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Preferential Acquisition and Activation of Plasminogen Glycoform II by PAM Positive Group A Streptococcal Isolates

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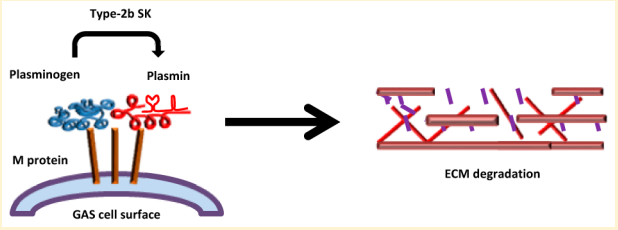
Supporting Information

ABSTRACT: Plasminogen (Plg) circulates in the host as two predominant glycoforms. Glycoform I Plg (GI-Plg) contains glycosylation sites at Asn_{289} and Thr_{346}, whereas glycoform II Plg (GII-Plg) is exclusively glycosylated at Thr_{346}. Surface plasmon resonance experiments demonstrated that Plg binding group A streptococcal M protein (PAM) exhibits comparative equal affinity for GI- and GII-Plg in the "closed" conformation (for GII-Plg, K_D = 27.4 nM; for GI-Plg, K_D = 37.0 nM). When Plg was in the "open" conformation, PAM exhibited an 11-fold increase in affinity for GII-Plg (K_D = 2.8 nM) compared with that for GI-Plg (K_D = 33.2 nM). The interaction of PAM with Plg is believed to be mediated by lysine binding sites within kringle (KR) 2 of Plg. PAM−GI-Plg interactions were fully inhibited with 100 mM lysine analogue ε-aminocaproic acid (εACA), whereas PAM−GII-Plg interactions were shown to be weakened but not inhibited in the presence of 400 mM εACA. In contrast, binding to the KR1−3 domains of GII-Plg (angiostatin) by PAM was completely inhibited in the presence 5 mM εACA. Along with PAM, *emm* pattern D GAS isolates express a phenotypically distinct SK variant (type 2b SK) that requires Plg ligands such as PAM to activate Plg. Type 2b SK was able to generate an active site and activate GII-Plg at a rate significantly higher than that of GI-Plg when bound to PAM. Taken together, these data suggest that GAS selectively recruits and activates GII-Plg. Furthermore, we propose that the interaction between PAM and Plg may be partially mediated by a secondary binding site outside of KR2, affected by glycosylation at Asn_{289}.



Multiple species of bacteria interact with the host protein plasminogen (Plg), and a growing body of evidence suggests the ability to bind Plg is central to multiple stages of bacterial pathogenesis.[1] Plg is a single-chain glycoprotein zymogen of the broad spectrum protease plasmin (Pln) that circulates in plasma and extracellular fluids at an approximate concentration of 2 μM.[2,3] Regulation of the Plg activation system is essential for the maintenance of homeostatic function, and the conversion of Plg into Pln is a major regulatory target. The conformation of the Plg molecule greatly influences the regulation of Plg activation. Intramolecular binding between lysine residues and the lysine binding sites (LBS) of kringle (KR) 4, KR5, and the N-terminal Pan-apple (PAp) domain maintains circulating Plg in a compact and internally rigid "closed" conformation that is highly resistant to activation.[2] Upon binding to specific cell surface receptors or ligands, such as fibrin, Plg adopts an "open" conformation that becomes more susceptible to activation.[4] It is now well recognized that these interactions play a central role in modulating key steps in Plg activation by host and bacterial Plg activators.[4,5]

Plg receptors and activators are expressed by a range of bacterial species, including *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Streptococcus pyogenes*.[5−9] The interaction of *S. pyogenes* [group A *Streptococcus* (GAS)] with Plg has been well characterized. GAS has evolved numerous highly specialized interactions with Plg/Pln that are critical for virulence. Central to this are the Plg binding group A streptococcal M-protein (PAM), and the secreted Plg activator, streptokinase (SK). The 43 kDa PAM protein binds to Plg with high affinity (K_D ∼ 1 nM).[10] Plg is known to interact with ligands via LBS within specific KR structures. KR1, KR4, and KR5 demonstrate the highest affinity for lysine-containing ligands, with KR2 displaying the weakest affinity.[11] The high-affinity interaction between PAM and Plg is unique, in that PAM lacks a typical C-terminal lysine, a common feature of most but not all Plg receptors. Early reports suggest that Lys_{98} and Lys_{111} mediate the binding of PAM to KR2 of Plg.[12] More recent work has shown that binding of Plg to PAM is primarily mediated by positively charged Arg_{101}, Arg_{114}, His_{102}, and His_{115} in the a1 and a2 repeat domains of PAM.[13] In addition to the expression of PAM, GAS also secrete SK, a potent Plg-activating protein. SK mediates Plg activation by the formation of a 1:1 (SK−Plg) complex, with substrate recognition occurring at KR5 of Plg.[14]

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SK produced by GAS displays considerable genetic and phenotypic diversity. Phylogenetic studies of *ska* sequences from GAS isolates have revealed two main sequence clusters (cluster type 1 and 2), with cluster type 2 sequences being further subdivided (cluster type 2a and 2b).[15,16] Recent studies analyzing these distinct SK variants from specific GAS isolates suggest that the phenotypic differences displayed by SK may directly affect GAS pathogenic outcomes.[17] Epidemiological studies have shown the type 2b *ska* lineage to be largely restricted to PAM positive GAS strains. In these isolates, PAM and SK play a cooperative role in the acquisition and activation of Plg/Pln. Specific ligand binding-induced conformational changes in Plg mediated by PAM and other host proteins such as fibrinogen are required for type 2b SK to form a functional activator complex with Plg. Furthermore, the combination of PAM and fibrinogen is required for these type 2b SK variants to display resistance to inhibition by circulating α_2-antiplasmin, which is a hallmark of the classical SK-mediated activation process.[18]

Glycosylation of Plg gives rise to two major species of the zymogen: glycoform I Plg (GI-Plg) and glycoform II Plg (GII-Plg). GI-Plg possesses carbohydrate chains N-linked to Asn_{289} and O-linked to Thr_{346}, while GII-Plg contains a sole O-linked carbohydrate at Thr_{346}. GI-Plg and GII-Plg exist in humans at an approximate ratio of 2:3.[4] Differences in carbohydrate content between the two glycoforms of Plg lead to disparities in the kinetic and activation properties of the zymogen.[19] The ability of highly specialized Plg binding pathogens such as GAS to interact with different glycoforms of Plg and the downstream consequences of these interactions have not been investigated.

Here, we examine the biochemical, structural, and functional differences between the interactions of GI-Plg and GII-Plg with GAS proteins, PAM and SK, with a view of resolving how binding and activation of these two different glycoforms may contribute to the interaction of GAS with the Plg activation system. This study highlights the preferential recruitment and activation of GII-Plg at the GAS cell surface, while proposing that N-linked glycosylation at Asn_{289} of GI-Plg obstructs activation by GAS. Furthermore, we propose that the interaction between PAM and GI-Plg and between PAM and GII-Plg may be mediated by a secondary binding site outside of KR2 that is disrupted by glycosylation at Asn_{289}.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Culture, and Expression Conditions. *Escherichia coli* strains and M15 and TOP10 containing pQE30-SK_{ALAB49} and pGEX2T-PAM_{NS13} expression plasmids, respectively, were cultured and used for expression of recombinant protein at 37 °C in Luria-Bertani broth supplemented with ampicillin (100 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) as previously described.[13,17] PAM positive GAS isolates NS13 and ALAB49 were used in this study, along with isogenic mutant GAS ALAB49Δ*pam*.[13,18,20] GAS isolates were cultured overnight at 37 °C on horse-blood agar (Biomerieux, Sydney, Australia) or in static liquid cultures of Todd-Hewitt broth (BD, Sydney, Australia) supplemented with 1% (w/v) yeast extract (Oxoid, Adelaide, Australia; THY medium).

Plg Glycoform and Angiostatin Purification. Human GI-Plg and GII-Plg were purified from plasma as previously described.[21] GII-angiostatin purification was achieved via elastase digestion of full-length GII-Plg that was buffer exchanged into 100 mM Tris and 100 mM ammonium bicarbonate (pH 8.5). Porcine pancreatic elastase (Sigma, Sydney, Australia) was incubated with GII-Plg at a ratio of 1:100 (w/w) for 5 h at 22 °C while being shaken. To stop the reaction, 0.5 mM PMSF was added, and the mixture was left to incubate for 30 min at 22 °C. Digestion mixtures were applied to a lysine-linked sepharose column. Purified angiostatin was eluted from the lysine-sepharose with 50 mM ε-aminocaproic acid (εACA) in 100 mM $NaPO_4$ and 100 mM NH_4CO_3 (pH 8).

εACA-Induced Conformational Change in GI-Plg and GII-Plg. Plg glycoforms were diluted to 1.5 μM in HEPES buffer [10 mM HEPES and 150 mM NaCl (pH 7.4)]. Methods were appended from ref 22. Briefly, 5 μL of each Plg glycoform at a concentration of 1.5 μM was added to a thin-walled 96-well unskirted polymerase chain reaction (PCR) microplate containing 2.5 μL of 40× SYPRO orange (Invitrogen, Carlsbad, CA) and 12.5 μL of HEPES buffer with varying concentrations (0−100 mM) of εACA. Plates were read on a LightCycler 480 II Real Time PCR system (Hoffman-La Roche, Basel, Switzerland) with excitation and emission set at 425 and 625 nm, respectively. Fluorescence measurements were expressed as the change in the initial fluorescence $[(F_{abs} - F_o)/F_o = \Delta F/F_o]$. As εACA was found to induce an immediate conformational change in Plg, intervals represent the end points of conformational change in relation to εACA concentration. To determine the contribution of εACA on hydrophobic exposure relative to maximal hydrophobic exposure, Plg glycoforms were also assessed at varying temperatures (37−70 °C) in the presence of varying εACA concentrations (0−100 mM). Plg samples were held for a period of 2 s at each temperature gradient. GI- and GII-Plg samples were measured and analyzed as described above.

Surface Plasmon Resonance (SPR). Recombinant histidine-tagged PAM was analyzed for its binding affinity for purified human GI-Plg, GII-Plg, and GII-angiostatin via single-cycle kinetics, on a series S Ni-NTA chip (GE Healthcare, Uppsala, Sweden), using a Biacore T200 (GE Healthcare) at 20 °C. All four flow cells were activated with 10 mM $NiCl_2$ for 60 s at a rate of 5 μL min^{-1} and washed with 3 mM EDTA for 60 s at a rate of 5 μL min^{-1}. PAM was captured at the surface of flow cells 2−4 until a level of 80−100 response units (RU) was reached. Flow cell 1 was used as a blank control. For competitive binding analysis, GI-Plg or GII-Plg was captured on flow cells 2−4 until saturation of PAM had occurred. Analytes were diluted in running buffer [PBS, 0.05% Tween 20, and 50 μM EDTA (pH 7.4)] or in running buffer containing varying concentrations (0−100 mM) of the lysine analogue εACA and/or benzamidine (Sigma). Kinetic assays were performed using varying concentrations of analyte (GI-Plg and GII-Plg, 0−200 nM; GII-angiostatin, 0−100 nM) over a series of five 120 s injections at a flow rate of 30 μL min^{-1} with a 600 s dissociation period. Regeneration of the flow cell surface was achieved with two separate injections consisting of 50 mM NaOH and 350 mM EDTA (pH 8.3) for 50 s at a rate of 30 μL min^{-1}. Affinity interactions were analyzed by nonlinear fitting of the single-cycle kinetic sensograms according to a 1:1 Langmuir binding model using Biacore T200 evaluation software (GE Healthcare).

Cell Surface Plg Glycoform Acquisition. To establish whether GAS preferentially captures GI-Plg or GII-Plg at the bacterial cell surface, PAM expressing GAS isolate NS13 was harvested at mid log phase (OD_{600} = 0.5), washed in PBS, and incubated in citrated normal human plasma as a source of Plg for 1 h at 37 °C. Following two washes in PBS, bound Plg was

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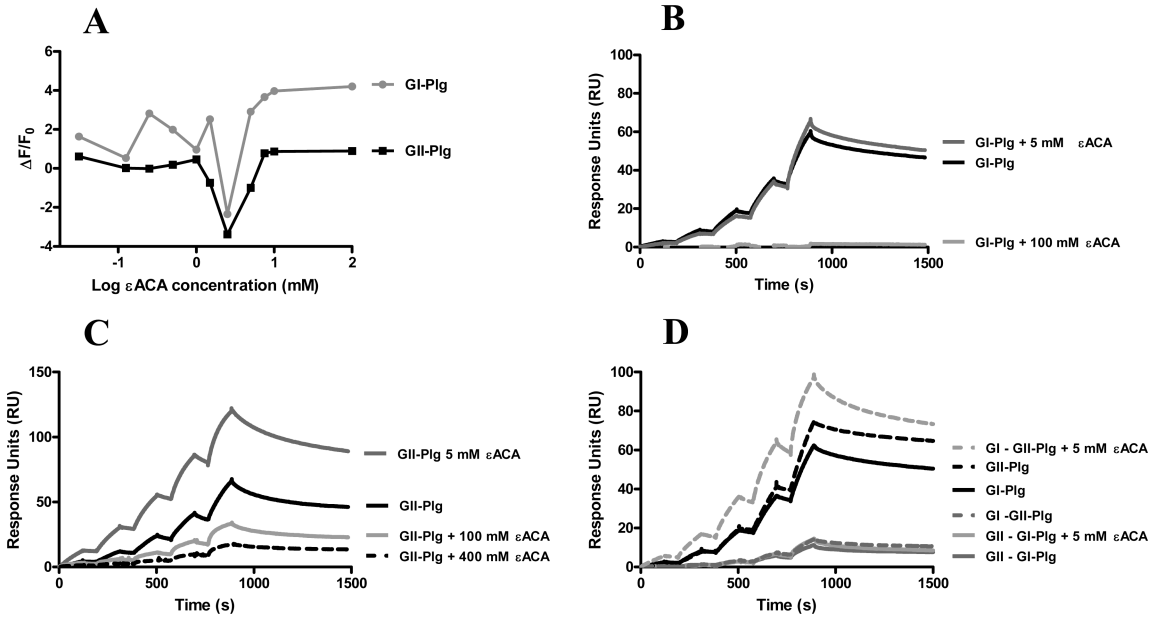

Figure 1. Effects of εACA on the structural conformation of GI- and GII-Plg and associated affinity for PAM. (A) Conformational changes in GI-Plg and GII-Plg induced by the lysine analogue, εACA. Changes in structural conformation were determined using the conformationally sensitive dye SYPRO orange. Analysis was undertaken at 37 °C using a LightCycler 480 II Real Time PCR system, with excitation and emission set at 425 and 625 nm, respectively. Intervals represent the end points of the conformational change in relation to εACA concentration. Fluorescence measurements were expressed as the change in the initial fluorescence $[(F_{abs} - F_o)/F_o = \Delta F/F_o]$. The "dip" observed in $\Delta F/F_o$ at a log εACA concentration of 0.39 correlates with an εACA concentration of approximately 2.5 mM. Single-cycle kinetic BIAcore sensograms analyzing the affinity of immobilized PAM for Plg glycoforms (B) GI-Plg and (C) GII-Plg in closed and open conformations. (D) Competitive binding analysis between GI-Plg and GII-Plg against immobilized PAM in the presence and absence of 5 mM εACA. Each run reflects five injections of Plg at 12.5, 25, 50, 100, and 200 nM. A dissociation period of 600 s followed the last injection. PAM−Plg interactions were analyzed by nonlinear fitting of the single-cycle sensograms according to a 1:1 Langmuir binding model using Biacore T200 evaluation software (Biacore AB).

eluted from the GAS cell surface using 100 mM glycine-HCl (pH 2.0) for 15 min at room temperature. Eluted Plg was then probed using rabbit anti-human Plg antibody (Calbiochem, San Diego, CA), followed by goat anti-rabbit IgG horseradish peroxidase conjugate (Invitrogen). Enhanced chemiluminescence detection (Pierce Biotechnology, Rockford, IL) was used to visualize Plg protein bands according to the manufacturer's instructions. Purified GI-Plg and GII-Plg samples were used as positive controls to allow identification of bound Plg glycoforms from plasma by GAS based on electrophoretic mobility.

Cell Surface Plg Activation Time Course. To assess the ability of GAS to acquire plasmin at the bacterial cell surface in the presence of each glycoform variant, a time course assay was performed. GAS strains NS13 and ALAB49 were harvested at mid log phase (OD_{600} = 0.5) and preincubated with 100 nM GI-Plg or GII-Plg at 37 °C for either 5 or 10 min. Following two washes in PBS, bacterial cells were resuspended in PBS containing 500 μM chromogenic substrate S-2251 and incubated overnight. GAS cell surface plasmin activity was measured at A_{405} using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA).

Nonproteolytic Active Site Generation in Plg. Nonproteolytic active site generation in GI-Plg and GII-Plg by type 2b SK was examined using the fluorescent active site titrant 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB) (Marker Gene Technologies Inc., Eugene, OR) in a POLARstar Omega fluorescence spectrophotometer (BMG LABTECH, Ortenberg, Germany). Plg glycoforms (200 nM) were added to a black 96-well microtiter plate containing 1 μM MUGB in assay buffer [50 mM Tris-HCl and 100 mM NaCl (pH 7.4)] and preincubated with PAM (200 nM) for 10 min at 37 °C. To initiate the reaction, SK was added to a final concentration of 400 nM in a total volume of 100 μL, and the development of fluorescence was monitored continuously with excitation at 355 nm and emission at 460 nm. Data were normalized by subtracting a control reaction of both Plg glycoforms without the addition of SK and 1 μM MUGB. Rate measurements were calculated and expressed as previously described.[18]

GI-Plg and GII-Plg Activation by Type 2b SK Variant Plg Complexes. The ability of type 2b SK variant Plg complexes to activate substrate Plg was studied by the addition of type 2b SK (final concentration of 5 nM) to assay buffer [10 mM HEPES, 150 mM NaCl, and 0.01% Tween 20 (pH 7.4)] containing an excess of GI-Plg or GII-Plg (500 nM) and chromogenic substrate S-2251 (500 μM) in a total volume of 100 μL. As type 2b SK does not possess the ability to activate Plg in the absence of PAM,[18] recombinant PAM was also added to each experiment at 500 nM. The parabolic generation of Pln was monitored by the change in absorbance at A_{405} and measured over 30 min at 37 °C as described above. For quantitative comparison of the Plg variant effect, the change in A_{405}, which is a function of S-2251 substrate cleavage by Pln generated during the activation of Plg by SK, was plotted against t^2. The velocities of these reactions were then calculated from the gradient of A_{405} versus t^2. Differences in GAS cell surface activation of GI-Plg and GII-Plg using GAS strains ALAB49 and isogenic mutant ALABΔ49 were determined as previously described.[18]

Statistical Analysis. Differences in binding affinity and Pln acquisition were determined by a one-way analysis of variance with Tukey's multiple-comparison test. Initial rates of proteolytic and nonproteolytic activation of GI-Plg and GII-

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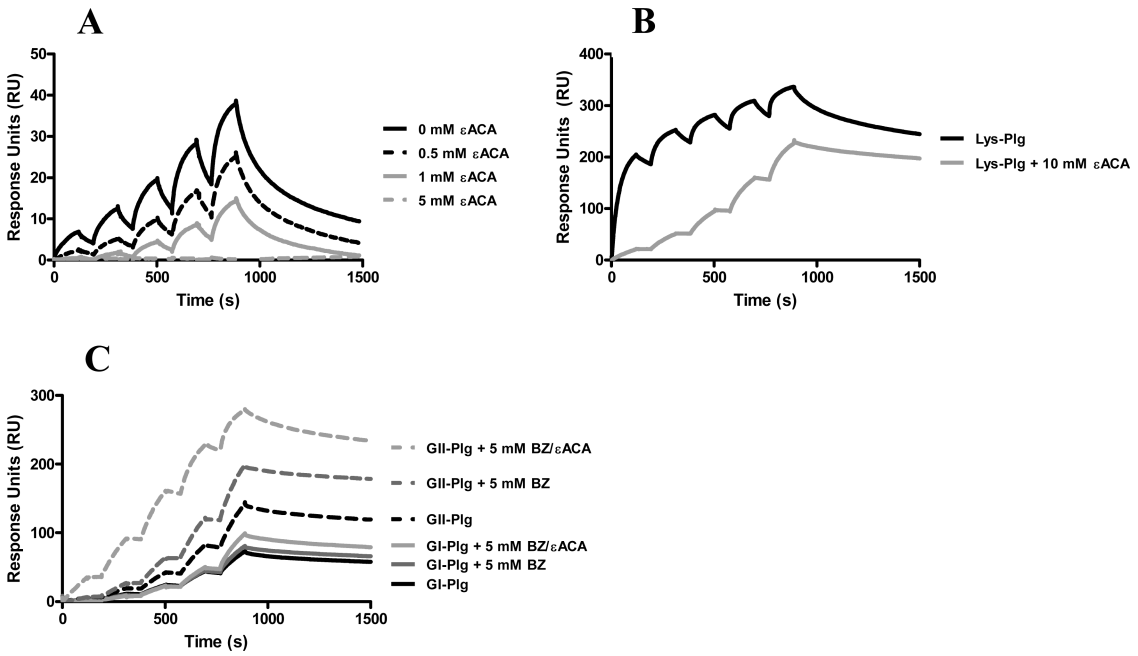

Figure 2. Isolating a potentially novel non-lysine-dependent PAM binding site in GI- and GII-Plg by SPR. (A) Affinity of immobilized PAM for GII-Plg-angiostatin (KR1−KR3) in the presence of varying concentrations of εACA (0−5 mM). (B) Affinity of immobilized PAM for Lys-plasminogen with and without 10 mM εACA. (C) Affinity of immobilized PAM for GI- and GII-Plg in the presence of KR5 binding competitor, benzamidine (BZ), and εACA. Each run reflects five injections of analyte at 12.5, 25, 50, 100, and 200 nM. A dissociation period of 600 s followed the last injection. PAM-KR1−KR3/GII-Plg/lys-Plg interactions were analyzed by nonlinear fitting of the single-cycle sensograms according to a 1:1 Langmuir binding model using Biacore T200 evaluation software (Biacore AB).

Plg were compared using a two-tailed unpaired Student's *t* test. Data sets were considered statistically significant at $p < 0.05$. All analysis, including data transformation and linear regression, was performed using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA).

Ethics Approval. Collection of blood was performed with the approval of the University of Wollongong Human Ethics Committee (HE08/250). Volunteers provided informed consent before donating blood samples.

RESULTS

εACA-Induced Protein Conformational Change in GI-Plg and GII-Plg. To examine the effect of εACA on protein domain unfolding in each Plg glycoform, GI-Plg and GII-Plg were monitored in the presence of εACA using the hydrophobic protein denaturation marker SYPRO orange as previously described.[22] Unfolding analysis determined that both GI-Plg and GII-Plg undergo a structural change in the presence of εACA. A dramatic conformational change was observed with both Plg glycoforms at a εACA concentration of 2.5 mM, indicating potential exposure of the typically compact KR2−KR5 and serine protease domains (Figure 1A). In the presence of ≥5 mM εACA, the level of hydrophobic exposure was consistently greater in GI-Plg than in GII-Plg. Higher levels of hydrophobicity displayed by GI-Plg versus those displayed by GII-Plg may be the result of the mobile KR3 domain in GI-Plg that has been implicated in allowing the molecule to more readily adopt an "open" conformation than its GII-Plg counterpart.[4] Subsequently, these data may also suggest that GII-Plg undergoes a smaller degree of εACA-induced conformational change than GI-Plg (Figure 1A). Although conformational changes were observed in both GI and GII-Plg, detected changes in conformation may not be proportional to absolute changes in hydrophobic exposure. To further examine the degree of εACA-induced conformational change relative to the maximal degree of hydrophobic exposure, GI- and GII-Plg conformational changes were assessed in the presence of increasing concentrations of εACA over an increase in temperature (Figure 1 of the Supporting Information). Temperature-dependent denaturation profiles determined that εACA (2.5−100 mM) was able to only partially induce hydrophobic exposure in GI-Plg and GII-Plg. Furthermore, the level of εACA-induced hydrophobic exposure relative to the maximal level of hydrophobic exposure was observed to be greater in GI-Plg than in GII-Plg, supporting the notion of a more flexible GI-Plg structure.

Affinity of PAM for GI and GII in Closed and Open Conformations. To analyze the biochemical interactions of PAM with GI-Plg and GII-Plg, PAM_{NS13} was cloned and expressed as a truncated recombinant protein, lacking the N-terminal signal sequence and C-terminal anchor domain.[8] SPR analysis indicated that PAM has a comparatively high affinity for closed forms of GI-Plg and GII-Plg with K_D values of 27.4 ± 1.6 and 37.0 ± 6.6 nM, respectively. Crystallization studies of full-length human Plg have revealed that only the LBS of KR1 is unprotected in closed Plg; however, upon adoption of an "open" conformation, KR5 moves away from the core of the Plg structure, allowing it to be transiently exposed to interact with lysine-containing ligands.[4] To assess the impact of Plg conformation on the interaction with PAM, an open conformation was induced by the addition of lysine analogue εACA to the binding buffer. As shown in Figure 1A at ≥5 mM εACA, Plg adopts a fully "open" conformation; however, large excesses of εACA will also block LBS and weaken interactions with ligands. In the presence of 5 mM εACA, both GI-Plg and GII-Plg maintained the ability to interact with PAM with a high affinity. However, binding of GI-Plg by PAM was completely

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inhibited in the presence of 100 mM εACA, whereas the GII-Plg−PAM complex was still able to form in 400 mM εACA, although with reduced affinity (Figure 1B,C). Unlike that of GI-Plg, the affinity of PAM for GII-Plg was increased approximately 11-fold in the presence of 5 mM εACA [for GI-Plg, K_D = 33.2 ± 4.4 nM (p > 0.05); for GII-Plg, K_D = 2.8 ± 0.6 nM (p = 0.0001)], suggesting that the conformational change to a potential "open" conformation (1) promotes an affinity between PAM and LBS of GII-Plg significantly higher than that between LBS and εACA or (2) exposes a secondary PAM binding site outside of the LBS of GII-Plg that cannot be outcompeted with εACA.

To further investigate the higher affinity displayed by PAM for "open" GII-Plg over GI-Plg, competitive binding analysis was undertaken via SPR, using both "closed" and "open" forms of each Plg glycoform. In the "closed" conformation, neither Plg glycoform was able to outcompete binding of the other to PAM. The low-level binding displayed by each "closed" Plg glycoform in the presence of immobilized PAM−GI-Plg or PAM−GII-Plg complex is likely to be attributed to the dissociation of respective PAM−Plg glycoforms, subsequently unmasking further binding sites. Interestingly, when in the "open" conformation, induced by the addition of 5 mM εACA, GII-Plg was able to outcompete GI-Plg−PAM binding interactions. Furthermore, once "open" conformation GII-Plg was bound to immobilized PAM, this interaction was unable to be disrupted by "open" form GI-Plg (Figure 1D). Collectively, these data suggest an interaction between PAM and GII-Plg that has an affinity higher than that of the interaction with GI-Plg when Plg is in the "open" conformation.

εACA Inhibits the Interaction between PAM and GII-Angiostatin. The KR2 LBS has been reported to mediate the interaction between PAM and Plg; however, our finding that 400 mM εACA is unable to fully disrupt the interaction between PAM and GII-Plg suggests the possibility of a secondary binding site within the Plg molecule. The first three KR domains of Plg (angiostatin) have been previously shown to interact with PAM via LBS.[10,23] To determine if a novel binding domain exists outside of KR1−KR3, GII-angiostatin−PAM binding kinetic analysis was undertaken with increasing concentrations of εACA (Figure 2A). In contrast to our findings with full-length GII-Plg, the interaction of GII-angiostatin with immobilized PAM was completely inhibited in the presence of 5 mM εACA. This supports previous findings that LBS within KR1−KR3 are involved in the PAM−Plg interaction. However, as comparative concentrations of εACA were unable to inhibit interactions of immobilized PAM with full-length GI-Plg and GII-Plg, these data also suggest that a secondary site outside of KR1−KR3 may mediate the interaction of PAM with Plg. This interaction may represent a non-LBS-mediated event, or alternatively a secondary higher-affinity interaction within full-length Plg.

PAM−Plg Binding Does Not Occur through PAp and KR5 Domains. Plg is known to adopt three different conformational states: the compact "closed" conformation, the partially extended intermediate conformation, and the fully extended "open" conformation.[24,25] The PAp domain serves as an important factor in the precise arrangement of "closed" Plg by interacting with KR5, preventing the reversible conformational change to intermediate state Plg and/or extended "open" form Plg.[4,25] Prior studies analyzing the interactions of PAM with the PAp−KR5 interface have been limited.[12,23] To determine whether PAM is able to bind outside of KR1−KR3 in the PAp domain, SPR analysis using Lys-Plg that lacks the PAp domain and exists in the intermediate conformation was undertaken in the presence and absence of 10 mM εACA. εACA has been previously shown to result in Lys-Plg adopting the extended "open" conformation.[24] In the absence of εACA, immobilized PAM was shown to bind Lys-Plg with a high affinity (K_D = 0.44 ± 0.03 nM), indicating the PAp domain is not required for high-affinity PAM−Plg interactions. In the presence of εACA, the binding affinity decreased 9-fold (K_D = 3.83 ± 0.1 nM) but was still high (Figure 2B). This decrease in affinity may be the result of blocking the PAM−K2 interaction, as seen for angiostatin. To further discount the PAp−KR5 interface as a factor in PAM−Plg recognition, benzamidine, a KR5 binding site competitor that can induce an intermediate conformation in Plg, was employed.[24,26] The addition of 5 mM benzamidine with and without equimolar concentrations of εACA could not inhibit binding in either Plg glycoform but was shown to significantly increase the affinity (p < 0.001) of PAM−GII-Plg complexes, which is similar to the effect seen in the presence of low concentrations of εACA. This is most likely due to the higher-affinity interaction of GII-Plg versus that of GI-Plg in the potential open conformation as a result of the secondary glycosylation site in GI-Plg (Figure 2C).

Selective Glycoform Recruitment at the GAS Cell Surface. To determine if PAM preferentially sequesters a particular glycoform of Plg at the GAS cell surface, PAM-expressing GAS strain NS13 was incubated in human plasma and screened using anti-human Plg Western blot analysis. The molecular masses of GI-Plg and GII-Plg are 93 and 89 kDa, respectively. Plg acquisition at the GAS cell surface was found to be specific to GII-Plg (Figure 3A), corresponding to an approximate molecular mass of 89 kDa. A 93 kDa band consistent with GI-Plg was absent from the elution sample.

Plg Activation at the GAS Cell Surface. For each glycoform, recruitment and activation at the GAS cell surface were qualitatively measured using the chromogenic substrate S-2251. S-2251 hydrolysis in this assay is given as a function of activated Pln bound to the GAS cell surface. SK expression during the course of the assay and subsequent formation of SK−Plg/Pln complexes at the cell surface are thought to have a minimal contribution to pNA generation and are equal between isolates. This is due to the large molar excess of exogenous Plg added to the experiment versus endogenous SK and evidenced by subsequent saturation of Pln activity between preincubation time points. PAM and type 2b SK-expressing GAS strains NS13 and ALAB49 were incubated in the presence of equimolar levels of either GI-Plg or GII-Plg for 5 or 10 min periods. At both time points, the level of acquisition of Plg by GAS strains NS13 and ALAB49 was significantly higher in the presence of GII-Plg than in the presence of GI-Plg [p < 0.05 (Figure 3B)]. This suggests that PAM-expressing GAS isolates preferentially recruit GII-Plg/Pln over GI-Plg/Pln at the cell surface.

SK-Mediated Nonproteolytic Active Site Generation and Plg Activation. SK from the type 2b-expressing GAS strain ALAB49 was examined for its ability to generate an active site in GI-Plg and GII-Plg. Nonproteolytic active site generation was examined using the fluorescent active site titrant MUGB as previously described.[17] The rate of active site generation by type 2b SK was significantly higher for GII-Plg (21.0×10^{-3} ΔF F_o^{-1} min^{-1}) than for GI-Plg (5.4×10^{-3} ΔF F_o^{-1} min^{-1}) [p = 0.01 (Figure 4A)]. Type 2b SK-mediated Plg activation in the presence of PAM was monitored using the chromogenic substrate S-2251. Type 2b SK was able to generate Pln activity

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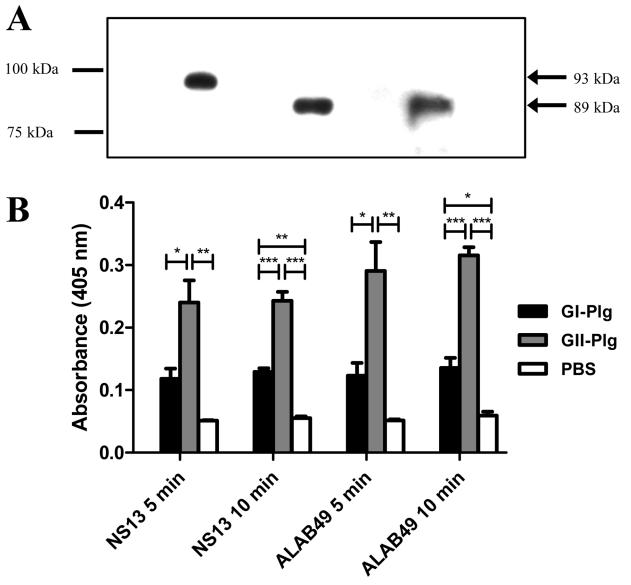

Figure 3. Selective Plg glycoform variant recruitment and Pln glycoform acquisition at the GAS cell surface. (A) GAS isolate NS13 was incubated in human plasma, and bound Plg was eluted using 100 mM glycine-HCl (pH 2.0). Purified controls GI-Plg (lane 1) and GII-Plg (lane 2) and eluted GAS cell surface Plg samples (lane 3) were visualized via an anti-human Plg Western blot. (B) GAS isolates NS13 and ALAB49 were incubated with GI-Plg, GII-Plg (100 nM), or PBS for 5 or 10 min intervals at 37 °C. GAS cell surface Pln acquisition was measured by the addition of S-2251, and the change in absorbance was monitored at 405 nm and 37 °C.

in both GI-Plg− and GII-Plg−PAM complexes. Pln activity generated by type 2b SK was observed to be significantly higher for GII-Plg-containing (3.0 $mAbs_{405}$ min^{-2}) than GI-Plg-containing samples (0.90 $mAbs_{405}$ min^{-2}) [$p < 0.001$ (Figure 4B)]. Using GAS strain ALAB49, cell surface, type 2b SK-dependent activation of Plg glycoform variants was shown to be significantly higher in GII-Plg (0.70 $mAbs_{405}$ min^{-2}) than in GI-Plg (0.49 $mAbs_{405}$ min^{-2}) ($p < 0.01$). GAS strain ALABΔ*pam* was unable to activate either Plg glycoform, confirming the importance of PAM in type 2b SK-mediated Plg activation (Figure 4C). Differences in the lag time of Pln generation displayed by both Plg glycoforms may be attributed to the delay in active site generation but could also be influenced by the differing affinities of PAM for each Plg glycoform.

DISCUSSION

The interaction of PAM and Plg combined with SK plays an important role in the pathogenicity of GAS. A key virulence mechanism of GAS is the bacterium's ability to sequester and activate Plg both extracellularly and at the GAS cell surface, aiding in the migration of the bacterium from cutaneous and mucosal surfaces to deeper tissue sites, resulting in severe invasive infection.[27] Plg has been previously shown to typically bind receptors via C-terminal lysine residues.[28,29] In contrast, binding of Plg to PAM has been attributed to a combination of internal lysine residues, and a series of positively charged arginine and histidine residues present within a1 and a2 repeat domains of PAM.[13] In this study, we have shown that PAM preferentially binds GII-Plg over GI-Plg and propose that both Plg glycoforms contain secondary PAM binding sites outside of KR2 that are impacted by glycosylation at Asn_{289}. Furthermore, GII-Plg was also shown to have a rate of activation higher than that of GI-Plg by type 2b SK.

GI-Plg and GII-Plg circulate in plasma at an approximate ratio of 2:3. Biochemical and affinity analysis presented in this study demonstrated that GAS preferentially recruits and activates GII-Plg over GI-Plg. Analysis of the interaction between PAM and GI-Plg or GII-Plg, in combination with Plg recruitment at the cell surface, demonstrated that PAM preferentially binds GII-Plg, the most abundant glycoform. Earlier work investigating the dynamics of interaction between GI-Plg and GII-Plg at the site of injury in the thoracic aorta of rabbits found that GII-Plg accumulates approximately 5 times faster than GI-Plg.[30] This result is consistent with an earlier report that determined that GII-Plg is synthesized and released by the liver 5 times faster than GI-Plg in eukaryotes.[31] The differences in abundance between the two glycoforms of Plg in both serum and at the site of cutaneous injury may have served as a selective pressure for preferential recruitment of GII-Plg by PAM.

PAM is one of the few Plg receptors that can bind Plg via residues other than lysine.[13] Crystallography studies of the first three KR domains of Plg (angiostatin) bound to VEK-30, a peptide derived from PAM, suggest that interactions between PAM and Plg occur exclusively at KR2.[23] Using GII-angiostatin, we have shown that binding of PAM to KR1−KR3 can be inhibited with the lysine analogue εACA, supporting the role of the KR2 LBS in the interaction between PAM and GII-Plg.[32] In contrast, whole molecule binding data using full-length GI-Plg and GII-Plg demonstrated that interactions with PAM in an α-helical coiled coil conformation could not be inhibited with comparative concentrations of εACA.[13] Berge and Sjöbring previously determined that interaction between PAM and radiolabeled Plg could be outcompeted with 100 mM εACA.[10] Although we cannot explain the discrepancies between these two data sets, observed differences may be a direct result of differences in binding methodology. During this study, SPR experiments captured and presented PAM in an orientation that allows the N-terminal portion of the protein to extend away from the BIAcore sensor surface. By capturing the C-terminus of PAM, we were able to closely model the presentation of PAM at the GAS cell surface, thus allowing for a more physiologically relevant comparison of binding with each Plg glycoform.

The affinity between PAM and GII-Plg was significantly increased in the presence of εACA, an inducer of "open" conformation Plg. Unlike GII-Plg, GI-Plg is glycosylated at Asn_{289} on KR3. Previous studies have shown that glycosylation at Asn_{289} results in a reduced affinity of KR1 for fibrin and α_2-antiplasmin.[33,34] A conformational change to full extended "open" form Plg has been suggested to be dependent on cooperative binding interactions between two ligands and Plg.[35] Christensen and Molgard proposed that cooperative binding involves two separate LBS where an initial low-affinity ligand interaction induces a rate-determining conformational change in Plg, allowing subsequent interaction with a secondary ligand of significantly higher affinity.[35] The presence of this additional glycosylation site at the KR3−SP interface that also lies in the vicinity of KR2 in GI-Plg may act to sterically hinder but not inhibit a secondary PAM interaction, potentially limiting the degree of conformational change undertaken by GI-Plg. Although glycosylation at Asn_{289} lies in the vicinity of KR2, we cannot discern if the KR2 function of GI-Plg is impaired by glycosylation at Asn_{289} in GI-Plg. PAM has been previously

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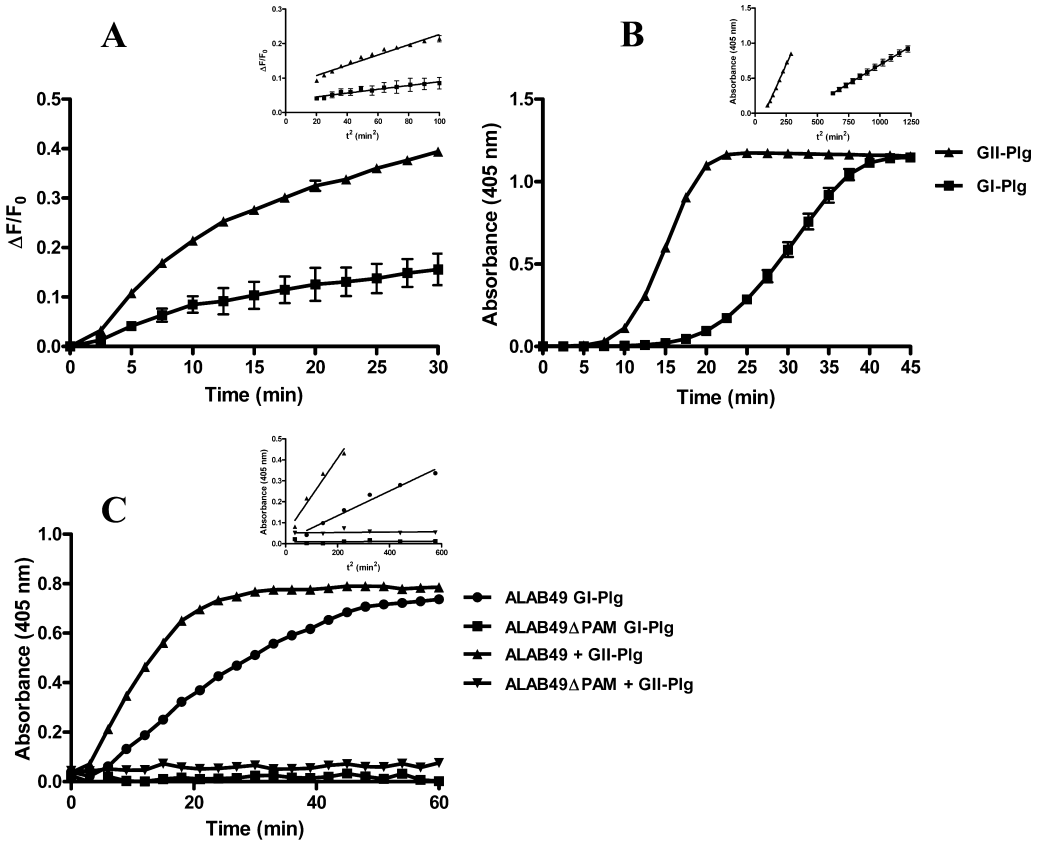

Figure 4. Nonproteolytic active site generation in Plg glycoforms by type 2b SK and the influence of the variant Plg conformation on SK-mediated Plg activation. (A) GI- and GII-Plg (200 nM) and PAM (200 nM) were added to 1 μM MUGB in assay buffer [50 mM Tris-HCl ad 100 mM NaCl (pH 7.4)] and preincubated at 37 °C for 10 min. To initiate the reaction, SK was added to a final concentration of 400 nM in a total volume of 100 μL, and the development of fluorescence was monitored continuously with excitation at 355 nm and emission at 460 nm. (B) SK (final concentration of 5 nM) was added to assay buffer [10 mM HEPES, 150 mM NaCl, and 0.01% Tween 20 (pH 7.4)] containing variant GI- or GII-Plg (500 nM), PAM (500 nM), and S-2251 (500 μM). (C) GAS cell surface Plg activation using GAS strains ALAB49 and ALABΔ*pam* containing GI- or GII-Plg (200 nM) and exogenous SK (5 nM) conducted in assay buffer [10 mM HEPES, 150 mM NaCl, and 0.01% Tween 20 (pH 7.4)]. The generation of Plg activation activity was monitored at an absorbance of 405 nm at 37 °C. Insets show transformed plots of absorbance at 405 nm vs t^2 with linear regression of the linearized region.

shown to bind KR1−KR3, but not truncated KR4 and KR5 fragments of Plg.[36] Taken together with SPR data discounting the role of the PAp and KR5 LBS in PAM−Plg binding interactions, we suggest a secondary binding interaction may be occurring between PAM and the SP domain of Plg, or via a cryptic binding site that is more accessible when GII-Plg is in the open conformation. Recent superposition experiments have revealed that SK can bind to the SP domain of Plg in a way that avoids the KR domains.[4] Therefore, we cannot rule out the possibility that at the SP domain in both glycoforms of Plg, two independent binding sites may exist where both PAM and SK are able to interact cooperatively.

Epidemiological studies have associated type 2b SK with skin tropic, PAM positive GAS isolates.[16] Type 2b SK variants have been previously shown not to induce the formation of an active site in Plg and have an affinity for Plg 25-fold than those of other SK polymorphic variants.[17] Unlike GII-Plg, GI-Plg contains a glycosylation site at Asn_{289} which does not allow for the formation of a hydrogen bond between KR3 and the Gly_{714} residue from the SP domain.[4] The absence of a hydrogen bond at the KR3−SP interface allows GI-Plg to more readily adopt an "open" conformation.[4,37] Consequently, this has been shown to be responsible for higher rates of activation of GI-Plg by select host and bacterial Plg activators whereby only in the presence of a lysine analogue is the activation rate of GII-Plg enhanced to the same extent as that of GI-Plg.[38] Only in the presence of either fibrinogen and/or PAM has type 2b SK been shown to create an active site in Plg.[15,18] In the presence of PAM, our results indicate that type 2b SK can both form an active site and mediate Plg activation significantly faster in GII-Plg than in GI-Plg. This suggests that differences in glycosylation profiles between the two glycoforms, particularly at Asn_{289} in GI-Plg, affect the potential of type 2b SK to generate active Pln. It is well established that PAM and type 2b SK play a cooperative role in Plg activation, depending on the ligand binding-induced conformational change in Plg. As stipulated above, differences in binding interaction between each Plg glycoform and PAM may prompt significant disparities in conformational changes between GI-Plg and GII-Plg, ultimately influencing activation in a manner that is unique to type 2b SK.

The acquisition of Plg/Pln by PAM and SK is a fundamental step in GAS virulence. In this study, we demonstrate for the first time that PAM expressing GAS selectively bind GII-Plg over GI-Plg with an affinity that is significantly higher than the affinity of GII-Plg for εACA. This suggests a role for either a non-LBS secondary interaction or the induction of a high-affinity LBS-dependent interaction in the open form of GII-Plg.

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Furthermore, we have identified significant differences in type 2b SK-mediated activation of Plg glycoforms. Recognizing the molecular mechanisms of how GAS sequesters Plg and how it is influenced by the distinct glycosylation profiles of Plg may significantly contribute to our understanding of GAS pathogenesis.

■ ASSOCIATED CONTENT

Supporting Information

SPR binding affinity data and additional GI- and GII-Plg unfolding experiments. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00130.

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Author Contributions

R.H.P.L. and D.L. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

GI-Plg, glycoform I plasminogen; GII-Plg, glycoform II plasminogen; SK, streptokinase; PAM, plasminogen binding group A streptococcal M protein; KR, kringle; LBS, lysine binding site; εACA, ε-aminocaproic acid.

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