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Substrates of the Plasminogen Activator Protease of *Yersinia pestis*

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32.1 Introduction

The plasminogen activator protease (Pla) of *Yersinia pestis* is a critical virulence determinant in the progression of both bubonic and pneumonic plague. A member of the ompT family of Gram-negative transmembrane proteases, Pla forms a conserved β -barrel fold in the bacterial outer membrane which permits the interaction of the protease with target substrates for cleavage. In vitro studies have identified numerous mammalian host targets, many of which comprise components of the coagulation and fibrinolytic cascades. Through the cleavage of host plasminogen, α 2-antiplasmin, plasminogen activator inhibitor-1 (PAI-1), thrombin-activatable fibrinolysis inhibitor (TAFI), and tissue factor pathway inhibitor (TFPI), Pla is hypothesized to disrupt coagulation pathways that are initiated as a natural host response to infection and inflammation. The clearance of fibrin clots, enhanced by the activities of Pla, may alleviate physical barriers to bacterial dissemination and inhibit the recruitment of immune cells. Subversion of innate immunity may be further potentiated through the Pla-dependent inactivation of complement protein C3 and cationic antimicrobial peptides. Currently, the precise molecular mechanisms of Pla-conferred virulence in vivo remain unclear and likely vary based upon route of infection. However, evidence supports Pla as a versatile virulence factor that may specifically cleave unique subsets of host proteins during the progression of bubonic and pneumonic plague.

32.2 Contribution of Pla to the Virulence of *Yersinia pestis*

The plasminogen activator protease (Pla) is encoded by the *Yersinia pestis*-specific 9.5-kb plasmid pPCP1 (Sodeinde and Goguen 1988). The acquisition of this plasmid is thought to have occurred as *Y. pestis* diverged from its genetic progenitor *Yersinia pseudotuberculosis* and has contributed significantly to the ability of this *Yersinia* species to cause the highly infectious and deadly disease plague (Achtman et al. 1999). Pla forms a β -barrel fold in the bacterial outer membrane that is conserved among other ompT family member proteases. Notably related in sequence and structure to OmpT of *Escherichia coli* and PgtE of *Salmonella enterica*, Pla has evolved unique substrate specificities that allows for the establishment of both the bubonic and pneumonic forms of plague.

Bubonic plague is initiated by infection of the mammalian subdermal or subcutaneous tissue with *Y. pestis*. Bacteria disseminate from the local site of infection into the lymphatics, and Pla has been implicated as a critical virulence factor in enhancing this process following transmission by flea bite or injection (Sebbane et al. 2006). In addition, mice infected

subcutaneously with *pla*-expressing *Y. pestis* develop widespread bacterial foci in the tissue with little visible recruitment of inflammatory cells. In contrast, the foci of Pla-deficient strains are rapidly and densely packed with infiltrating inflammatory cells, which may restrict the presence of free bacteria (Sodeinde et al. 1992). Deletion of *pla* severely attenuates infection with *Y. pestis* by this route, increasing the LD₅₀ by approximately one million fold (Sodeinde et al. 1992). Coagulation occurs as a result of infection and also contributes to enhanced inflammation through the recruitment of immune cells to bacterial foci. Fibrin deposition stimulates the expression of pro-inflammatory cytokines and chemokines by mononuclear and endothelial cells, respectively (Szaba and Smiley 2002). Bubonic plague ultimately results in systemic spread of *Y. pestis*, and death of the animal is likely due to the overwhelming numbers of *Y. pestis* bacilli in the blood.

Pla is also required for the development of primary pneumonic plague, but unlike during bubonic infection, Pla is dispensable for dissemination from the initial site of infection (in this case, the lungs) (Lathem et al. 2007). Intranasal infection with *Y. pestis* leads to the rapid development of a fulminant pneumonia involving tissue damage, edema, and significant immune cell influx consisting primarily of neutrophils. Pneumonic plague is a biphasic syndrome: the early phase of disease is characterized as relatively noninflammatory, but as the infection progresses and bacterial load increases, massive inflammatory lesions are formed and the synthesis of pro-inflammatory cytokines is upregulated (Lathem et al. 2005). In the absence of Pla, a quiescent or anti-inflammatory state is maintained in the lungs, bacterial numbers do not appreciably increase, and the infection does not progress to a debilitating and deadly pneumonia. While Pla is necessary for the progression of both bubonic and pneumonic plague, it seems to have opposing contributions to virulence depending on the route of infection. During bubonic plague, Pla is necessary for bacterial dissemination and inhibition of immune cell recruitment, yet does not affect bacterial growth at the local site of infection. In contrast, following intranasal infection, Pla is necessary for outgrowth of the bacteria in the lungs by aiding either growth or survival in this environment, yet is dispensable for dissemination. It remains unclear whether Pla actively recruits immune cells and directly causes inflammation in the lung through substrate cleavage, or whether host responses simply correlate with the growing bacterial burden and injury within the lungs.

The precise mechanisms by which Pla contributes to virulence during bubonic and pneumonic plague are largely unknown, although infections with catalytically inactive Pla point mutants confirm that the proteolytic activity of Pla is necessary for its contribution to virulence (Degen et al. 2007; Lathem et al. 2007). Of specific interest is how Pla mediates the massive inflammatory response observed during the later stages of pneumonic disease. In addition to its proteolytic activities, Pla possesses several non-enzymatic functions in vitro including binding to extracellular matrix (ECM) proteins, enhancing invasion into epithelial cells, and mediating the delivery of type-III secretion system (T3SS) effector proteins into host cells (Cowan et al. 2000; Felek et al. 2010). Interactions with host innate immune cells occur via the binding of Pla to the murine lectin receptor DEC-205 to facilitate attachment, invasion, and bacterial dissemination following a pneumonic infection (Zhang et al. 2008). In vivo studies are necessary to determine if these additional activities of Pla are also necessary for *Y. pestis* to cause plague.

32.2.1 Substrates of Pla

Work from multiple laboratories has identified numerous host substrates cleaved by Pla in vitro, yet in vivo data on the interactions of these factors with Pla and their contributions to virulence remain limited. There is no known Pla consensus cleavage motif, but cleavage is thought to preferentially occur between consecutive basic residues (as determined for OmpT using small peptide libraries with internally quenched fluorophores or phage-displayed

synthetic peptides) (Dekker et al. 2001, McCarter et al. 2004). Given the vastly different presentations of plague, it is likely that Pla acts upon different yet overlapping subsets of substrate proteins depending on route of infection.

32.3 Pla Cleaves Host Proteins Involved with Coagulation and Fibrinolysis

The coagulation system is likely a major physiological target of Pla activity in vivo as altered fibrin(ogen) deposition is detected during both bubonic and pneumonic plague (Degen et al. 2007; Lathem et al. 2007). As a consequence of trauma, injury, or infection, host coagulation cascades are initiated leading to the recruitment and deposition of fibrin at the local site of infection. During bubonic plague, these deposits are thought to physically inhibit the spread of subcutaneous or intradermal *Y. pestis*. Fibrin(ogen) also potentiates inflammatory signaling during bacterial infections by stimulating the chemotactic recruitment and activation of polymorphonuclear neutrophils through interactions with the $\alpha_M \beta_2$ integrin leukocyte receptor contained within the γ chain of fibrinogen (Flick et al. 2004, Forsyth et al. 2001). As a means of facilitating dissemination through these host defense barriers, Pla is able to cleave several host proteins that may potentiate fibrinolysis (Fig. 32.1).

32.3.1 Plasminogen

One of the first Pla substrates to be identified was the circulating fibrinolytic regulator plasminogen (Beesley et al. 1967). Pla efficiently cleaves the zymogen plasminogen into its active plasmin form, a potent serine protease with the ability to degrade polymerized fibrin clots. Pla activates plasminogen through cleavage at a single site between residues Arg561 and Val562 of the zymogen, the same residues at which endogenous plasminogen activators cleave (Robbins et al. 1967; Sodeinde et al. 1992). During bubonic plague, fibrinolysis mediated by the Pla-dependent generation of plasmin may enhance bacterial dissemination and escape from immobilizing fibrin clots. The degradation of these clots may also prevent or reduce immune cell recruitment at the site of bacterial colonization. In addition to polymerized fibrin, plasmin has also been shown to activate latent pro-matrix metalloproteinases (proMMPs) and to cleave ECM components such as laminin (Haiko et al. 2009). These activities may be linked, as MMPs cleave collagen within ECMs that form tissue barriers and hinder bacterial migration. While the proteolytic targets of plasmin critical for *Y. pestis* virulence remain to be fully defined in vivo, it is likely that multiple pathways are involved to facilitate dissemination.

The critical role of plasminogen as a Pla substrate during bubonic plague is supported by experiments utilizing plasminogen-deficient mice. These mice are more resistant than wild-type animals to subcutaneous *Y. pestis* infection: in the absence of plasminogen, the LD₅₀ of a wild-type strain of *Y. pestis* increases 100-fold compared to control mice (Degen et al. 2007). However, infection with Pla-deficient *Y. pestis* leads to an even greater attenuation in virulence, suggesting that interactions with plasminogen may not fully explain the virulence conferred by Pla. Inflammatory cell recruitment was also seen in the hepatic lesions of *Y. pestis*-infected plasminogen-deficient mice regardless of *pla* expression, suggesting that both Pla and plasminogen are needed to avoid immune cell recruitment and resulting inflammation (Degen et al. 2007). During pneumonic plague, infection with *Y. pestis* is also associated with pulmonary fibrin deposition, however the pattern of deposition varied depending on *pla* expression (Lathem et al. 2007). It is still unknown whether uncontrolled plasminogen activation in the lungs and fibrinolysis is required for pneumonic plague progression.

32.3.2 α_2 -Antiplasmin

α_2 -Antiplasmin is a serine protease inhibitor (serpin) that specifically and irreversibly binds in an equimolar ratio with circulating soluble plasmin to inhibit its proteolytic activity (Wiman and Collen 1978). α_2 -Antiplasmin primarily inhibits free circulating plasmin, but is also able to inhibit cell-bound plasmin with slower kinetics (Ponting et al. 1992). Pla cleaves α_2 -antiplasmin, preventing this serpin from inhibiting plasmin (Kukkonen et al. 2001). Interestingly, the related *E. coli* omptin OmpT is unable to bind or inactivate α_2 -antiplasmin, which is dependent on the presence of specific residues in the outer loops of Pla (Kukkonen et al. 2001). The concerted action of Pla to simultaneously activate plasminogen while inactivating α_2 -antiplasmin may lead to the uncontrolled production of active plasmin and resulting clearance of fibrin depositions.

32.3.3 Plasminogen Activator Inhibitor-1

Plasminogen activator inhibitor-1 (PAI-1) is the principal inhibitor of tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). These endogenous activators of plasminogen are mainly released from vascular endothelial cells in response to the inflammation caused by sepsis or trauma (Levi et al. 2004). To maintain a delicate hemostatic balance, the serpin PAI-1 is simultaneously released by these cells to counteract the fibrinolytic response. PAI-1 rapidly inhibits tPA and uPA by inserting plasminogen-mimic residues into their catalytic sites to form inert, covalent complexes (Bergmann and Hammerschmidt 2007; Vaughan 2005). This interaction leads to a decrease in plasmin-dependent fibrinolysis and MMP activation in vivo (Ghosh and Vaughan 2011).

Pla cleaves PAI-1 between residues R346 and M347, which serves as the functional bait peptide bond for the inhibitor (Haiko et al. 2010). The resulting PAI-1 fragments are unable to inhibit the enzymatic activity of uPA. As with the Pla substrates described earlier, the predicted overall outcome of PAI-1 cleavage is enhanced plasmin formation and fibrinolysis. The Kop omptin protease of Gram-negative *Klebsiella pneumoniae* is similarly able to inactivate PAI-1 (Haiko et al. 2010). In response to infection with this pathogen, PAI-1 deficiency results in a decreased level of neutrophil influx to the pulmonary compartment during pneumonia. This impairment of host defenses leads to increased bacterial out-growth, enhanced dissemination, and decreased survival of infected mice (Renckens et al. 2007).

32.3.4 Thrombin-Activatable Fibrinolysis Inhibitor

Cleavage of thrombin-activatable fibrinolysis inhibitor (TAFI) provides another means by which Pla may enhance fibrinolysis. TAFI is an anti fibrinolytic plasma protein that is processed from proenzyme to mature TAFIa form by thrombin–thrombomodulin complexes. TAFIa inhibits fibrinolysis and plasmin formation by cleaving at C-terminal lysine residues within partially degraded fibrin. This disrupts the fibrin region necessary for the binding of host tPA and plasminogen (Valls Seron et al. 2010). By preventing binding of these fibrinolytic factors, plasmin generation is unable to escalate and fibrinolysis is diminished. Pla is able to cleave TAFI near its C-terminus, preventing activation to TAFIa during subsequent incubation with thrombin–thrombomodulin (Valls Seron et al. 2010). This activity may have a pro-fibrinolytic effect in vivo, although biological significance of this interaction remains to be elucidated. In addition to the direct inactivation of TAFI by Pla, TAFIa can also be inactivated through proteolysis by plasmin (Marx et al. 2002).

32.3.5 Tissue Factor Pathway Inhibitor

In contrast to the cleavage of substrates described earlier, tissue factor pathway inhibitor (TFPI) cleavage by Pla is predicted to have procoagulant consequences. Early work with *Y.*

pestis described a coagulase activity under certain conditions that is dependent on the expression of *pla* (Sodeinde and Goguen 1988). Recent work identifying TFPI as a Pla substrate may provide a possible biochemical basis for this unexplained activity (Yun et al. 2009). TFPI is an anticoagulant protein that reversibly binds to coagulation factor Xa (FXa). This bimolecular TFPI–FXa complex is a potent inhibitor of the procoagulant complex TF:FVIIa (the primary initiator of coagulation in vivo), which acts to block further coagulation at this point in the cascade (Kasthuri et al. 2010). TFPI-mediated inhibition is further enhanced by the protein S cofactor and is functionally anticoagulant even at low nanomolar concentrations (Hackeng et al. 2006; Kasthuri et al. 2010). Most TFPI in vivo is bound to the endothelium while soluble TFPI can be found circulating in plasma and released by activated platelets.

It was recently reported that TFPI is highly sensitive to proteolysis by Pla, disrupting the TFPI-mediated inhibition of clot formation (Yun et al. 2009). Cleavage of TFPI by Pla occurs between residues K249 and G250. Cleavage at this location is predicted to separate the C-terminal tail region from the inhibitory Kunitz-1 and -2 domains and explains the inactivation of TPFI by preventing its association with FXa (Lockett and Mast 2002). In this way, TFPI inactivation is expected to enhance coagulation, in contrast to the cleavage of plasminogen, α_2 -antiplasmin, PAI-1, and TAFI, which are thought to increase fibrinolysis. To explain this potential conflict, Yun et al. hypothesize that through TFPI cleavage, *Y. pestis* initially promotes activation of the clotting system to form a localized barrier to protect replicating bacteria from detection by host inflammatory cells early during infection (Yun et al. 2009). As such, interactions of Pla with the hemostatic system are likely to be complex and may be temporally diverse, promoting clot formation early during infection and fibrinolysis at later stages. Conversely, the high sensitivity of TFPI to proteolytic inactivation by bacterial omptins may represent an evolutionary movement by the host to intensify protective pro-coagulation in response to bacterial infection (Yun et al. 2009). It remains to be seen whether TFPI cleavage in vivo contributes to host defense by delaying fibrinolysis or whether TFPI cleavage provides a survival advantage to *Y. pestis*.

32.4 Pla Inactivates Complement

As a central component of innate immune defenses, complement is a common target for inactivation or modification by bacterial virulence factors. Omptins were first discovered to inactivate complement when Pla was shown to cleave the central complement component 3 (C3) (Sodeinde et al. 1992). Cleavage of C3 is predicted to inhibit trafficking of inflammatory cells to foci of infection by disrupting complement prior to the production of the chemoattractant molecule C5a. Cleavage may also promote the serum resistance of *Y. pestis* by reducing bacterial opsonophagocytosis. The potential outcomes resulting from C3 inactivation by *Y. pestis* correlate with Pla-dependent effects during bubonic infections.

PgtE of *S. enterica* serovar Typhimurium, a closely related ortholog of Pla, was found to cleave the complement components C3b, C4b, and C5 that result in the increased serum resistance of *Salmonella* (Ramu et al. 2007; Sodeinde and Goguen 1989). C3b and C4b are convertase components critical for the initiation of the complement cascade, while conversion of C5 to C5a acts as a potent chemoattractant for phagocytes (Walport 2001; Ward and Newman 1969). While complement cleavage appears to be a conserved activity of omptins, this activity may be more important during *Salmonella* infections, as *Y. pestis* strains lacking Pla remain highly serum resistant. As with many other substrates of Pla, the biological relevance of C3 cleavage in the context of plague infection remains to be determined.

32.5 Pla Cleaves Cationic Antimicrobial Peptides

Cationic antimicrobial peptides (CAMPs) are small amphipathic structures that permeabilize bacterial lipid bilayers, resulting in the lysis of affected cells (Bishop and Finlay 2006). In addition to this direct role within the innate immune response, CAMPs also have chemoattractant properties that recruit neutrophils, monocytes, and T cells in response to infection. Work by Galvan et al. recently demonstrated that *Y. pestis* is susceptible to killing by the human cathelicidin LL-37 and rat cathelicidin rCRAMP (Galvan et al. 2008). During pneumonic plague, the small airways of the lung are predicted to contain high concentrations of CAMPs produced by alveolar epithelial cells exposed to inflammatory proteins (Galvan et al. 2008). Interestingly, mutant strains of *Y. pestis* lacking the gene encoding the F1 pilus (*cafI*) are resistant to killing, while strains lacking both *cafI* and *pla* are again susceptible to killing. The use of catalytically inactive *pla* point mutants suggests that Pla provides a protective effect by inactivating these cathelicidins through proteolytic cleavage (Galvan et al. 2008). Inactivation, however, only occurs in the absence of F1, which may either interfere with the active site conformation of Pla or simply sterically restrict interactions with CAMPs. Although CAMP cleavage in vivo remains to be demonstrated, it is possible that Pla primarily acts upon antimicrobial peptides in the flea gut where F1 is not produced but CAMPs are likely expressed, or potentially by *cafI*A::IS 1541 mutants of *Y. pestis* that can arise during mammalian infection and are no longer able to produce the F1 pili (Bulet and Stocklin 2005; Cornelius et al. 2009). CAMPs contain several basic residue repeats, perhaps making them more susceptible to cleavage by omptin proteins. This activity may have originated in ancestral *Salmonella*, where a survival benefit exists for PgtE-expressing *Salmonella* able to cleave cathelicidins LL-37 and CRAMP present inside macrophage vacuoles (Guina et al. 2000).

32.6 Pla Cleaves Type-III Secretion System effectors

Yersinia Yop effectors are injected into the cytoplasm of host cells via the pCD1-based type-III secretion system, the cumulative effects of which block bacterial phagocytosis, disrupt host cell actin cytoskeleton and NF- κ B signaling, and suppress the production of pro-inflammatory cytokines and chemokines (Matsumoto and Young 2009). Pla degrades most Yops in vitro including the Yops B, C, D, E, F, H, J, and M, but is unable to degrade LcrV (Perry and Fetherston 1997; Sodeinde et al. 1988). Little is known about how or why Pla degrades Yops, though this activity could be an artifact of forced secretion conditions in the absence of host cells. It was recently shown that extracellular Yops of *Y. pseudotuberculosis* can be translocated into host cells by a T3SS-dependent mechanism; if this phenomenon also occurs during *Y. pestis* infections, the presence of Pla may impact the efficiency of this process by degrading these extracellular Yops (Akopyan et al. 2011). Since Yops function within host cells, it is unknown whether Pla coordinates the degradation of extracellular Yops that may otherwise compromise innate immunity evasion.

32.7 Concluding Remarks

Pla acts as a versatile virulence factor that cleaves a variety of substrates to facilitate the progression of bubonic and pneumonic plague. It is clear that coagulation is initiated as a natural host defense to infections to both physically contain bacteria and also to recruit and activate immune cells. Current data suggest that Pla utilizes a multifaceted approach to initiate fibrinolysis to disrupt these and other host defenses. While numerous substrates have been identified in vitro, the discovery of additional Pla targets may add to our understanding of Pla as a complex virulence factor and its role during both bubonic and pneumonic plague. Additional in vivo studies will be necessary to determine the biological contribution of various substrate cleavage events during disease.

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Fig. 32.1.

Pla disrupts host coagulation and fibrinolysis. Pla may temporally enhance coagulation and fibrin deposition by inactivating host TFPI upstream of extrinsic coagulation pathway factors. The effects of Pla on multiple host substrates may also amplify fibrinolysis. The activation of plasminogen and simultaneous inactivation of PAI-1, α_2 -antiplasmin (A2AP), and TAFI may collectively facilitate dissemination through physical barriers and enhance immune cell evasion