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Abstract

The migration of the human pathogen *Streptococcus pyogenes* (group A streptococcus) from localized to deep tissue sites may result in severe invasive disease, and sequestration of the host zymogen plasminogen appears crucial for virulence. Here, we describe a novel plasminogen-binding M protein, the plasminogen-binding group A streptococcal M protein (PAM)-related protein (Prp). Prp is phylogenetically distinct from previously described plasminogen-binding M proteins of group A, C, and G streptococci. While competition experiments indicate that Prp binds plasminogen with a lower affinity than PAM (50% effective concentration = 0.34 μ M), Prp nonetheless binds plasminogen with high affinity and at physiologically relevant concentrations of plasminogen (K_d = 7.8 nM). Site-directed mutagenesis of the putative plasminogen binding site indicates that unlike the majority of plasminogen receptors, Prp does not interact with plasminogen exclusively via lysine residues. Mutagenesis to alanine of lysine residues Lys96 and Lys101 reduced but did not abrogate plasminogen binding by Prp. Plasminogen binding was abolished only with the additional mutagenesis of Arg107 and His108 to alanine. Furthermore, mutagenesis of Arg107 and His108 abolished plasminogen binding by Prp despite the presence of Lys96 and Lys101 in the binding site. Thus, binding to plasminogen via arginine and histidine residues appears to be a conserved mechanism among plasminogen-binding M proteins.

Keywords

Plasminogen, PAM, CMMB

Disciplines

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The Plasminogen-Binding Group A Streptococcal M Protein-Related Protein Prp Binds Plasminogen via Arginine and Histidine Residues_

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ABSTRACT

The migration of the human pathogen *Streptococcus pyogenes* (group A streptococcus) from localized to deep tissue sites may result in severe invasive disease, and sequestration of the host zymogen plasminogen appears crucial for virulence. Here, we describe a novel plasminogen-binding M protein, the plasminogen-binding group A streptococcal M protein (PAM)-related protein (Prp). Prp is phylogenetically distinct from previously described plasminogen-binding M proteins of group A, C, and G streptococci. While competition experiments indicate that Prp binds plasminogen with a lower affinity than PAM (50% effective concentration = 0.34 μ M), Prp nonetheless binds plasminogen with high affinity and at physiologically relevant concentrations of plasminogen (K_d = 7.8 nM). Site-directed mutagenesis of the putative plasminogen binding site indicates that unlike the majority of plasminogen receptors, Prp does not interact with plasminogen exclusively via lysine residues. Mutagenesis to alanine of lysine residues Lys⁹⁶ and Lys¹⁰¹ reduced but did not abrogate plasminogen binding by Prp. Plasminogen binding was abolished only with the additional mutagenesis of Arg¹⁰⁷ and His¹⁰⁸ to alanine. Furthermore, mutagenesis of Arg¹⁰⁷ and His¹⁰⁸ abolished plasminogen binding by Prp despite the presence of Lys⁹⁶ and Lys¹⁰¹ in the binding site. Thus, binding to plasminogen via arginine and histidine residues appears to be a conserved mechanism among plasminogen-binding M proteins.

INTRODUCTION

The gram-positive bacterium *Streptococcus pyogenes* (group A streptococcus [GAS]) is the major pathogenic agent of a wide variety of skin and mucosal infections in humans. Current estimates indicate that approximately 1.78 million new cases of severe streptococcal infection emerge each year (5). A key feature of invasive GAS infections is the ability of the organism to migrate from cutaneous and mucosal surfaces to deep tissue sites, resulting in severe invasive disease. While the exact mechanisms by which GAS cross host tissue barriers are yet to be fully elucidated, sequestration of plasminogen by GAS is being increasingly implicated in the pathogenesis of this organism (32). Indeed, for a subset of GAS isolates, acquisition of plasminogen via cell surface receptors appears to be crucial for virulence (6, 30). Plasminogen is a single-chain glycoprotein found in plasma and extracellular fluids at concentrations of approximately 2 μ M (8). Specific mammalian plasminogen activators cleave plasminogen at a single site (Arg⁵⁶⁰-Val⁵⁶¹), resulting in the formation of the two-chain plasmin molecule, which contains a serine protease active site in the C-terminal region. Plasmin(ogen) typically interacts with its ligands via lysine binding sites located in the five kringle domains of the N terminus of plasminogen (21). Plasmin(ogen) receptors and activators have been found to be expressed by a large number of bacteria, including group A, C, and G streptococci (15). In addition to the secreted nephritogenic plasminogen-binding protein (22), three cell surface-expressed GAS plasminogen-binding proteins have been identified. These are streptococcal surface enolase (SEN) (18), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (4, 19), and the plasminogen-binding group A streptococcal M protein (PAM) (3). Furthermore, the secreted protein streptokinase enables GAS to activate plasminogen

to plasmin (15). Plasmin has the ability to degrade fibrin clots, connective tissue, and the extracellular matrix (8, 21). Thus, sequestration of this proteolytic system by GAS may have significant pathological consequences in the host. Coded for by the *emm* gene, M protein is one of the major virulence factors of GAS. The *emm* family of genes consists of *emm*, *mrp*, and *enn* genes (12). The N termini of M proteins consist of a highly variable region, followed by a series of repeat domains designated a, b, c, and d. The number and type of repeats vary significantly between different M proteins (7, 16). Here we describe a novel, high-affinity plasminogen-binding M protein, the PAM-related protein Prp. Associated with an *S. pyogenes emm* 98.1 strain isolated from a severe invasive infection and which displays high levels of cell surface plasminogen binding (17), Prp is phylogenetically distinct from previously characterized plasminogen-binding M proteins. Using site-directed mutagenesis we show that, like PAM, Prp binds plasminogen via arginine and histidine residues, rather than lysine residues, which commonly mediate the interaction of plasminogen with its many other receptors.

MATERIALS AND METHODS

Bacterial strains and culture methods. Group A streptococcal strains were grown on horse blood agar plates (American Diagnostica) or cultured overnight at 37°C in Todd-Hewitt broth (Difco Laboratories) containing 1% yeast extract. All streptococcal strains used in this study were collected from the Northern Territory of Australia and have been described previously (17, 26). *Escherichia coli* INV α containing pCR2.1/Prp constructs (17) or *E. coli* TOP10 containing expression plasmids was grown on Luria-Bertani (LB)

agar plates or cultured in LB broth supplemented with ampicillin (100 µg/ml) as described previously (25). Plasmid DNA was extracted for PCR and DNA sequence analysis using the Wizard Plus SV DNA purification kit (Promega).

Phylogenetic analysis. In order to characterize the evolutionary relationships between Prp and naturally occurring variants of the PAM protein, the full amino acid sequences of 12 previously identified PAM proteins and Prp (17) were aligned using ClustalW (31). Evolutionary gene trees were then estimated using MrBayes version 3.1 (13, 24). For the MrBayes analysis, four simultaneous chains were run, with trees sampled every 100 generations for a total of 500,000 generations. Plots of likelihood scores against generation were used to identify when the analysis had reached stationarity. Trees sampled prior to stationarity (the first 40 trees) were discarded. The amino acid model was empirically chosen by MrBayes, using the prset aamodelpr = mixed command. This permits jumping between nine alternative amino acid substitution models. The WAG model (33) was empirically chosen by MrBayes. The majority rule consensus of all trees generated after stationarity was used to estimate the posterior probabilities of the various nodes in the most likely tree.

DNA sequence analysis, expression, and purification of recombinant M proteins.

The Prp gene from GAS strain NS88.2 had previously been cloned into the vector pCR2.1 (17). Primer sequences used for DNA sequence analysis are available upon request. DNA sequence reactions were undertaken using terminator ready reaction mix (PE Applied Biosystems). DNA sequencing gels were prepared as per the manufacturer's instructions and electrophoresed using a Perkin-Elmer ABI PRISM 377 sequencer. Sequence data were analyzed using ABI Prism DNA sequencing analysis software

(Perkin-Elmer). To facilitate functional analysis, Prp was cloned into the expression vector pGEX-2T essentially as previously described (26). Briefly, the Prp gene was amplified from a pCR2.1 construct by using *Pfu* polymerase (Stratagene). PCR cycling parameters consisted of 30 cycles of 97°C, 55°C, and 72°C for denaturation, annealing, and extension reactions, respectively. Amplicons were cloned into pGEX-2T (29), resulting in an N-terminal fusion with glutathione S-transferase, and constructs were transformed into *E. coli* TOP10 (Invitrogen) by standard procedures (25). The presence of both a His6 tag and a glutathione S-transferase tag on the recombinant protein enabled purification by two methods. DNA sequence analysis was used to confirm the lack of PCR errors in the cloned amplification product. Recombinant proteins were expressed and purified as described previously (26). Each step of the protein purification process was monitored by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (14), with protein visualized using Coomassie blue R250 staining.

Site-directed mutagenesis. Sequence alignment of Prp with other plasminogenbinding M proteins identified a potential plasminogen binding site within the molecule. In order to confirm the role of specific residues within this region in the interaction of Prp with plasminogen, site-specific mutations were introduced into the putative binding site. The wild-type pGEX-2T construct (100 ng) was used as template DNA to create site-directed mutants with the QuikChange site-directed mutagenesis kit (Stratagene). PCR mixtures consisted of 1×PFU reaction buffer (Stratagene), 0.25mM deoxynucleoside triphosphates (Boehringer Mannheim, Germany), 2.5U of *Pfu* Ultra polymerase (Stratagene), and 125 ng of each primer, made up to a volume of 50 µl with distilled water. Oligonucleotide primers (Sigma-Aldrich) were designed as per the manufacturer's instructions

(Stratagene). In general, primers consisted of sequence encoding the desired mutation, flanked on either side by 15 nucleotides of wild-type sequence. The specific primer sequences are available upon request. For site-directed mutants containing more than two mutations not encoded by a single primer, alanine residues were sequentially introduced using previously mutated DNA as a template. Following an initial denaturation step (95°C, 30 s), PCR cycling parameters consisted of 16 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 10 min. An additional 7-min extension at 55°C was then performed. PCR was conducted with a Cooled Palm 96 thermocycler (Corbett Research). Nonamplified template DNA was removed by incubation of reaction mixtures for 1 h at 37°C following the addition of DpnI (10 U/50-µl reaction mixture). *E. coli* TOP10 (Invitrogen) was transformed with 150 ng of DpnI-digested PCR product by standard procedures (25).

CD spectroscopy. To highlight potential variation in protein secondary structure as a result of site-directed mutagenesis, far-UV circular dichroism (CD) spectra were obtained for both wild-type and mutant recombinant proteins. CD spectra were acquired using a Jasco J-810 spectropolarimeter (Jasco, Canada) at room temperature. CD spectral data were recorded from 190 to 250 nm in a 1-cm-path-length cell containing 1.5 ml of protein solution at a concentration of 0.04 mg/ml in 10 mM sodium phosphate buffer (pH 7.4). Recorded data represent the averages of six scans, corrected for buffer baseline. Molar residue ellipticity ($[\theta]$) was calculated using the following formula: $[\theta] = \theta \times 100 \times \text{molecular weight/concentration (mg/ml)} \times \text{distance} \times \text{number of amino acids}$ (28). The percentage of α -helices was estimated from the ellipticity at 222 nm using the following formula: $\text{percent } \alpha\text{-helix} = -(\theta_{222 \text{ nm}} - 4,800)/45,400$ (20).

Plasminogen purification and labeling. Glu-plasminogen, the circulating form of plasminogen, was purified from human plasma by using lysine Sepharose 4B affinity chromatography as described previously (1, 26). Purified plasminogen was biotinylated by the addition of 10% (vol/vol) 1 M NaHCO₃ (pH 9), and a 40 molar excess of biotin-X-*N*-hydroxysuccinimide in dimethyl sulfoxide (Sigma-Aldrich). The reaction mixture was incubated at 4°C overnight with mixing. Free biotin was separated from biotinylated plasminogen by PD-10 gel filtration chromatography (Amersham Biosciences).

Plasminogen binding analysis. Solid-phase plasminogen binding assays were performed in order to assess both the affinity of Prp for plasminogen and the impact of introduced mutations on protein function, essentially as previously described (26). Ninety-six-well microtiter plates (Greiner Bio-one, Germany) were coated with 150 nM recombinant protein (50 µl in 0.1 M NaHCO₃) at 4°C overnight. Following three washes with PiNT (50 mM Na₂HPO₄, 150 mM NaCl, 0.05% Tween 80, pH 7.5), plates were blocked with 50 µl of blocking solution (1% skim milk powder in PiNT) for 1 h at 37°C. Wells were washed as described above, and 500 nM biotinylated glu-plasminogen was diluted in a threefold titration across the plate with blocking buffer in the presence or absence of a 50-fold molar excess of unlabeled glu-plasminogen. Plasminogen was allowed to bind to immobilized proteins for 2 h at room temperature. For competition assays, decreasing concentrations of unlabeled fluid-phase PAMNS13 (25 µM to 0.14 nM) or wild-type Prp were allowed to compete with immobilized proteins for binding to biotinylated glu-plasminogen. Competitor was titrated threefold across the microtiter plate prior to the addition of biotinylated glu-plasminogen to all wells, at a final concentration of 500 nM. The assay mixture was incubated for 2 h at room temperature. Following the plasminogen

incubation step, microtiter plates were washed three times, and 50 μ l of neutravidin conjugated to horseradish peroxidase (Progen, Australia) diluted 1:5,000 with blocking solution was added to all wells and incubated for 2 h at room temperature. After five washes with PiNT, the reactions were developed by the addition of 50 μ l o-phenylenediamine (Sigma-Aldrich) substrate (8 mM Na₂HPO₄ [pH 5.0], 2.2 mM o-phenylenediamine, 3% H₂O₂). Color development was stopped by the addition of 50 μ l of 10 M hydrochloric acid, and the plates were read at 490 nm using a Spectramax 250 plate reader (Molecular Devices). Data were normalized against the highest and lowest absorbance value for each assay, and nonlinear regression analysis was performed using GraphPad Prism (v4.00; GraphPad Software, CA). For the calculation of equilibrium binding dissociation constants (*K_d*), a one- versus two-site binding analysis was conducted and the best-fit curve fitted to the data. For competition experiments, a one-site competition curve was fitted to the data, from which the effective concentration of competitor required to inhibit binding by 50% was calculated.

Statistical analysis. For plasminogen binding experiments, a one-way analysis of variance was initially used on all data, followed by an unpaired *t* test with Welsch's correction to determine if there was any significant difference in the *K_d* values obtained for various proteins in this study.

Nucleotide sequence accession number. The Prp gene sequence has been submitted to the NCBI database and assigned GenBank accession number AY351856.

RESULTS

Phylogenetic analysis. Isolated from a bacteremia infection, GAS strain NS88.2 encodes a PAM-related M protein (Prp) that is only 66.4% identical to PAM if the conserved C-repeat domain is excluded. Phylogenetic analysis of Prp and a subset of group A, C, and G streptococcal plasminogen-binding M proteins indicates that Prp is a phylogenetically distinct molecule (Fig. 1). All of the PAM variants share a common ancestor that does not include Prp, suggesting that Prp diverged early during the evolution of PAM and the PAM-related proteins.

Plasminogen binding analysis. In order to confirm that Prp functions as a high-affinity plasminogen receptor, plasminogen binding analysis of recombinant Prp was undertaken. Recombinant Prp was expressed and purified using glutathione-agarose and Ni-nitrilotriacetic acid agarose affinity chromatographies. Recombinant PAMNS13 and the NS696 M1 protein were also purified for use as positive and negative controls, respectively, in plasminogen binding studies (Fig. 2A). PAMNS13 is 100% identical to the prototype PAM protein in the a1 and a2 repeat region and has previously been shown to interact with glu-plasminogen with high affinity, while the NS696 M1 protein displays only nonspecific binding affinity for plasminogen (26). Ligand blot analysis indicated that the 43-kDa recombinant Prp molecule interacts with biotinylated glu-plasminogen (Fig. 2B). The interaction between recombinant Prp and glu-plasminogen was further characterized using solid-phase plasminogen binding assays. Immobilized Prp bound plasminogen in a dose-dependent fashion, and saturable binding was achieved with 500 nM plasminogen after 2 h (Fig. 3A). This is comparable to the binding of plasminogen by

the positive control protein PAMNS13 (Fig. 3B). Only nonspecific binding was found for the negative control NS696 M1 protein (Fig. 3C).

Best-fit nonlinear regression analysis determined the equilibrium dissociation constant (K_d) for the interaction of Prp with glu-plasminogen to be 7.6 nM. The interaction between Prp and plasminogen is therefore significantly lower than that of PAMNS13 with plasminogen ($K_d = 1.6$ nM; $P = 0.02$). To further explore the relative affinity of Prp for plasminogen compared to PAM, Prp binding to biotinylated plasminogen was competed with various concentrations of unlabeled fluidphase PAMNS13. The effective concentration of competitor required to inhibit plasminogen binding by 50% was found to be 0.34 μ M, as determined by fitting a one-site competition curve (Fig. 4). This confirms that Prp has a lower affinity for plasminogen than PAM. Nonetheless, the binding of plasminogen by Prp with a K_d of 7.6 nM represents a high-affinity, physiologically relevant interaction.

Binding site characterization. The major site of variation between Prp and the prototype PAM protein lies between residues 29 and 100 in the N terminus of Prp. This sequence includes a region of 21 amino acids, with 52% identity to the plasminogen binding site of PAM (17), which may function as a plasminogen binding domain within the Prp molecule. This putative plasminogen binding domain appears to consist of a single binding site comprised of 21 amino acids (Fig. 5A), in contrast to the $\alpha 1$ and $\alpha 2$ repeats found to mediate plasminogen binding by PAM (3). In order to confirm the role of this potential binding site in the interaction of Prp with plasminogen and to identify the specific residues which mediate binding, a series of site-directed mutants in which

selected binding site residues were mutated to alanine was constructed. The binding-site sequences of the site-directed mutants are given in Fig. 5B. Plasminogen typically binds to internal and C-terminally lysine residues, and thus lysine residues Lys⁹⁶ and Lys¹⁰¹ were mutated to alanine. Additionally, PAM is able to mediate high-affinity plasminogen binding via internal residues Arg¹⁰¹, His¹⁰², Arg¹¹⁴, and His¹¹⁵ (27). Therefore, residues Arg¹⁰⁷ and His¹⁰⁸ were also selected for mutagenesis. The impact of mutations in the plasminogen binding domain of Prp on protein structure was analyzed using far-UV CD spectroscopy. All site-directed mutants used in this study were found to have a CD emission spectrum similar to that of wild-type Prp, displaying two characteristic minima at approximately 210 nm and 220 nm and a maximum peak at 190 nm (data not shown). This is similar to the CD spectra of other streptococcal M proteins, which are coiled-coil α -helical proteins (20). Thus, even after mutagenesis these proteins appear to maintain an α -helical secondary structure. Additionally, for all proteins the two minima are of a similar magnitude, which is indicative of coiled-coil proteins (9, 10). The percent α -helicity ranged from 37% to 43%. These data confirm that these proteins are structurally similar and that site-directed mutagenesis has resulted in minimal changes to secondary structure.

In order to determine the impact of site-directed mutagenesis on plasminogen binding by Prp, plasminogen binding analysis was conducted. Wild-type Prp and Prp[K⁹⁶ K¹⁰¹] both interacted with biotinylated glu-plasminogen in a ligand blot analysis. No interaction with Prp[K⁹⁶K¹⁰¹R¹⁰⁷H¹⁰⁸] or Prp [R¹⁰⁷H¹⁰⁸] was seen (Fig. 6). This suggests that the interaction of Prp with glu-plasminogen is mediated by residues Arg¹⁰⁷ and His¹⁰⁸. To

further characterize the role of these residues in plasminogen binding and to determine the specificity of Prp[K⁹⁶K¹⁰¹] plasminogen binding, solid-phase plasminogen binding assays were conducted using the site-directed mutant Prp proteins. None of the site-directed mutants interacted in a specific, saturable fashion with 500 nM plasminogen (Fig. 7). Prp[K⁹⁶K¹⁰¹] approached but did not reach saturation (Fig. 7). Indeed, in the presence of 500 nM plasminogen, wild-type Prp was found to interact specifically with approximately 80% of the available labeled plasminogen. In contrast, Prp[K⁹⁶K¹⁰¹] shows only 50% specific binding at the same plasminogen concentration. This suggests that this protein is still able to mediate a lower-affinity interaction with plasminogen, in spite of the absence of lysine residues. Only nonspecific binding was found for site-directed mutants Prp[K⁹⁶K¹⁰¹R¹⁰⁷H¹⁰⁸] and Prp[R¹⁰⁷H¹⁰⁸]. Thus, it appears that residues Arg¹⁰⁷ and His¹⁰⁸ are indispensable for mediation of the high-affinity interaction of Prp with plasminogen.

DISCUSSION

Prp functions as a high-affinity receptor for glu-plasminogen, as evidenced by the plasminogen binding data presented here. To date, PAM has been reported to have the highest affinity of the known GAS plasminogen receptors for glu-plasminogen (K_d 1 nM) (3, 26). While the interaction of Prp with gluplasminogen is of a lower affinity than that of PAM (K_d = 7.8nM), Prp binds plasminogen specifically. Furthermore, circulating concentrations of glu-plasminogen are approximately 2 μ M, and thus the interaction of Prp with plasminogen is within physiologically relevant concentrations of the zymogen (8).

Plasminogen typically binds to receptors via C-terminal or internal lysine residues, as exemplified by the previously characterized GAS plasminogen receptors SEN and GAPDH. Binding of plasminogen to SEN occurs via residues Lys⁴³⁴ and Lys⁴³⁵ at the C terminus of the SEN molecule, while binding of plasminogen to GAPDH occurs via the C-terminal lysine Lys³³⁴ (4, 18). In contrast, plasminogen binding to PAM has been attributed to internal lysine residues Lys⁹⁸ and Lys¹¹¹ (3, 34, 35). However, it has recently been demonstrated that PAM mediates its interaction with plasminogen via arginine and histidine residues within two internal repeat domains (27). Mutation of lysine residues within a putative Prp plasminogen binding site (Lys96 and Lys101) did not result in the abrogation of plasminogen binding. While mutation of these residues reduced the affinity of Prp for plasminogen, binding was fully eliminated only following the mutation of residues Arg¹⁰⁷ and His¹⁰⁸ to alanine. Indeed, the finding that plasminogen binding was eliminated even in the presence of lysine residues highlights the role of residues Arg¹⁰⁷ and His¹⁰⁸ in mediating the interaction of Prp with plasminogen. Changes in plasminogen binding by site-directed mutants do not appear to result from protein structural changes, as far-UV CD analysis of site-directed mutants indicates that these molecules are structurally similar to the wild-type Prp. The percent α -helicity for mutants reported here was between 37% and 43%. CD analysis of other streptococcal M proteins has found them to contain between 23% and 70% α -helices (20). This demonstrates that despite the phylogenetic differences between PAM and Prp, both interact with plasminogen via a common mechanism which is distinct from the interaction of other plasminogen-binding proteins.

X-ray crystallography analysis of a 30-amino-acid peptide has shown that arginine and histidine residues, when correctly presented in an alpha-helical molecule, are able to make numerous salt bridge and hydrophobic electrostatic interactions with recombinant kringle 2 of plasminogen, forming a pseudoligand similar to the lysine analogue ϵ -amino caproic acid (23). It is conceivable that the residues Arg¹⁰⁷ and His¹⁰⁸ within Prp interact with plasminogen in a similar fashion. Thus, the binding site of Prp can be localized to a single domain within the N terminus of the protein encompassing residues Arg¹⁰⁷ and His¹⁰⁸.

Recent findings that the acquisition of plasminogen by *S. pyogenes* may be crucial for the virulence of certain strains of GAS (6, 30), and the ability of multiple GAS proteins to facilitate this process, necessitate a deeper understanding of the mechanisms via which GAS interacts with plasminogen. This study identifies a novel plasminogen-binding M protein which interacts with plasminogen via arginine and histidine residues within a single binding domain. While lysine residues within this domain do appear to contribute partially to this interaction, they are not the primary residues by which plasminogen binds, in contrast to findings for other GAS plasminogen receptors. The finding that multiple M proteins with plasminogen binding ability have evolved separately is indicative of the selective advantage that plasminogen binding confers upon GAS and supports the suggestion that interaction with the host plasma protein plasminogen may be important for the virulence of certain strains of GAS. Plasminogen binding by arginine and histidine residues has, to date, been reported only for GAS plasminogen-binding M proteins. This interaction may represent a novel way in which the bacterium binds to the

low-affinity kringle 2 of plasminogen, thereby avoiding competition with other abundant host proteins and inhibitors. The apparent advantage conferred on GAS by its ability to interact with the host plasminogen activation system via a number of receptors has widespread implications for both the study and treatment of GAS pathogenesis and infection.

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FIGURE LEGENDS

FIG. 1. Phylogeny generated by Bayesian analysis of the amino acid sequences of 13 PAM genes (PAM_{NS13}, GenBank accession no. AY351851; PAM_{NS455}, GenBank accession no. AY351857; PAM_{NS265}, GenBank accession no. AY351855; PAM_{NS223}, GenBank accession no. AY351854; PAM_{NS253}, GenBank accession no. AY351853; PAM_{NS53}, GenBank accession no. AY351852; PAM_{NS32}, GenBank accession no. AY351850; PAM_{NS50.1}, GenBank accession no. AY351849; PAM_{NS59}, GenBank accession no. AY351848; PAM_{NS1133}, GenBank accessionno. AY351847; PAM_{NS10}, accession no. GenBank AY351846; PAM_{NS221}, GenBank accession no. DQ136319; NS696 M1 protein, GenBank accessionno. AY351858; and Prp, GenBank accession no. AY351856). MLC36 (GenBank accession no. Z32677) and MLC72 (GenBank accession no. Z32678) are plasminogen-bindingMprotein sequences from group C andgroup G streptococci, respectively (2). The M1 (11) and NS696 M1 proteinsequences were included as outgroups.

FIG. 2. SDS-PAGE and ligand blot analysis of recombinant M proteins. (A) SDS-12% polyacrylamide gel showing the purified recombinant proteins PAM_{NS13} (lane 1), Prp (lane 2), and NS696 M1 (lane 3). Molecular mass markers are given in kilodaltons. (B) Ligand blot analysis employing biotinylated glu-plasminogen of purified recombinant proteins PAM_{NS13} (lane 1), Prp (lane 2), and NS69 6 M1 (lane 3). Molecular mass markers are given in kilodaltons.

FIG. 3. Saturation binding analysis of biotinylated glu-plasminogen binding to immobilized recombinant proteins. Biotinylated glu-plasminogen binding to immobilized recombinant proteins Prp (A), PAM_{NS13} (B), and NS696 M1 protein (C) was measured in the absence (▼, total binding) and presence (◆, nonspecific binding) of a 50-fold molar excess of unlabeled glu-plasminogen. Specific binding (■) was determined by subtracting nonspecific binding from total binding at each concentration. A one-site hyperbolic binding function was fitted to the data ($P < 0.05$), from which the binding dissociation constants were determined. Error bars represent the standard errors of the means.

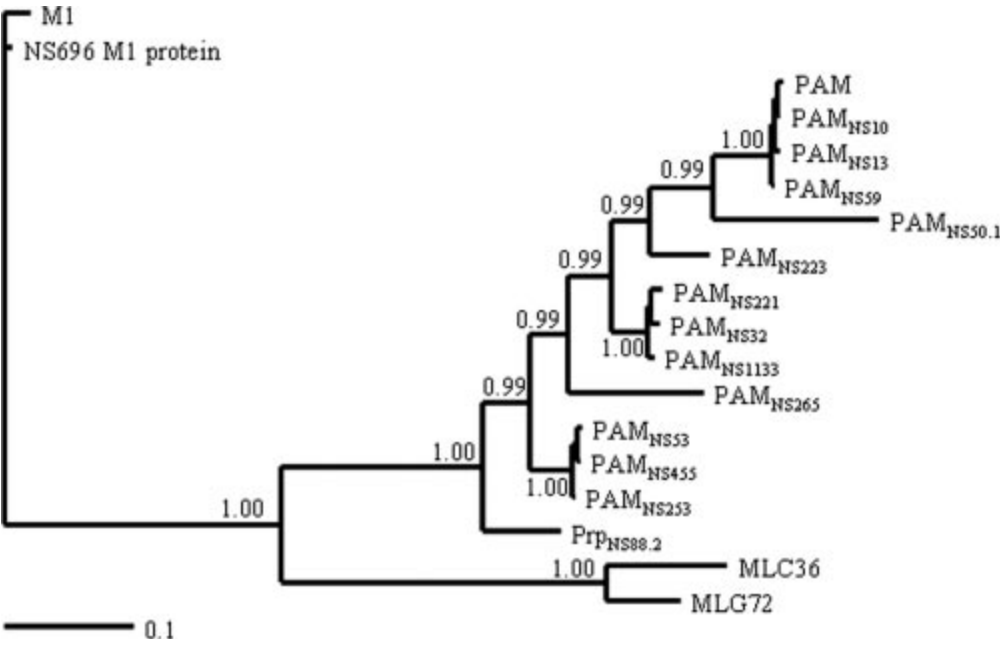
FIG. 4. Competition of glu-plasminogen binding to immobilized recombinant Prp with fluid-phase PAM_{NS13}. Binding of biotinylated glu-plasminogen to immobilized Prp was measured in the presence of various concentrations of unlabeled fluid-phase PAM_{NS13}. Data points are the mean values of triplicate readings, with error bars indicating standard errors of the means. One-site competition analysis was used to determine the concentration of PAM_{NS13} required to inhibit binding of biotinylated glu-plasminogen by 50%.

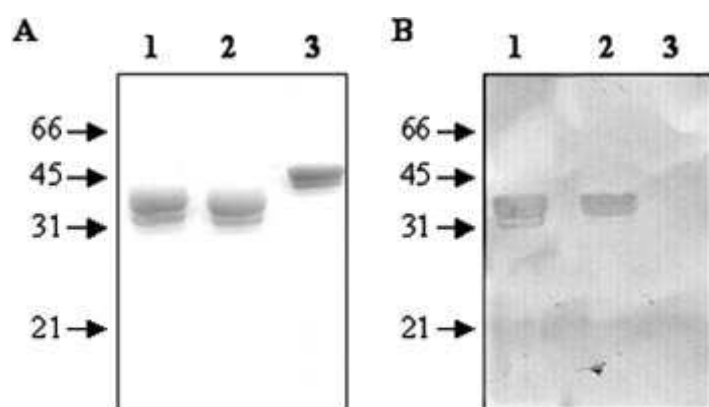
FIG. 5. (A) Translated DNA sequences of the plasminogen-binding region (a1/a2 repeats) of the prototype PAM binding site with PAM_{NS13} and a putative 21-amino-acid residue Prp plasminogen-binding site. *, residues identical to those of the PAM sequence; -, gaps in the alignment. (B) Alignment of the plasminogen binding domain of wild-type Prp with those of the three site-directed mutants constructed in this study. Mutated residues are indicated in boldface.

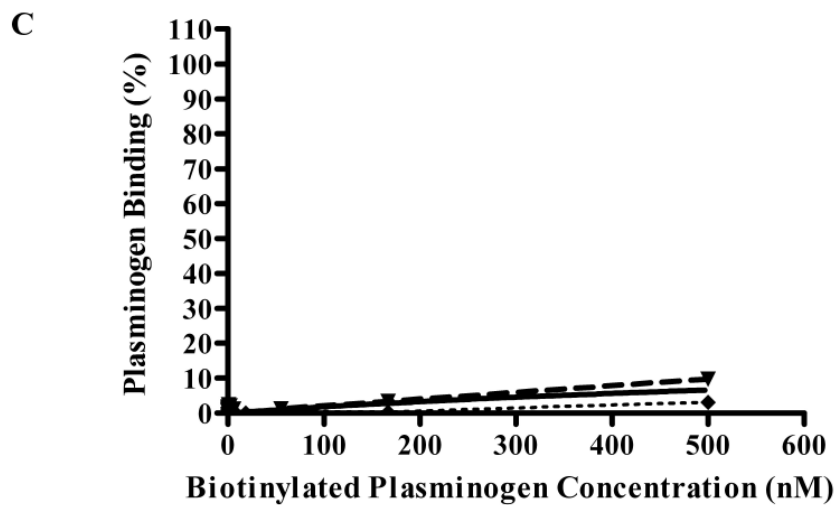
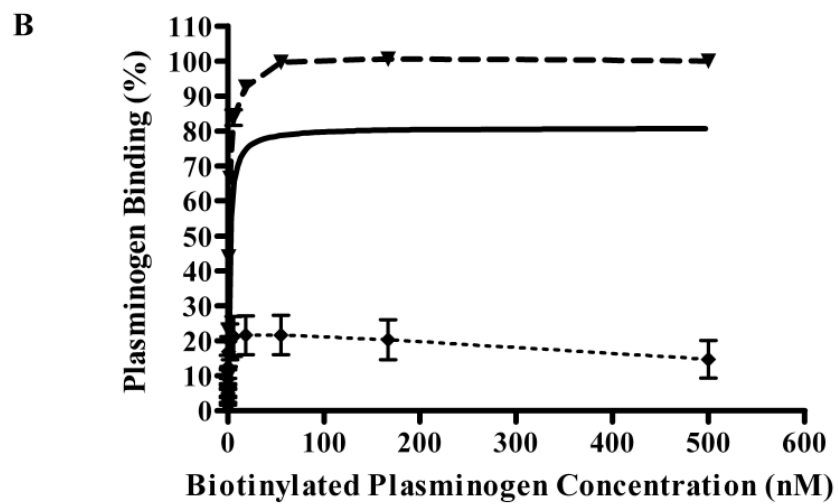
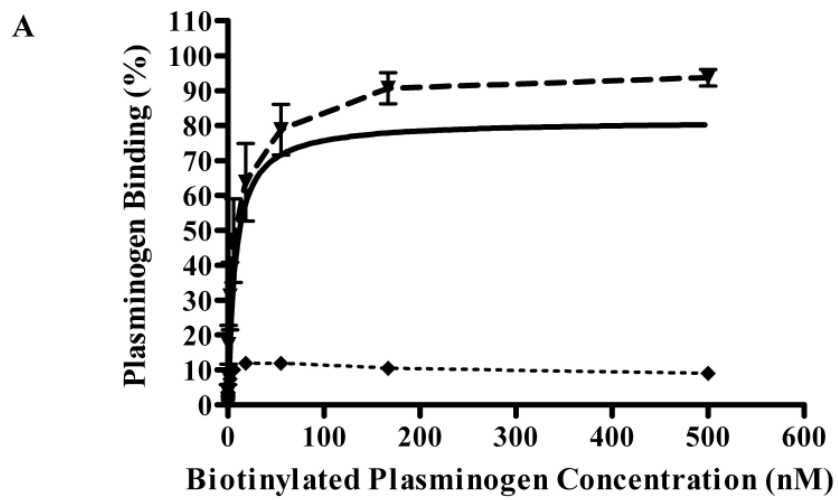
FIG. 6. SDS-PAGE and ligand blot analysis of Prp site-directed mutants. (A) SDS-12% polyacrylamide gel showing the purified recombinant proteins Prp (lane 1), Prp[K⁹⁶K¹⁰¹R¹⁰⁷H¹⁰⁸] (lane 2), Prp[K⁹⁶K¹⁰¹] (lane 3), and Prp[R¹⁰⁷H¹⁰⁸] (lane 4). Molecular mass markers are given in kilodaltons. (B) Ligand blot analysis employing biotinylated glu-plasminogen of purified recombinant proteins Prp (lane 1), Prp[K⁹⁶K¹⁰¹R¹⁰⁷H¹⁰⁸] (lane 2), Prp[K⁹⁶K¹⁰¹] (lane 3), and Prp[R¹⁰⁷H¹⁰⁸] (lane 4). Molecular mass markers are given in kilodaltons.

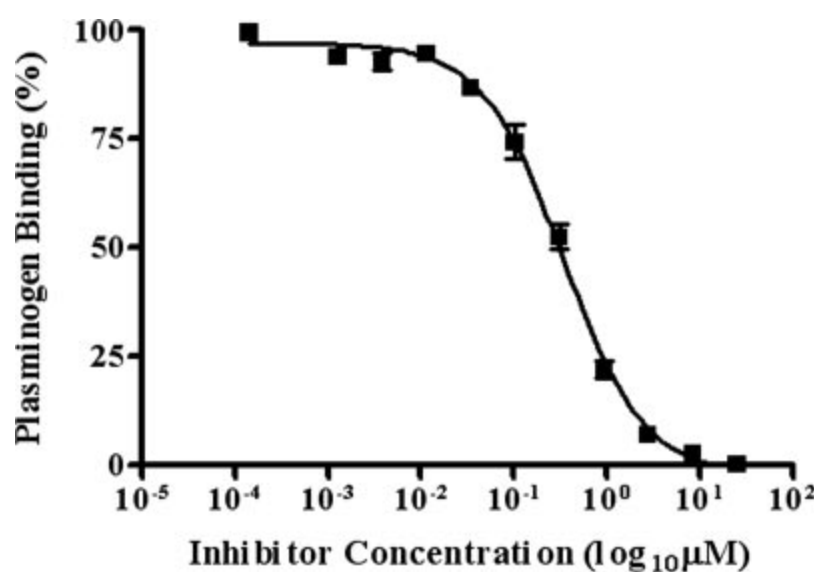
FIG. 7. Specific binding of biotinylated glu-plasminogen to immobilized recombinant proteins Prp (■), Prp[K⁹⁶K¹⁰¹R¹⁰⁷H¹⁰⁸] (▼), Prp[K⁹⁶K¹⁰¹] (●), and Prp[R¹⁰⁷H¹⁰⁸] (F). Specific binding was determined by subtracting nonspecific binding from total binding at each concentration. A one-site hyperbolic binding function was fitted to the data ($P < 0.05$), from which the binding dissociation constants were determined. Error bars represent the standard errors of the means ($n=3$).

FIGURES









A	a1	a2	
PAM	DAELQRLKNERH---E	EAELERLKSERHD	Percent Identity
NS13	*****	*****	100%
Prp	ER**EDL*-----	D***K**NE****	52%

B	Recombinant Protein	Plasminogen Binding Site
	Prp	ERELEDLKDAELKRLNEERHD
	Prp [K ⁹⁶ K ¹⁰¹]	ERELEDLADAELARLNEERHD
	Prp [K ⁹⁶ K ¹⁰¹ R ¹⁰⁷ H ¹⁰⁸]	ERELEDLADAELARLNEEAAD
	Prp [R ¹⁰⁷ H ¹⁰⁸]	ERELEDLKDAELKRLNEEAAD

