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 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, mpr, 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 nonetheless binds plasminogen with high affinity and at physiologically relevant concentrations of plasminogen (Kd = 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.

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* 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 preset 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 N88S.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 termini 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 94°C for 1 min, 68°C for 10 min. An additional 7-min extension at 55°C was then used using previously mutated DNA as a template. Following an initial denaturation step reactions not encoded by a single primer, alanine residues were sequentially introduced available upon request. For site-directed mutants containing more than two mutations, side by 15 nucleotides of wild-type sequence. The specific primer sequences are 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 PNT (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 PNT) 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 PAM384 (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 μl of neutravidin conjugated to biotin-X-glu-plasminogen (Pharmacia, Australia) diluted 1:5,000 with blocking solution was added to all wells and incubated for 2 h at room temperature. After washes with PNT, the reactions were developed by the addition of 50 μl 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 μl of 10 M hydrochloric acid, and the plates were read at 490 nm using a Spectramax 250 plate reader (Molecular Devices).

RESULTS

Phylogenetic analysis. Isolated from a bacteremia infection, GAS strain N88S.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-nitrioltriacetic acid agarose affinity chromatography. Recombinant PAM384 and the NS696 M1 protein were also purified for use as positive and negative controls, respectively, in plasminogen binding studies (Fig. 2A). PAM384 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 \( (K_d = 1.6 \text{ nM}; P = 0.02) \). 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 fluid-phase PAMNS13. The effective concentration of competitor required to inhibit plasminogen binding by 50% was found to be 0.34 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 a1 and a2 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-terminal 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.

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[K96K101] 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

![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[K96K101] (lane 2), Prp[K96K101R107H108] (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[K96K101] (lane 2), Prp[K96K101R107H108] (lane 3), and Prp[R107H108] (lane 4). Molecular mass markers are given in kilodaltons.](http://jb.asm.org/)

![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%.](http://jb.asm.org/)

![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.](http://jb.asm.org/)
of Prp with glu-plasminogen is mediated by residues Arg\(^{107}\) and His\(^{108}\). To further characterize the role of these residues in plasminogen binding and to determine the specificity of Prp[\(K^{96}K^{101}\)] 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[\(K^{96}K^{101}\)] 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[\(K^{96}K^{101}\)] 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[\(K^{96}K^{101}R^{107}H^{108}\)] and Prp[\(R^{107}H^{108}\)]. Thus, it appears that residues Arg\(^{107}\) and His\(^{108}\) 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 (\(K_a \sim 1\) nM) (3, 26). While the interaction of Prp with glu-plasminogen is of a lower affinity than that of PAM (\(K_a = 7.8\) nM), Prp binds plasminogen specifically. Furthermore, circulating concentrations of glu-plasminogen are approximately 2 \(\mu\)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 Lys\(^{334}\) and Lys\(^{345}\) at the C terminus of the SEN molecule, while binding of plasminogen to GAPDH occurs via the C-terminal lysine Lys\(^{338}\) (4, 18). In contrast, plasminogen binding to PAM has been attributed to internal lysine residues Lys\(^{98}\) and Lys\(^{111}\) (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 (Lys\(^{96}\) and Lys\(^{101}\)) 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 Arg\(^{107}\) and His\(^{108}\) to alanine. Indeed, the finding that plasminogen binding was eliminated even in the presence of lysine residues highlights the role of residues Arg\(^{107}\) and His\(^{108}\) 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 \(\alpha\)-helix 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% \(\alpha\)-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 \(\varepsilon\)-amino caproic acid (23). It is conceivable that the residues Arg\(^{107}\) and His\(^{108}\) 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 Arg\(^{107}\) and His\(^{108}\).

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.

ACKNOWLEDGMENTS
This work was funded by the National Health and Medical Research Council [grant 303401]. M. L. Sanderson-Smith is the recipient of an Australian Research Council Postgraduate Award.

We thank T. Trewick for her assistance with the circular dichroism analysis.

REFERENCES