner that is dose-dependent. As the MOI increases, the density of the cleaved GFP band increases. However, we can make no conclusions regarding variations between the W83 wild type and the W83Δ0553 mutant based on this data.

**Modulation of Host IL-8 Secretion is Altered in the PG0553 Knockout**

\emph{Pg} induces chronic inflammation in the oral cavity partially through the action of proteases on host tissue and the production of highly antigenic LPS. This inflammatory response is mediated by chemokines and cytokines secreted by the host cell. \emph{Pg} is known to have a significant impact on cytokine induction via interactions between \emph{Pg} surface proteins and host pattern-recognition receptors (170). Disregulation of the cytokine network is a powerful mechanism for evading the host immune response. \emph{Pg} modulates host cytokines by multiple mechanisms. For example, IL-6, a highly inflammatory cytokine that mediates the acute phase immune response, is rapidly and efficiently cleaved by all three gingipains, but most efficiently by surface-associated Kgp (171). This generates an inflammatory gradient mediated by \emph{Pg} that is thought to be a major contributor to the pathogenesis of periodontal disease (41, 172, 173). Another
mechanism \textit{Pg} uses is the induction of IL-8, a powerful inflammatory mediator that induces neutrophil infiltration as part of the acute phase immune response. \textit{Pg} is known to induce IL-8 secretion in host endothelial cells through interactions between HtpG (PG0045) and TLR4 and CD91 (174). Chronic inflammation and neutrophil infiltration can result in damage to the vascular endothelium and is a potential mechanism for \textit{Pg} to contribute to atherosclerosis. HtpG is homologous to human heat shock protein Hsp90, which interacts with signal transduction pathways to induce a stress response, including the production of inflammatory cytokines. \textit{Pg} can also modulate the IL-8 chemokine response through gingipain-mediated mechanisms (175).

To determine if the W83\textDelta0553 knockout impacted cytokine cleavage or induction, ELISAs were used to assess levels of secreted IL-6 and IL-8 in \textit{Pg}-infected HCAEC supernatants (Figure 4-6). Unexpectedly, no difference was observed in secreted IL-6, as in both the wild type and the W83\textDelta0553 mutant, IL-6 levels were at or below the uninfected control. Whatever impact the absence of PG0553 may be having on the processing of the RgpA and Kgp polypeptides it does not appear to affect the ability of the gingipains to cleave IL-6, possibly because RgpB is also able to efficiently cleave IL-6 (171). It is also possible that Kgp, the most potent agent of IL-6 cleavage, was able to overcome the impact of the PG0553 knockout in this experimental setting.

As expected, strain W83 activated IL-8 secretion in HCAEC. Interestingly, IL-8 secretion in the W83\textDelta0553 mutant-infected HCAEC was significantly reduced, resembling the uninfected control (though still significantly higher than uninfected cells). IL-8 secretion is induced in vascular endothelial cells by HtpG (174) and modulated by the gingipains (175). Our current model predicts that PG0553 is necessary for the post-
translational modification of PG0232 into an active carboxypeptidase (CPG70), which processes the RgpA and Kgp polypeptides and facilitates the formation of the gingipain protease-adhesin complex on the bacterial cell surface. These results potentially reflect a loss or disruption of gingipain-mediated IL-8 modulation attributable to alterations in bacterial cell surface-associated gingipains.

However, it is also possible that PG0553 is involved in the post-translational modification of multiple proteins, including PG0045 (HtpG). How PG0045 is processed is currently unknown, but cytosolic PG0045 is detectable at 68kD while secreted PG0045 is detected at 40-44kD (176). The observed levels of IL-8 secretion in HCAEC bear a striking resemblance to similar experiments performed with a PG0045 knock out (176) and are consistent with PG0553 playing a role in the functional activation of PG0045.

**Methods**

**Cell Culture**

HCAEC (Cambrex) were maintained at 37°C with 5% CO2 in EBM-2 supplemented with EGM-2-MV singlequots (Cambrex) as described by the manufacturer. Cells were harvested and used to seed 24 well plates at 5X10^5 cells/per well. The plates were incubated for approximately 24 hours until HCAEC confluence reached 70-90%.

**Bacterial Cell Culture**

Cultures of *P. gingivalis* W83 and W83Δ0553 were grown anaerobically overnight in tryptic soy broth supplemented with 0.5% yeast extract, 0.05% L-cysteine hydrochloride, 5 µg/mL hemin and 1 µg/mL vitamin K1. These cultures were diluted to
1X10^7 CFU/mL in pre-warmed, supplemented EBM-2 media for inoculation at an MOI of 100.

**Adherence Assay**

HCAEC were treated for 30 minutes with cytochalasin D, a potent inhibitor of actin polymerization, at a concentration of 5 ug/mL in EBM-2 media. This treatment blocked the internalization of Pg, making it possible to analyze only the bacteria that successfully adhered to the HCAEC cell surface. The treated cells were then inoculated with wild type W83 and the W83Δ0553 mutant at a concentration of 1X10^7 CFU/mL. After 1 hour of exposure to Pg at 37°C, the HCAEC were gently washed and then lysed with sterile water. The lysate was plated on blood-agar medium containing 10 ug/mL gentamycin. CFU were enumerated for each strain to quantify adherence. This experiment was repeated three times with three replicates per experiment.

**Invasion Assay**

Invasion of HCAEC by the W83 wild type and the W83Δ0553 mutant were tested using a conventional antibiotic protection assay. HCAEC were inoculated with Pg at a concentration of 1X10^7 CFU/mL. The HCAEC were exposed to Pg for 1.5 hours at 37°C. The cells were then treated with EBM-2 media containing 300ug/mL gentamycin and 400 ug/mL metronidazole for 1 hour to kill any extracellular bacteria. The HCAEC were also exposed to Pg for 0.5, 1.0 and 2.5 hours with no antibiotic protection. At each time point, the HCAEC were lysed with sterile water and the lysate was plated on blood-agar medium containing 10 ug/mL gentamycin. This experiment was repeated three times with five replicates per experiment.
Autophagy Activation in SAOS-2

SAOS-2 cells were stably transfected with GFP-LC3. These cells were cultured and maintained in EBM-2 media as previously described for HCAEC. GFP-expressing SAOS-2 were seeded onto coverslips in 12-well plates at 5X10^5 cells/per well. Experimental conditions were inoculated with *Pg* at an MOI of 100 without antibiotic protection. Fed controls were treated with EBM-2 media alone. Starved controls were treated with KOH media. After 1 hour, the cells were fixed, mounted and imaged on a Leica DM IRBE microscope at 400X magnification. A cell was assessed as ‘undergoing autophagy’ if it contained five or more labeled autophagic vacuoles. Approximately 500 cells were counted in each condition.

Autophagy Activation and Trafficking in HCAEC

HCAEC were maintained as previously described and seeded onto coverslips in 12-well plates at 5X10^5 cells/per well. The HCAEC were transiently transduced with GFP-LC3 for 48 hours. Transduced cells were inoculated with *Pg* at an MOI of 100 without antibiotic protection for 4 hours. The infected HCAEC were then fixed and mounted with DAPI and imaged on a Leica DM IRBE microscope at 600X magnification. DAPI staining was used to quantify intracellular bacteria. GFP-LC3 was used to assess the activation of autophagy in the HCAEC. Colocalization was used to assess trafficking to autophagic vacuoles.

Western Blots

HCAEC and SAOS-2 cells expressing GFP-LC3 were seeded into 12-well plates at 5X10^5 cells/per well. Cells were inoculated with *Pg* at an MOI of 100 and incubated without antibiotic protection for 1 hour. Following inoculation period, 200 µL of Laemmli sample buffer containing protease inhibitor cocktail (Sigma-Aldrich) at a 1:100 dilution
was added to each well. After freezing overnight, the samples were boiled for 5 min, run on a 10% polyacrylamide gel, and blotted onto a PVDF membrane for 1 hr using a semi-dry BioRad TransBlot blotter. After blocking with 5% nonfat dried milk in PBS with 0.1% Tween 20 (PBSTw) for 1 hour at 25°C, the membrane was incubated with a mouse anti-GFP antibody (Sigma-Aldrich) at a 1:5,000 dilution for 18 h at 4°C. Horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich) was employed at a 1:10,000 dilution in 2% milk in PBSTw for 2 h at 25°C. After several washes with PBSTw, the blots were transferred to a chemiluminescent solution and imaged on x-ray film.

**ELISAs**

HCAEC were maintained and seeded into 24-well plates as previously described. Cells were inoculated with *Pg* at an MOI of 100 for 1.5 hours at 37°C. Untreated controls were treated with EBM-2 alone. The cells were then treated with EBM-2 media containing 300ug/mL gentamycin and 400 ug/mL metronidazole for 24 hours at 37°C. Supernatants were collected and stored under liquid nitrogen.

Supernatant samples were assessed for cytokine secretion using the IL-6 (CHC1263) and IL-8 (CHC1303) human antibody pair kits from Invitrogen. ELISA assays were conducted according to the manufacturer’s instructions and output was generated using the BioRad plate reader.

**Discussion**

We demonstrated previously that PG0553 had a significant impact on intracellular protease activity in *Pg* as measured in the soluble lysate fraction. A potential mechanism of PG0553 activity is the facilitation of PG0232 processing in association with the por secretion pathway. This hypothesis is supported by the
absence of active CPG70 activity observed in the casein zymograms (Figure 4-4) and the impact on CTD family proteins observed in the PG0553 knockout. CPG70, the truncated and proteolytically active form of PG0232, processes the C-terminal lysine residues of RgpA and Kgp polypeptides prior to secretion (138). Without this processing step, formation of the gingipain protease-adhesin complex on the bacterial cell surface of *Pg* is inhibited and the structure of surface peptides are altered (138). We hypothesized that the impact of this conformational change, which may not impact the gingipain protease domain following por secretion and therefore be difficult to detect in enzymatic assays, could be observed more accurately in cellular assays that reproduce some of the pressures of a host environment.

This work investigated the interactions between W83Δ0553 and host cells *in vitro*. Proteases and surface adhesins, such as the RgpA and Kgp polypeptides, play a major role in host cell interactions. Subtle alterations in the structure of these surface-associated protein complexes have the potential to significantly impact multiple aspects of virulence in *Pg*. We observed impairment in adherence, cellular invasion, autophagy activation, trafficking and IL-8 induction in host cells coincubated with W83Δ0553 in a manner that is consistent with impairments in the known functions of CPG70 and the gingipains.

The specific effector molecules used by *Pg* to activate autophagy in the host cell are currently unknown, but the SAOS-2 system indicates that *Pg* does not require contact or internalization in order to impact host autophagy. Sterile-filtered *Pg* supernatants are able to activate autophagy in endothelial cells, strongly suggesting that the effector is secreted (Progulske-Fox lab, unpublished data). Additional studies
performed in the Progulske-Fox lab demonstrate that the gingipains are also necessary to activate autophagy in SAOS-2 (unpublished). Active gingipains are also necessary to prevent fusion of the autophagosome with the lysosome in HCAECs (165). The PG0553 knockout is able to activate autophagy in the host cell and traffic to autophagosomes, but to a lesser extent than the wild type. On average, 38±13% of intracellular W83 and 17±8% of W83Δ0553 were localized to autophagic vacuoles in HCAEC. This is consistent with subtle alterations in the structure of bacterial cell surface-associated gingipain complexes impacting the role the gingipains play in manipulating the host autophagic pathway.

HtpG (PG0045) is a chaperone protein expressed by *Pg* that functions as a potent activator of inflammatory cytokines in the host (174). It was first identified as an antigen associated with periodontal health, with high levels of anti-HtpG IgG found to be predictive of periodontal health in patients (177). Characterization of HtpG in *Pg* found that the protein was localized to the cytosolic fraction with a molecular weight of 68 kD, but localized to the cell membrane and vesicle fractions with a molecular weight of 44 kD, and to a lesser extent 40 kD (176). This suggests that PG0045 must be processed by *Pg* to form a truncated, metabolically active HtpG molecule prior to secretion. The significant reduction in IL-8 secretion in HCAEC coincubated with the W83Δ0553 mutant suggests that PG0553 may be associated with the processing of PG0045; similar to the role it is predicted to play in the N-terminal cleavage of PG0232 to form an active carboxypeptidase. This phenotype is also potentially attributable to alterations in the gingipains, which are known to modulate the IL-8 response in endothelial cells (175).
The phenotype of the W83Δ0553 mutant is subtle and diverse, but it is consistent with a reduction in CPG70 activity impacting the function of the gingipains. The RgpA and Kgp polypeptides are processed by CPG70, facilitating the formation of the gingipain protease-adhesin complex on the bacterial cell surface. Functional gingipains have been demonstrated experimentally to impact host cell adherence and internalization (178), activation of autophagy (Porgulske-Fox lab, unpublished), persistence within autophagic vacuoles (165), and modulation of the host IL-8 response (175); functions that were reduced or inhibited in the PG0553 knockout. It is also possible that PG0553 is involved with the post-translational modification of additional effector proteins, similar to its predicted function in facilitating the N-terminal cleavage and activation of PG0232. PG0045 (HtpG) is significantly processed prior to secretion, and a lack of PG0045 activity would also explain the reduction in IL-8 secretion observed in Figure 4-6. Further experimentation is required to more precisely define the role of PG0553 in the complex virulence systems of Pg.
Figure 4-1. Model of *Pg* invasion of HCAEC (37).
Figure 4-2. Interactions between *P. gingivalis* and HCAEC host cells are impaired in the W83Δ0553 mutant. A) Adherence Assay. HCAEC pre-treated with cytochalasin D to inhibit bacterial cell uptake were inoculated with *P. gingivalis*. Infected cells were washed and lysed. The lysate was plated and adherent bacteria were quantified by enumerating CFU. The W83Δ0553 mutant was found to be significantly impaired. Adherence was reduced 1.5-fold. B) Invasion Assay. HCAEC were inoculated with *P. gingivalis* and lysed at various time points. The lysate was plated and adherent and invasive bacteria were quantified by enumerating CFUs. The W83Δ0553 mutant was found to be significantly impaired at 0.5 (3.4-fold reduction) and 1.0 hours (2.6-fold reduction). At 2.5 hours, both with and without antibiotic protection, there was no significant difference in recoverable CFU. These results are the average of five biological replicates (n=5) and are plated in duplicate. Error bars indicate the standard deviation. *p<0.001, **p<0.0001
Figure 4-3. Activation of host cell autophagy is reduced in the W83Δ0553 mutant in a SAOS-2 model. SAOS-2 cells were transfected with GFP-LC3 to label autophagic vacuoles. *Pg* does not invade SAOS-2 but is able to activate autophagy in inoculated cells. After one hour of inoculation, the cells were fixed and imaged. Fed (A) and starved (B) controls were compared to cells coincubated with W83Δ0553 (C) and W83 wild type (D). A cell was assessed as ‘undergoing autophagy’ if it contained five or more labeled autophagic vacuoles. Approximately 500 cells were counted in each condition. The proportion of cells with ongoing autophagy was determined and analyzed by chi-square analysis. Individual conditions were compared using Fisher’s Exact t-test. (E). Cells treated with *Pg* W83 and the W83Δ0553 mutant were undergoing autophagy at significantly higher rates than the fed controls (p<0.0001, p=0.0016). However, W83Δ0553 was significantly impaired when compared to the wild type in this model (p=0.004). These results are representative of two separate experiments.
Figure 4-4. Activation of host cell autophagy and trafficking to autophagic vacuoles is reduced in the W83Δ0553 mutant in a HCAEC model. HCAEC were transduced with Ad GFP-LC3 (green) to label autophagic vacuoles. The cells were spin-inoculated with *Pg* to synchronize invasion. After four hours of coincubation with W83 (A) or W83Δ0553 (B) the cells were fixed with 4% PFA and stained with DAPI (red) to label the bacterial DNA. (C) The number of autophagic vacuoles per cell were counted and evaluated using Mann Whitney t-test (n=30) and were found to be significantly reduced in W83Δ0553 (p=0.0035). Error bars indicate standard deviation. (D) Intracellular bacteria were counted and classified as either LC3+ or LC3- to quantify colocalization. The proportion of intracellular bacteria trafficking through the autophagic pathway was analyzed by chi-square analysis. W83Δ0553 was found to be significantly impaired in trafficking (p<0.0001), though autophagy activation and trafficking to autophagic vacuoles is still occurring. Panel 1: DNA staining alone. Panel 2: GFP labeling alone. Panel 3: Merged. Results are representative of two separate experiments.
Figure 4-5. Measuring autophagy activation in host cells by Western Blot. Western blots against GFP in GFP-LC3 SAOS-2 at an MOI of 100 (A) and GFP-LC3 HCAEC at the indicated MOIs (B). LC3 is incorporated into the autophagic membrane as autophagosomes form. As autophagosomes mature, the GFP marker is clipped off. This clip can be measured by western blot as an indicator of ongoing autophagy. These experiments confirm that autophagy is being activated in both the wild type and the mutant. Arrows at approximately 44kD indicate the LC3-GFP conjugate. Arrows at approximately 26 kD indicate the GFP alone.
Figure 4-6. Measuring inflammatory cytokine secretion by ELISA. Secretion of key inflammatory cytokines was measured in *Pg*-infected HCAEC supernatants using Invitrogen human antibody kits. The W83Δ0553 mutant was found to be less immunogenic than the wild type, as indicated by significantly reduced levels of IL-8 secretion (p<0.001). This is consistent with W83Δ0553 being impaired in the secretion of key virulence factors. Results are the average of five biological replicates (n=5) that were plated in duplicate. Results were analyzed using Student’s t-test. Error bars indicate standard deviation.
CHAPTER 5
DISCUSSION OF RESULTS AND PLANS FOR FUTURE EXPERIMENTS

PG0553 is important for proteolytic activity in *Pg* strain W83, potentially through interactions with carboxypeptidase, PG0232, and the por secretion system. Knocking out the *PG0553* gene resulted in significant alterations in protease activity as measured by FITC-casein processing, GpK-prna processing and by zymogram analysis. The protease phenotype observed in W83Δ0553 is associated with altered host cell interactions, including impaired cellular adherence and invasion, altered activation of autophagy and trafficking, and reduced induction of IL-8 secretion in HCAEC. Thus, the available data suggests that PG0553 plays a biologically significant role in *Porphyromonas gingivalis* virulence, but additional research is required to confirm the function of PG0553.

Perhaps the most important hurdle that must be overcome in order to validate the function of PG0553 is generating a purified sample of the protein. Purified PG0553 would enable co-precipitation experiments to directly identify binding partners by mass spectrometry, as well as the production of specific antibodies for immunoprecipitation and western blot analysis. Unfortunately attempts to express PG0553 in a pET vector have been confounded by instability in *E. coli*, resulting in an unusually high rate of mutations and sequence inversions. There are several options that could potentially overcome this limitation, but each requires a significant investment of time and resources because of the necessary materials and nature of the approaches, which require significant trial and error to optimize.

However, a truncated version of *PG0553* was successfully cloned into pET-blue and expressed in *E. coli*, but it is impossible to predict the significance of the truncated
region due to the lack of information regarding the active domains and secondary structure of PG0553. However, this PG0553 fragment could still potentially be used to generate a specific antibody against PG0553. In addition, systems other than *E. coli* are available for protein over-expression, but would likely require significant troubleshooting to function ideally with *Pg* proteins. For example, insect cell expression systems are available which are known to be more tolerant of proteins that are toxic or unstable in *E. coli*. With sufficient funding it is also possible to commercially synthesize a protein expression vector containing PG0553, by-passing the cloning steps which generated errors in the PG0553 sequence. PG0553 is not predicted to be membrane-bound and could potentially be isolated directly from *Pg* lysates if the predicted molecular weight corresponds to mature PG0553. However, it is not yet known if PG0553 itself is processed by other proteases to become metabolically active. A specific antibody against PG0553 would greatly facilitate isolation from endogenous lysates.

Even in the absence of purified protein or antibodies against PG0553 there are several experiments which could be performed to generate support for the proposed model of PG0553 interactions with gingipains processing and por secretion. First, the current model predicts that PG0553 functions in the periplasm in association with PG0232 and PorSS. The bacterial cell lysates used to assess protease activity do not distinguish between periplasm and cytoplasm, between supernatant and secreted vesicles, or between membrane-bound and surface proteins. A more thorough fractionation would generate a specific localization of PG0553-associated alterations in protease activity. The current model predicts that impaired protease function would be primarily localized to the periplasm.
Future experiments designed to analyze protease activity in *Pg* should consider two important factors. Firstly, the longer it takes to process a sample the less reliable and consistent the readings will be due to the ongoing activity of proteases within the sample. All samples should be kept on ice whenever possible and processed with haste to prevent deterioration of the proteases of interest by the action of other proteases. Protease inhibitors could also be added to the samples to slow the degradation process, but could prove problematic if they have a non-specific effect that carries over to the assay in question. Second, the growth phase of the bacterial cells has a significant impact on the level of protease activity. While the experiments described in this manuscript accounted for this source of potential variation by diluting overnight cultures to an OD of 0.5 and doubling the OD before performing the experiments in question, the substantial error bars observed in several experiments suggests that this was not sufficient to synchronize the bacterial cultures in exponential phase. In the future, it would be advisable to allow for multiple doublings, possibly diluting the overnight culture to an OD of 0.25 or 0.12 and allowing it to grow to an OD of 1.0. This will create a more homogenized sample and likely reduce variation between experiments.

The current model also predicts that PG0553 is associated with the processing of PG0232, which is truncated at the N-terminus to form the active carboxypeptidase CPG70 (139). In the casein zymogram (Figure 3-3A), the band of activity associated with CPG70 was absent in W83Δ0553. Based on this, it is reasonable to expect a significant reduction or complete ablation of CPG70 activity in the W83Δ0553 mutant. This would produce a phenotype similar to that of the PG0232 knockout strain constructed by the Reynolds lab in *Pg* W50 to assess the role of CPG70 in gingipain
processing (138, 139). Through 2D electrophoresis and MALDI mass spectrometry analysis of *Pg* surface protein extracts, Veith et al. (148) identified peaks of different mass on the surface of the CPG70 knockout as the unprocessed C-terminal peptides of RgpA and Kgp. CPG70 is responsible for cleavage at several C-terminal lysine residues, generating a distinguishable difference in mass in the gingipains secreted by the mutant. (138) If the alteration in CPG70 activity is also responsible, either wholly or in part, for the phenotype observed in the W83Δ0553 mutant, then similar variations should be detectable between cell surface extracts of W83 and W83Δ0553. By generating antibodies against CPG70, we could also directly observe changes in CPG70 processing in the PG0553 knockout via western blot. Though costly, this technique would be the most convincing method of confirming the alterations in CPG70 processing indicated by zymogram analysis in W83Δ0553.

The Reynolds lab also found that the CPG70 mutant was avirulent in a murine lesion model when compared to the W50 wild type (139). In the work presented here, knocking out *PG0553* in *Pg* strain W83 significantly altered host cell interactions, but the phenotype observed in the *in vitro* cellular assays was subtle. Though occurring at a slower rate, gingipains were still being produced and secreted; autophagy was still being activated; host cell invasion was still occurring. There is significant potential for the subtle alterations in gingipain conformation predicted by the current model to have a more dramatic impact *in vivo*. It would be very interesting to test the virulence of the *PG0553* knockout strain in a similar murine model. If virulence is significantly decreased *in vivo*, it would strongly support the predicted role of PG0553 in gingipain processing.
PG0553 also has the potential to mediate the post-translational modification of other CTD family proteins, such as PG1837, major hemagglutinin HagA in *Pg*. Antibodies against HagA and the gingipains, as well as other CTD proteins of interest such as HBP35 and CPG70, could be used to directly measure changes in CTD protein processing in the *PG0553* knockout via western blot. Specific antibodies could also be used in immunoprecipitation experiments to determine how these important virulence factors interact with PorSS and PG0553.

When we examined cytokine induction in HCAEC, W83Δ0553 demonstrated a similar phenotype to the HtpG knockout strain developed by Shelburne et al in 2007 (174). HtpG is a secreted factor in *Pg* that induces IL-8 secretion in vascular endothelial cells through interactions with TLR4 and CD91 (174). W83Δ0553 did not strongly induce IL-8 secretion in host HCAEC, with cytokine levels of infected supernatants resembling the uninfected control. HtpG is also processed significantly in *Pg* prior to secretion, with bands detected by western blot in the cytosolic fraction of *Pg* at a molecular weight of 68 kD and in the membrane/vesicle fraction at 44 and 40 kD (176). While HtpG is not a CTD family protein, we cannot rule out the potential for PG0553 to impact the processing of additional secreted factors. Confirming a relationship between PG0553 and HtpG would establish a role for PG0553 that extends beyond por-mediated secretion.

Again, purified PG0553 protein would be a powerful tool for confirming an interaction between PG0553 and PG0045. However, the HtpG mutant developed by Shelburne et al. (174) suggests how W83Δ0553 may impact host cell cytokine secretion differently from W83 if PG0553 is associated with PG0045 processing and activation.
THP-1 cells were treated with wild type *Pg* W83 and W83ΔHtpG and the concentration of various cytokines were measured in the host cell supernatants. In addition to IL-8, *Pg* W83 induced the secretion of IL-1α, IL-1β and TNFα. W83ΔHtpG induced significantly less secretion of all four cytokines than the wild type (174). If PG0553 is involved with processing PG0045 into active HtpG, then we would expect to see a reduction in these cytokines as well when measured by ELISA according to our protocol.

In our model, knocking out PG0553 impairs activation of the carboxypeptidase CPG70, resulting in the secretion of unprocessed RgpA and Kgp polypeptides. Protein complexes derived from RgpA and Kgp polypeptides are major outer membrane components in *Pg* that are critical for virulence. Subtle changes in these complexes have the potential to significantly alter the properties of the *Pg* outer membrane, which functions as the mediator of host cell interactions. The significance of these phenotypic characteristics is difficult to demonstrate using *in vitro* cellular assays, which do not reproduce the physical and immunological pressures of an *in vivo* model. However, there is much that can be done experimentally *in vitro* to validate the proposed model. Further research is highly recommended to fully elucidate the role of PG0553 in the increasingly complex and fascinating system of virulence factors in *Pg*.

Characterizing the W83Δ0553 mutant and determining the biological significance of PG0553 has been a challenging process. As the pieces come together, a complex picture is forming where PG0553 functions as an intermediary for the recruitment and processing of key effector proteins in the periplasm prior to PorSS-mediated secretion. Protein secretion in gram-negative species is directly related to the pathogenicity of the organism and is a major field of study. Increasing our understanding of how virulence
factors are processed and secreted improves our ability to control pathogenic species and increases our ability to identify virulence-associated proteins in less-characterized species.

The PorSS complex is a novel protein secretion system that is unique to *Pg* and is critical for virulence. While it is known that CTD family proteins secreted through PorSS are heavily processed, the mechanism of this processing and it is regulated have not yet been identified. This is the first time a potential mediator of this process has been described. The characterization of PG0553 and its role in por secretion presented here represents a significant contribution to our understanding of this novel system.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Amanda Barrett first became interested in a scientific career while volunteering as an undergraduate student in the Ostrov lab in the Pathology Department of the University of Florida. After being immersed in biomedical research for nearly three years, she obtained her bachelor's degree in psychology from the University of Florida in May of 2006 and promptly joined the IDP graduate research program. After a year of rotations within the program, she settled into the lab of Dr. Ann Progulske-Fox in the Oral Biology Department. She has spent her graduate career studying Porphyromonas gingivalis in the context of cardiovascular disease and characterizing the roles of specific genes associated with the invasion of human coronary artery endothelial cells by Pg. She graduated from the IDP program in May of 2013 with a PhD in Medical Science, specializing in Immunology and Microbiology. Her cats are very proud of her. Her family and her boyfriend probably are too.

TL;DR