Divergence in the Plasminogen-binding Group A Streptococcal M Protein Family

FUNCTIONAL CONSERVATION OF BINDING SITE AND POTENTIAL ROLE FOR IMMUNE SELECTION OF VARIANTS

Received for publication, August 9, 2005, and in revised form, November 23, 2005 Published, JBC Papers in Press, November 30, 2005, DOI 10.1074/jbc.M508758200

Martina Sanderson-Smith†, Michael Batzloff†, Kabada S. Sriprakash†, Mark Dowton†, Marie Ranson†, and Mark J. Walker†–2

From the †School of Biological Sciences, University of Wollongong, Wollongong, New South Wales 2522 and ‡Queensland Institute for Medical Research, Herston, Queensland 4006, Australia

Group A streptococci (GAS) display receptors for the human zymogen plasminogen on the cell surface, one of which is the plasminogen-binding group A streptococcal M protein (PAM). Characterization of PAM genes from 12 GAS isolates showed significant variation within the plasminogen-binding repeat motifs (a1/a2) of this protein. To determine the impact of sequence variation on protein function, recombinant proteins representing five naturally occurring variants of PAM, together with a recombinant M1 protein, were expressed and purified. Equilibrium dissociation constants for the interaction of PAM variants with biotinylated Glu-plasminogen, were expressed and purified. Equilibrium dissociation constants for the interaction of PAM variants with biotinylated Glu-plasminogen ranged from 1.58 to 4.99 nM. Effective concentrations of prototype PAM required for 50% inhibition of plasminogen binding to immobilized PAM variants ranged from 0.68 to 22.06 nM. These results suggest that although variation in the a1/a2 region of the PAM protein does affect the comparative affinity of PAM variants, the functional capacity to bind plasminogen is conserved. Additionally, a potential role for the a1 region of PAM in eliciting a protective immune response was investigated by using a mouse model for GAS infection. The a1 region of PAM was found to protect immunized mice challenged with a PAM-positive GAS strain. These data suggest a link between selective immune pressure against the plasminogen-binding repeats and the functional conservation of the binding domain in PAM variants.

Streptococcus pyogenes (group A streptococcus (GAS))3 is a Gram-positive bacterium responsible for a wide variety of skin and mucosal infections in humans. 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. The mechanisms by which GAS initiates pathogenesis have yet to be fully elucidated; however, one proposed mechanism is via subversion of the host plasminogen activation system by GAS. The plasminogen zymogen, once converted to the active protease plasmin, has the ability to degrade fibrin clots, connective tissue, and the extracellular matrix (6, 7). Thus activation of this proteolytic system by GAS may have significant pathological consequences in the host. Four GAS plasminogen-binding proteins have been identified, including the plasminogen-binding group A streptococcal M protein (PAM) (2).

Isolated initially from M53 serotype GAS, PAM is a 42-kDa protein that binds both plasmin and plasminogen with high affinity (Kd ~1 nM) (2). The major plasminogen-binding site of PAM is located in the N-terminal variable region of the protein and is comprised of two characteristic tandem repeats designated a1 and a2. Similar binding motifs have been identified in M-like proteins of other GAS isolates associated with both invasive and noninvasive disease (2, 8, 9). PAM lacks the typical C-terminal lysine residues of many plasminogen receptors (3, 10, 11). Rather, internal lysine residues in the a1/a2 repeat regions of PAM (Lys98 and Lys111), along with internal His102, Arg101, and Glu104 residues in a1, mediate binding to kringle 2 of plasminogen (12–15).

There is a mounting body of evidence to suggest a role for the plasminogen activation system in streptococcal virulence, and it has recently been hypothesized that PAM-dependent plasminogen binding may confer a selective advantage on GAS during host colonization and infection (16). The multiplicity of potential virulence factors associated with GAS that interact with the plasminogen activation system necessitates a deeper understanding of the GAS-plasminogen relationship. This study aims to investigate the potential effect of sequence variation among naturally occurring PAM variants on plasminogen binding function. Variation within the functional domain of PAM may result from selective pressure by the host immune response. If this is the case, it is expected that the plasminogen binding domain within PAM will be highly immunogenic, and this response will be restrictive for propagation of GAS strains in systemic infection. To examine this hypothesis, we report here that the immune response against the plasminogen-binding domain of PAM is functionally opsonic for GAS in a phagocytosis assay and thus could result in selective pressure for generation of new variants.

* This work was supported in part by National Health and Medical Research Council Grant 303401. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of an Australian Research Council postgraduate award.

§ Two who correspondence should be addressed: School of Biological Sciences, University of Wollongong, Wollongong, New South Wales 2522, Australia. Tel.: 61-2-4221-3439; Fax: 61-2-4221-4135; E-mail: mwalker@uow.edu.au.

‡ The abbreviations used are: GAS, group A streptococcus; PAM, plasminogen-binding group A streptococcal M protein; PFGE, pulsed field gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; KLH, keyhole limpet hemocyanin; THB, Todd Hewitt broth; BSA, bovine serum albumin; CFU, colony forming units; Ni-NTA, nickel-nitrilotriacetic acid.
Characterization of PAM Variants

TABLE 1

| Primer | PCR amplification primers |
|--------|--------------------------|
| N5113pGEX2TP | 5’-GGGGAGTTCGCGCTCTTTTCTG-3’ |
| N5113pGEX2TR | 3’-GATCCCGTGATCTTCGCTTACCACT-5’ |
| N5113pGEK2TF | 5’-GGGGAGTTCGCGCTCTTTTCTG-3’ |
| N5113GEX2TF | 3’-GATCCCGTGATCTTCGCTTACCACT-5’ |

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) containing 1% yeast extract. Cultures were inoculated with single GAS colonies. All streptococcal strains used in this study were collected from the Northern Territory of Australia and have been described previously (9, 17). Escherichia coli INV F containing pCR2.1/PAM constructs (9) or E. coli TOP10 containing expression plasmids were grown on Luria Bertani (LB) agar plates or cultured in LB broth supplemented with ampicillin (100 μg/ml) as described previously (18).

Pulsed Field Gel Electrophoresis (PFGE) and Phylogenetic Analysis—To determine whether variation within the PAM genes of streptococcal isolates was indicative of wider chromosomal variation, PFGE of the chromosomal DNA of 12 PAM-positive GAS isolates was performed. Fifteen PAM-negative isolates were also included to enable comparison of chromosomal variation between PAM-positive and PAM-negative isolates. Pulsed field samples were prepared and electrophoresed as described previously (17). PFGE restriction fragment patterns were analyzed using Diversity software (version 2.1; Bio-Rad). Genetic similarities were compared by clustering methods (unweighted pair group method with arithmetic means) using the Dice coefficient. To characterize the evolutionary relationships between the PAM genes used in the study, the full amino acid sequence of 12 previously identified PAM proteins (9) was aligned by using ClustalW (19). Evolutionary gene trees were then estimated using MrBayes version 3.1 (20, 21). 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 converged. Trees sampled prior to convergence were discarded (the first 40 trees). The amino acid model was empirically chosen by MrBayes, using the preset amodelpr = mixed command. This permitted jumping between nine alternative amino acid substitution models. The WAG model (22) was empirically chosen by MrBayes. The majority rule consensus of all trees generated after convergence 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 PAM-like genes from 12 GAS strains had been cloned previously into the vector pCR2.1 (9). DNA sequence analysis was performed using the primers listed in Table 1, as well as M13LacZ universal forward and reverse primers (PerkinElmer Life Sciences), which anneal to pCR2.1, and sequence reactions undertaken using terminator ready reaction mix (PerkinElmer Life Sciences). DNA sequencing gels were prepared as per the manufacturer’s instructions and electrophoresed using an ABI PRISM 377 sequencer (PerkinElmer Life Sciences). Sequence data were analyzed using ABI Prism™ DNA sequencing analysis software (PerkinElmer Life Sciences). The sequences were submitted to the NCBI data base, and the following GenBank accession numbers were obtained: PAMNS13, AY351851; PAMNS455, AY351857; PAMNS265, AY351855; PAMNS223, AY351854; PAMNS253, AY351853; PAMNS353, AY351852; PAMNS32, AY351850; PAMNS651, AY351849; PAMNS509, AY351848; PAMNS1133, AY351847; PAMNS10, AY351846; PAMNS221, DQ136319; and NS696 M1 protein gene AY351858. Five naturally occurring variants of the prototype PAM gene were selected for functional studies, including one with 99.7% identity to the prototype PAM and 100% identity in the plasminogen-binding domain (PAM513). The NS696 M1 protein, from an emn1 gene type (NS696) gene was selected as a negative control for plasminogen binding assays. These genes were amplified from pCR2.1 constructs using Ffu polymerase (Strategene) with the oligonucleotide primers listed in Table 1. BamHI and EcoRI restriction sites were incorporated into the oligonucleotide primers. The amplification products did not encode a signal peptide at the N terminus nor the LPXTG motif at the C terminus for each recombinant protein. Additionally, a C-terminal hexahistidyl tag (His6) was incorporated into the reverse primer sequence. PCR cycling parameters consisted of 30 cycles of 97, 55, and 72 °C for denaturation, annealing, and extension reactions, respectively. Amplicons were cloned into pGEX-2T (23) resulting in an N-terminal fusion with glutathione S-transferase, and the constructs were transformed into E. coli TOP10 (Invitrogen) using standard procedures (18). 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 essentially as described previously (23), with the following modifications. 1 liter of bacterial culture was incubated at 37 °C with shaking at 225 rpm until the culture reached A600 nm 0.6, at which point protein expression was induced for 4 h by the addition of 0.1 mM isopropyl β-D-1 thiogalactopyranoside. Protein was expressed in the presence of 1

3218 JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 281 • NUMBER 6 • FEBRUARY 10, 2006
mm phenylmethylsulfonyl fluoride (PMSF) to reduce protein degradation. Cells were harvested by centrifugation and resuspended in ice-cold PBS containing 1% Triton X-100. Cell lysis was achieved by sonication of the cell suspension with a Branson sonifier 250 (30% duty cycle, microtip output control 2) for 2 min and the addition of a 0.1× volume of lysis buffer (25 mM Tris-Cl, pH 8.0, 10 mg/ml lysozyme). Following centrifugation, recombinant protein was purified from the cleared lysate using a glutathione-agarose (Sigma) column. Prior to elution from the column, the glutathione S-transferase tag was removed from the N terminus of recombinant fusion proteins by the addition of 1 column volume of thrombin solution (1 unit of thrombin/μl, PBS, pH 8.0). The column was incubated for 5 h at room temperature, and the cleaved recombinant protein was eluted in PBS, pH 8.0. To remove thrombin from the protein solution, the C-terminal His6 tag was utilized to purify the protein further by using a Ni-NTA column. Protein solution was added to an equilibrated Ni-NTA-agarose (Qiagen, Germany) column and washed with 10 column volumes of PBS. Bound protein was eluted under native conditions with elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 8.0), and the imidazole was removed by dialysis against 10 liters of 1× PBS. Each step of the protein purification process was monitored by 12% SDS-PAGE analysis (24), with protein visualized using Coomassie R-250 staining.

Plasminogen Purification and Labeling—Glu-plasminogen was purified from human plasma using lysine-Sepharose-4B affinity chromatography as described previously (25), with the following modifications. Human plasma (300 ml) was diluted with an equal volume of distilled water containing 10 mM EDTA and 2 mM PMSF. Precipitated proteins were allowed to settle, and the cleared plasma was batch-incubated with 60 ml of lysine-Sepharose 4B (Amersham Biosciences) for 2 h with gentle agitation. Following centrifugation, the resin was poured into a column and washed overnight with PBS, pH 7.4, containing 10 mM EDTA and 2 mM PMSF. The column was then washed with salt wash buffer (0.05 M Na2HPO4, 5 mM NaCl, pH 7.5), and the plasminogen eluted with PBS, pH 7.4, containing 200 mM e-amino caproic acid (Sigma). e-Amino caproic acid was removed by dialysis at 4 °C against 10 liters of PBS. Purified plasminogen was biotinylated by the addition of 10% (v/v/1 M NaHCO3, pH 9, and a 40-fold excess of biotin-X-NHS in dimethyl sulfoxide (Sigma). The reaction was incubated at 4 °C overnight with mixing. Free biotin was separated from biotinylated plasminogen by PD-10 gel filtration chromatography (Amersham Biosciences) (25).

Plasminogen Binding Analysis—Ligand blotting analysis of recombinant proteins was conducted using biotinylated Glu-plasminogen as described previously (25). To characterize further the interaction between the recombinant PAM variants and Glu-plasminogen, solid phase microtiter plasminogen binding assays were performed. 96-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 NaH2PO4, 150 mM NaCl, 0.05% Tween 80, pH 7.5), plates were blocked with 50 μl of blocking solution (1% skim milk powder, PiNT) for 1 h at 37 °C. Wells were washed as above, and 500 nm biotinylated Glu-plasminogen was diluted in a 3-fold 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-0.14 nM) were allowed to compete with immobilized proteins for binding to biotinylated Glu-plasminogen. Competitor was titrated 3-fold across the microtiter plate prior to the addition of biotinylated Glu-plasminogen to all wells, at a final concentration of 500 nm. The assay was incubated for 2 h at room temperature. Following the plasminogen incubation step, microtiter plates were washed three times, and 50 μl of neutrophin conjugated to horseradish peroxidase (Progen, Australia) diluted 1:5000 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 μl of o-phenylenediamine (Sigma) 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).

Data were normalized against the highest and lowest absorbance value for each assay, and nonlinear regression analysis performed using GraphPad® Prism (version 4.00, GraphPad software). For the calculation of equilibrium binding dissociation constants (Kd), a one versus two site binding analysis was conducted, and the best fit curve was 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% (EC50) was calculated.

Immunization and Challenge of Mice—Quackenbush (outbred) mice (n = 20; Animal Resources Centre, Western Australia) were immunized with a commercially synthesized peptide representing the α1 repeat of PAM (NH2-CDAELQRLKNERHE-COOH) conjugated to keyhole limpet hemocyanin (KLH; Chiron Mimotopes, San Diego) with PepM or with controls. PepM, a pepsin extract of M protein, was prepared from isolate NS13 as described previously (26). PepM was used as a positive control for the immunization procedure as it has been demonstrated that PepM extracts induce type-specific opsonic antibodies that confer protection upon subsequent challenge with a homologous GAS strain (27, 28). Peptide-KLH conjugate, PepM, KLH control, or PBS control was administered subcutaneously in a volume of 50 μl at the tail base. Each mouse received a total of 30 μg of immunogen emulsified 1:1 in complete Freund’s adjuvant (Difco) on day 1 and booster injections at days 21 and 28 with antigen in PBS. Mice were bled on days 20, 27, and 35 post-primary immunization. All experimental protocols described in this report complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, Australia) and institutional ethics requirements.

The NS13 GAS strain was passaged in mice to enhance virulence before challenge. GAS was cultured overnight in THB with 1% neopeptone (THBN; Difco), washed twice in THBN, and resuspended in 25% of the original volume. The inoculum dose (CFU/ml) was determined using a previously determined standard curve of A500 nm against CFU/ml. Following overnight incubation at 37 °C, colony counts were determined. Groups of immunized (n = 20) and control (n = 20) mice were challenged intraperitoneally with GAS 10 days after the final immunization. One mouse from each of the PepM and KLH control groups died during the challenge procedure.

Detection of Murine Antibodies and Indirect Bactericidal Assay—Peptide-specific murine serum IgG antibodies and antibody isotypes were determined by enzyme-linked immunosorbent assay as described previously (29, 30). Plates were coated with either a1 peptide conjugated to bovine serum albumin (BSA) or BSA alone. The absorbance of BSA control wells was subtracted from the absorbance of corresponding a1 peptide-BSA wells to determine the specific anti-a1 peptide response. Titer was defined as the highest dilution that gave an absorbance of more than three standard deviations above the mean absorbance of control wells containing nonimmunized mouse sera.

Murine sera were assayed for the ability to opsonize GAS in vitro as described previously (31). Briefly, GAS strains NS13 and NS455 were grown overnight at 37 °C in 5 ml of THB, followed by serial dilution to
FIGURE 1. Evolutionary analysis of PAM-positive GAS isolates. A, dendrogram generated by diversity software showing the genetic relationships between 12 PAM-positive GAS isolates and 15 PAM-negative GAS isolates. The dendrogram was constructed by cluster analysis of the PFGE patterns obtained after macrorestriction with SmaI enzyme, using the unweighted pair group method with arithmetic means. PFGE fingerprint patterns are shown next to the corresponding branches of the dendrogram. The strain number is followed by vir type, emm sequence type, emm pattern, and PAM status (9). Patterned branches represent the three different clusters of GAS isolates found in the dendrogram (hatched, cluster 1; shaded, cluster 2; cross-hatched, cluster 3). B, phylogeny tree generated by MrBayes analysis of the amino acid sequences of 13 PAM genes (PAMNS13, GenBankTM accession number AY351851; PAMNS455, GenBankTM accession number AY351857; PAMNS265, GenBankTM accession number AY351855; PAMNS223, GenBankTM accession number AY351854; PAMNS253, GenBankTM accession number AY351853; PAMNS53, GenBankTM accession number AY351852; PAMNS32, GenBankTM accession number AY351850; PAMNS59, GenBankTM accession number AY351849; PAMNS1133, GenBankTM accession number AY351847; PAMNS10, GenBankTM accession number AY351846; PAMNS221, GenBankTM accession number DQ136319; NS696 M1 protein, GenBankTM accession number Z32677) and MLC36 (GenBankTM accession number Z32677) and MLC72 (GenBankTM accession number Z32678) are plasminogen-binding M protein sequences from group C and group G streptococci respectively (35). The M1 protein sequence (42) was included as an outgroup and for comparison with the NS696 M1 protein.
Characterization of PAM Variants

10<sup>-5</sup> in PBS. For each murine anti-peptide serum sample, 50 μl of fresh heat-inactivated serum was mixed with 50 μl of the bacterial dilution and incubated for 20 min at room temperature. Following this incubation, 400 μl of nonopsonic heparinized human blood was added and incubated at 37 °C with end-over-end mixing. All human blood was tested prior to performing the assay to ensure that it could support the growth of the GAS strain to at least 32 times the colony-forming units (CFU) of the inoculum in a 3-h incubation at 37 °C (31). Human blood was obtained according to institutional and National Health and Medical Research Council ethical requirements.

The mixtures were incubated end-over-end at 37 °C for 3 h, and 50 μl from each tube was plated in duplicate on 2% blood THB agar plates. The plates were incubated overnight at 37 °C, and the number of colonies on each plate was determined. Opsonic activity of the anti-peptide sera (% reduction in mean CFU) was calculated as (1 − (CFU in the presence of anti-peptide sera)/(mean CFU in the presence of normal mouse sera)) × 100.

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 Welch’s correction to determine whether there was any significant difference in the 𝐾<sub>d</sub> values for plasminogen binding by the PAM variants and the PAM<sub>N53</sub>. For immunization and challenge experiments, a Kruskal-Wallis test was used to determine whether there was any significant variation in the median titers or opsonization induced by the four groups of antisera. Dunn’s Multiple Comparison test was used for individual comparison of two groups of antisera. Difference in survival curves was determined by log rank test.

RESULTS

The subset of 27 GAS strains used in this study consisted of 20 different vir types and 20 different emm sequence types (9). PFGE analysis (Fig. 1A) revealed 22 different fingerprint patterns. The 12 PAM-positive isolates displayed 7 different fingerprint patterns, suggesting that this group represents a genetically diverse subset of isolates. In general, PFGE clustering was concordant with both vir and emm type. No congruence was seen between the chromosomal patterns for the emm locus (emm pattern) (32) and PFGE fingerprint, with isolates of all emm patterns found to be distributed throughout the dendrogram. This was anticipated, as there are only five possible emm patterns. All PAM-positive isolates used in this study were found to contain an emm pattern D chromosomal arrangement, as has been described previously (8, 9, 33). PAM-positive isolates were found to cluster into three groups. Cluster 1 contained PAM-positive isolate NS265; cluster 2 contained the PAM-positive isolates NS223, NS50.1, NS59, NS13, and NS10; and cluster 3 contained the PAM-positive isolates NS221, NS455, NS253, NS32, and NS1133. Isolates NS10, NS13, and NS59 (cluster 2) and NS455 and NS253 (cluster 3) appear to be clonal as they display identical PFGE patterns, vir types and emm sequence types. A small number of changes in the PAM sequences of these isolates do not appear to be mirrored by changes to emm sequence type or vir type. Similarly, isolates NS1133 and NS32 (cluster 3) display identical PFGE patterns and emm sequence types and as such are likely to be clonal. Phylogenetic analysis of the amino acid sequences of the PAM genes from the PAM-positive isolates showed only minor congruence with PFGE analysis (Fig. 1B). The PAM proteins were found to cluster in three groups; however, this clustering was not concordant with that seen in the PFGE analysis, suggesting that horizontal gene transfer of PAM is occurring in GAS isolates. This is most evident in the case of GAS isolate NS265.

![FIGURE 2. SDS-PAGE analysis of recombinant M proteins. A, translated DNA sequences of the plasminogen-binding region (a1/a2 repeats) of PAM variants, aligned with the amino acid sequence of the prototype PAM-binding site (2). *, residues identical to those of the PAM sequence; ---, gaps in the alignment. B, 12% SDS-polyacrylamide gel showing the six purified recombinant proteins used in this study. Lane 1, NS696 M1 protein; lane 2, PAM<sub>N53</sub>; lane 3, PAM<sub>N455</sub>; lane 4, PAM<sub>N1133</sub>; lane 5, PAM<sub>N535</sub>; and lane 6, PAM<sub>N53</sub>. Molecular mass markers are given in kDa. C, ligand blot analysis employing biotinylated Glu-plasminogen of purified variant PAM proteins. Lane 1, NS696 M1 protein; lane 2, PAM<sub>N265</sub>; lane 3, PAM<sub>N455</sub>; lane 4, PAM<sub>N1133</sub>; lane 5, PAM<sub>N535</sub>; and lane 6, PAM<sub>N53</sub>. Molecular mass markers are given in kDa.](image)

TABLE 2

Characteristics of PAM variants

| Recombinant protein | 𝐾<sub>d</sub> (nM) | EC<sub>50</sub> (μg/ml) | Homology to prototype PAM a1/a2 repeat motif | Homology to entire prototype PAM |
|---------------------|----------------|----------------|---------------------------------|-------------------------------|
| **emm type**        | **prot**      | **prot**      | **prot**                        | **prot**                      |
| NS13                | 1.58          | 22.06         | 100                             | 99.7                          |
| NS455               | 3.47          | 5.77          | 69                              | 84.5                          |
| NS1133              | 4.68          | 0.68          | 79                              | 87.6                          |
| NS53                | 4.86          | 1.96          | 69                              | 88.7                          |
| NS535               | 4.99          | 0.81          | 65                              | 84.5                          |
| NS265               |               |               |                                 |                               |
| NS696 M1            |               |               |                                 |                               |
| Nonspecific binding only | Not determined | | 21                             | 71.9                          |

FEBRUARY 10, 2006•VOLUME 281•NUMBER 6 JOURNAL OF BIOLOGICAL CHEMISTRY 3221
Characterization of PAM Variants

PAM_{NS13} lies close to PAM sequences from cluster 3 isolates (PAM_{253}, PAM_{259}, and PAM_{455}); however, GAS strain NS265 is genetically distinct from the other PAM-positive isolates subjected to PFGE. The positioning of PAM_{NS223} from cluster 2, in close proximity to PAM proteins from cluster 3 GAS isolates, also suggests horizontal transfer of the PAM gene among GAS strains belonging to distinct PFGE clusters.

To analyze the plasminogen binding characteristics of naturally occurring PAM variants, the PAM genes from five GAS strains, which had been cloned previously into pCR2.1 (9), were subcloned into the expression vector pGEX-2T. Genes were selected for expression based on variation both within the plasminogen-binding a1/a2 repeat domain (Fig. 2A and Table 2) and other regions within the protein (Fig. 1A and Table 2). The gene encoding M1 protein, with no significant identity to the prototype PAM a1/a2 repeat, was selected as a negative control. Following expression in E. coli, recombinant proteins within the expected size range (PAM_{NS13} 42 kDa; PAM_{NS53,4} 2 kDa; PAM_{NS265} 45 kDa; PAM_{NS455} 32 kDa; PAM_{NS1133} 43 kDa; and NS696 M1 protein, 47 kDa) were purified using glutathione-agarose and Ni-NTA-agarose (Fig. 2B). PAM_{NS265} and PAM_{NS455} proteins appear as doublet bands following SDS-PAGE, which is characteristic of some M proteins (34). All recombinant proteins reacted with biotinylated Glu-plasminogen in a ligand blot analysis, except for the NS696 M1 protein (Fig. 2C). For PAM_{NS265} and PAM_{NS455}, both the doublet species reacted with the Glu-plasminogen.

The interaction between recombinant PAM variants and Glu-plasminogen was further characterized using saturation plasminogen binding assays. Recombinant proteins immobilized to 96-well plates were incubated with increasing concentrations of biotinylated Glu-plasminogen, in the presence or absence of a 50-fold molar excess of unlabeled Glu-plasminogen. The recombinant PAM proteins bound plasminogen in a dose-dependent fashion, and saturable binding was achieved with 500 nM plasminogen for 5 of the 6 recombinant proteins after 2 h (Fig. 3). Only nonspecific binding was found for NS696 M1 protein. Nonlinear regression analysis was used to determine the affinity of each recombinant protein for Glu-plasminogen (Table 2). Equilibrium dissociation constants (K_d) were calculated using a best fit nonlinear regression curve. K_d values were found to be consistent with those found for the prototype PAM protein and plasminogen binding M proteins of group C and G streptococci (2, 12, 35, 36) and ranged from 1.58 to 4.99 nM, indicating that each of the PAM variants maintained the ability to bind plasminogen with high affinity. Although the range in affinity was small, PAM_{NS13}, which has 100% identity to the prototype PAM in the a1/a2 region, was found to have significantly higher affinity for plasminogen than the other PAM variants (PAM_{NS53} p = 0.001; PAM_{NS265} p = 0.002; PAM_{NS1133} p = 0.005; and PAM_{NS455} p = 0.008).

Plasminogen binding experiments indicated that limited sequence variation within the a1 and a2 repeats reduces but does not abolish plasminogen binding affinity by PAM variants. To explore further the PAM variants’ relative affinity for plasminogen, competition binding experiments were performed. Recombinant PAM variants were immobilized and then incubated with biotinylated Glu-plasminogen at a saturating concentration (500 nM). Unlabeled fluid phase competitor (PAM_{NS13}) was added at varying concentrations to the immobilized protein prior to the addition of labeled plasminogen. PAM_{NS13} was selected as the competitor, because it was found to have the highest affinity for plasminogen, and it has a plasminogen-binding site that shows 100% identity to the prototype PAM a1 and a2 repeats (Fig. 2A) (2, 12, 35, 36). The effective concentration of competitor required to inhibit plasminogen binding by 50% (EC_{50}) was determined by fitting a one-site competition curve (Fig. 4). EC_{50} values ranged from 0.68 to 22.06 μM (Table 2). These data also suggest that variation within the plasminogen binding region of these PAM variants decreased the comparative affinity for plasminogen. As expected, there was generally an inverse correlation between K_d and EC_{50} values (Table 2). Additionally, when immobilized, PAM_{NS13} plasminogen-binding out-competed binding to biotinylated Glu-plasminogen by fluid phase PAM variants (PAM_{NS53}, PAM_{NS455}, PAM_{NS1133}, and PAM_{NS265}). No competition of PAM_{NS13} plasminogen binding was found in the presence of a 50-fold molar excess of any of the PAM variants examined (data not shown), further indicating that the prototype PAM sequence (PAM_{NS13}) provides a higher affinity binding site for plasminogen.

Naturally occurring variation in the a1 region of PAM studied here does not abrogate plasminogen binding function. Although there are several potential explanations for substantial sequence divergence in the major functional domain of a protein, we hypothesized that the plasminogen-binding region of PAM may be an important target for immune recognition by the host. New variants may be selected for by host immune pressure. Thus, the potential for the a1 region of PAM to elicit protective immunity in a mouse model for GAS infection was investigated. Immunization of mice with a KLH-conjugated peptide encompassing the a1 region of PAM_{NS13} elicited a significantly higher titer of a1 peptide-specific IgG than KLH alone (p < 0.001) or PBS (p < 0.001) controls (Fig. 5A). The anti-a1 peptide-KLH sera also showed significant opsonizing activity toward GAS in an in vitro bacterial assay in human blood when compared with the KLH (p < 0.01) and PBS (p < 0.001) control antisera (Fig. 5B). Additionally, anti-a1 peptide-KLH sera showed no opsonizing activity against GAS expressing variant PAM_{NS455}, when compared with KLH antisera (p > 0.05), indicating that variation in the a1 region of PAM can result in a change in immune recognition (Fig. 5C).

Mice immunized with the a1 peptide-KLH showed increased survival when challenged with GAS strain NS13 compared with both the PBS (p = 0.0071) and KLH (p = 0.0055) immunized control mice (Fig. 5D), indicating that the a1 repeat of PAM may represent a protective epitope against group A streptococcal infection.

DISCUSSION

Plasminogen receptors and activators have been found to be expressed by a large number of pathogenic bacteria, including group A, C, and G streptococci (37). The finding that such a wide variety of microbial pathogens interact with the host plasminogen activation system suggests that the acquisition of plasminogen is a major contributing factor in bacterial virulence. PAM is a cell surface-exposed, high affinity plasminogen receptor expressed by GAS thought to be associated primarily with impetigo (8). However, the PAM genotype has been found to be associated with isolates from a variety of disease states (9) and appears to play an integral role in the plasminogen-dependent virulence of PAM-positive GAS. In a recent study it was found that a PAM-positive genotype confers high Glu-plasminogen binding ability on GAS when compared with PAM-negative GAS (9). Furthermore, in studies using mice expressing a human plasminogen transgene, a PAM knockout mutant was found to have markedly reduced virulence when compared with the wild type GAS strain (4), indicating that the association of plasminogen at the bacterial cell surface via PAM is an important virulence mechanism for a subset of GAS isolates. The present study characterizes the interaction of human plasminogen with five naturally occurring PAM variants and indicates that variation in the plasminogen binding region of PAM may reflect the targeting of this region by the host immune response.

Phylogenetic analysis of 27 GAS isolates by PFGE indicated that the
PFGE profile was concordant with vir type and emm sequence type. However, there was little association between emm pattern and PFGE profile. Previous studies of the allelic profiles of neutral housekeeping genes have found a lack of concordance between emm pattern and the genetic relatedness of strains (38), and as such, this finding is not unexpected. All the PAM-positive isolates used in this study were found to be emm pattern D. To date, the PAM gene has been found to be primarily associated with emm pattern D isolates, with this chromosomal arrangement thought to be a marker for skin tropic GAS isolates (8, 33). Comparison of the PFGE dendrogram with a phylogenetic analysis of 13 PAM protein sequences provided evidence to suggest that there has been horizontal gene transfer of the PAM gene during GAS evolution.
Horizontal gene transfer is considered to be a major source of genetic variation within emm and emm-like genes and appears to have been a common event during GAS evolution (39).

Solid phase plasminogen binding assays indicated that despite variation in the plasminogen binding domain of PAM, all naturally occurring variants maintained the ability to bind plasminogen with relatively high affinity.

**FIGURE 4.** Competition of Glu-plasminogen binding to immobilized recombinant PAM variants with fluid phase PAMNS13. Binding of biotinylated Glu-plasminogen to immobilized PAMNS13 (A), PAMNS53 (B), PAMNS265 (C), PAMNS455 (D), and PAMNS1133 (E) was measured in the presence of varying concentrations of unlabeled fluid phase PAMNS13. Data points are the mean values of triplicate readings, with error bars indicating S.E. One-site competition analysis was performed on data for all recombinant proteins; this analysis was used to determine the concentration of PAMNS13 required to inhibit binding of biotinylated Glu-plasminogen by 50% (EC50).
affinity. Although the binding characteristics of fragments representing different a1/a2 repeat sequences have been investigated previously (13, 15), the functional characteristics of M proteins, including fibrinogen and plasminogen binding, may depend largely on their overall structure (36, 40), highlighting the importance of using full-length protein in functional studies. Binding dissociation constants for the interaction of PAM variants and biotinylated plasminogen were within the same range as reported previously for PAM and plasminogen binding M proteins of groups C and G streptococci (2, 12, 35). The degree of sequence similarity in the plasminogen binding regions of the variants used in this study to that of the prototype PAM ranged from 100 to 65%. Overall, this variation has not abolished affinity for plasminogen. The circulating concentration of Glu-plasminogen is $K_d$ (6), and as such, the $K_d$ values reported in this work ranging from 1.58 to 4.99 nM are within the physiological range and thus are of functional significance.

The maintenance of binding function in the presence of sequence divergence may relate to the conservation of key binding site residues. Studies involving the interaction of a polypeptide sequence encompassing the a1 and a2 region of PAM in addition to six residues preceding the a1 repeat (designated VEK-30) with kringle 2 of plasminogen highlighted the importance of Lys98, Arg101, His102, Glu104, and Lys111 in the PAM-plasminogen interaction (13, 15). In the PAM variants used in this study, Lys98, Lys111, Arg101, and His102 were conserved in all sequences. Glu104 was present in all proteins except PAMNS1133. It is likely that the conservation of these key residues provides the necessary binding site integrity for the interaction with plasminogen despite other sequence variation within this region of PAM. Nonetheless, although PAMNS1133 and PAMNS455 contain identical a1 and a2 repeats, the $K_d$ and EC$_{50}$ values of these proteins vary, suggesting that the overall M protein structure subtly contributes to the capacity to bind plasminogen. PAMNS113 displays the highest affinity for plasminogen, contains a binding site identical to that of the prototype PAM sequence, and was able to compete plasminogen binding to other PAM variants at low concentrations, suggesting that this binding site sequence represents the ideal motif for the interaction of plasminogen with PAM.

M protein is one of the major virulence factors of GAS and consists of a highly variable N-terminal domain, as well as A and B repeat blocks that generate type-specific immunity in the host. Variation within the repeat sequences of M proteins as a result of both point mutations and duplication/deletion events may function as a means of developing antigenic variation (41–43). Furthermore, it has been found that a difference of only four amino acids in the N terminus of M3 variants was enough to alter the antigenic profile of the protein, resulting in altered immune recognition (44). A potential role for the variable a1 region of PAM in eliciting protective immunity in the host was investigated using a mouse model for GAS infection. The plasminogen binding a1 repeat region of PAM is an orthologue of the A repeat region in other M proteins. The immunogenic properties of the a1 domain have not been reported previously. Type-specific anti-M antibodies bind to exposed epitopes within the N terminus of M protein, thus inhibiting the phagocytic role of this protein and initiating opsonization (41). If the binding of plasminogen by PAM promotes deep tissue dissemination of
Characterization of PAM Variants

GAS strains, it may be argued that the antibody response against the plasminogen binding domain of PAM could promote clearance of the infecting GAS in order for it to offer selective pressure for novel variants. The results presented here clearly show that in a murine model, the a1 domain is highly immunogenic, and the antibodies against this domain are opsonic, resulting in phagocytic clearance. It has also been demonstrated that antiserum raised against the prototype a1 sequence does not opsonize GAS expressing the variant protein PAMN5A55. It is possible then that divergence in the a1 and a2 repeats of PAM may have resulted as a response to selective pressure from the host immune system.

Although the functional domain of the PAM protein is highly divergent, this variation has only limited impact on the PAM-plasminogen interaction. Such a finding is indicative of the physiological significance of this interaction and the ability of GAS to subvert the host plasminogen activation system. Furthermore, our results are consistent with host immuno-selective pressure as a potential mechanism for divergence in the functional domain of PAM. The multifactorial nature of GAS-host interactions, and the apparent advantage conferred on GAS by its ability to interact with the host plasminogen activation system (1–5), has widespread implications for the treatment of streptococcal infection.

REFERENCES

1. Lottenberg, R., DesJardin, L. E., Wang, H., and Boyle, M. D. (1992) J. Infect. Dis. 166, 436–440
2. Berge, A., and Sjobring, U. (1993) J. Biol. Chem. 268, 25417–25424
3. Pancholi, V., and Fischetti, V. A. (1998) J. Biol. Chem. 273, 14503–14515
4. Sun, H., Ringdahl, U., Homeister, J. W., Fay, W. P., Engleberg, N. C., Yang, A. Y., Rozek, I. S., Wang, X., Sjobring, U., and Ginsburg, D. (2004) Science 305, 1283–1286
5. Walker, M. J., McArthur, J. D., McKay, F., and Ranson, M. (2005) Trends Microbiol. 13, 308–313
6. Dano, K., Andreassen, P. A., Grondal-Hansen, J., Kristensen, P., Nielsen, L. S., and Skriver, L. (1985) Adv. Cancer Res. 44, 139–266
7. Ponting, C. P., Marshall, J. M., and Cederholm-Williams, S. A. (1992) Blood Coagul. Fibrinolysis 3, 605–614
8. Svensson, M. D., Sjobring, U., and Bessen, D. E. (1999) Infect. Immun. 67, 3915–3920
9. McKay, F. C., McArthur, J. D., Sanderson-Smith, M. L., Gardam, S., Currie, B. J., Sririprakash, K. S., Fagan, P. K., Towers, R. J., Batzloff, M. R., Chhatwal, G. S., Ranson, M., and Walker, M. J. (2004) Infect. Immun. 72, 364–370
10. Pancholi, V., and Fischetti, V. A. (1992) J. Exp. Med. 176, 415–426
11. Lottenberg, R., Broder, C. C., Boyle, M. D., Kain, S. J., Schroeder, B. L., and Curtiss, R. III (1992) J. Bacteriol. 174, 5204–5210
12. Wistedt, A. C., Ringdahl, U., Muller-Esterl, W., and Sjobring, U. (1995) Mol. Microbiol. 18, 569–578
13. Schenone, M., Warder, S. E., Martin, J. A., Prorok, M., and Castellino, F. J. (2000) J. Pept. Res. 56, 438–445
14. Ringdahl, U., Svensson, M., Wistedt, A. C., Renné, T., Kellner, R., Muller-Esterl, W., and Sjobring, U. (1998) J. Biol. Chem. 273, 6424–6430
15. Rios-Steiner, J. L., Schenone, M., Rocha, I., Tundisins, A., and Castellino, F. J. (2001) J. Mol. Biol. 308, 705–719
16. Kalia, A., and Bessen, D. E. (2004) J. Bacteriol. 186, 110–121
17. Ramachandran, V., McArthur, J. D., Behm, C. E., Gutzeit, C., Dowton, M., Fagan, P. K., Towers, R., Currie, B., Sririprakash, K. S., and Walker, M. J. (2004) J. Bacteriol. 186, 7601–7609
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, pp. 74–82, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
19. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
20. Rouquist, F., and Hueslenbeck, J. P. (2003) Bioinformatics 19, 1572–1574
21. Hueslenbeck, J. P., Ronquist, F., Nielsen, R., and Bollback, J. P. (2001) Science 294, 2310–2314
22. Whelan, S., and Goldman, N. (2001) Mol. Biol. Evol. 18, 691–699
23. Smith, D. B., and Johnson, K. S. (1988) Gene (Amst.) 67, 31–40
24. Laemmli, U. K. (1970) Nature 227, 680–685
25. Andronicos, N. M., Ranson, M., Bognar, J., and Baker, M. S. (1997) Biochim. Biophys. Acta 1337, 27–39
26. Beache, E. H., Stollerman, G. H., Chiang, Y. E., Chiang, T. M., Seyer, J. M., and Kang, A. H. (1977) J. Exp. Med. 145, 1469–1483
27. Beache, E. H., Stollerman, G. H., Johnson, R. H., Ofek, I., and Bisno, A. L. (1979) J. Exp. Med. 150, 862–877
28. Olive, C., Batzloff, M. R., Horvath, A., Wong, A., Clair, T., Yarwood, P., Toth, I., and Good, M. F. (2002) Infect. Immun. 70, 2734–2738
29. Hayman, W. A., Brandt, E. R., Relf, W. A., Cooper, J., Saul, A., and Good, M. F. (1997) Int. Immunol. 9, 1723–1733
30. Hunter, W. M., and Greenwood, F. C. (1962) Nature 194, 495–496
31. Brandt, E. R., Hayman, W. A., Currie, B., Carapetis, J., Wood, Y., Jackson, D. C., Cooper, J., Melrose, W. D., Saul, A. J., and Good, M. F. (1996) Immunology 89, 331–337
32. Hollingshead, S. K., Readdy, T. L., Yung, D. L., and Bessen, D. E. (1993) Mol. Microbiol. 9, 569–578
33. Bessen, D. E., Sotir, C. M., Readdy, T. L., and Hollingshead, S. K. (1996) J. Infect. Dis. 173, 896–900
34. Cunningham, M. W. (2000) Clin. Microbiol. Rev. 13, 470–511
35. Ben Nar, A., Wistedt, A., Ringdahl, U., and Sjobring, U. (1994) Eur. J. Biochem. 222, 267–276
36. Wistedt, A. C., Kotarsky, H., Marti, D., Ringdahl, U., Castellino, F. J., Schaller, J., and Sjobring, U. (1998) J. Biol. Chem. 273, 24420–24424
37. Lahteenmaki, K., Kussela, P., and Korhonen, T. K. (2001) FEBS Microbiol. Rev. 25, 531–552
38. Kalia, A., Spratt, B. G., Enright, M. C., and Bessen, D. E. (2002) Infect. Immun. 70, 1971–1983
39. Whatmore, A. M., Kapur, V., Sullivan, D. J., Musser, J. M., and Kehoe, M. A. (1994) Mol. Microbiol. 14, 619–631
40. Cedervall, T., Johansson, M. U., and Akerstrom, B. (1997) Biochemistry 36, 4987–4994
41. Jones, K. F., Hollingshead, S. K., Scott, J. R., and Fischetti, V. A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8271–8275
42. Harbaugh, M. P., Podbielski, A., Hugl, S., and Cleary, P. P. (1993) Mol. Microbiol. 8, 981–991
43. Relf, W. A., Martin, D. R., and Sririprakash, K. S. (1994) Gene (Amst) 144, 25–30
44. Beres, S. B., Syba, G. L., Sturdevant, D. E., Granville, C. N., Liu, M., Rickles, S. M., Whitney, A. R., Parkins, L. D., Hoe, N. P., Adams, G. J., Low, D. E., DeLeo, F. R., McGee, A., and Musser, J. M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 11833–11838