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M protein mediated plasminogen binding is essential for the virulence of an invasive Streptococcus pyogenes isolate

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Abstract

The human protease plasmin plays a crucial role in the capacity of the group A streptococcus (Streptococus pyogenes; GAS) to initiate invasive disease. The GAS strain NS88.2 was isolated from a case of bacteremia from the Northern Territory of Australia, a region with high rates of GAS invasive disease. Mutagenesis of the NS88.2 plasminogen binding M protein Prp was undertaken to examine the contribution of plasminogen binding and cell surface plasmin acquisition to virulence. The isogenic mutant NS88.2prp was engineered whereby four amino acid residues critical for plasminogen binding were converted to alanine codons in the GAS genome sequence. The mutated residues were reverse complemented to the wildtype sequence to construct GAS strain NS88.2prpRC. In comparison to NS88.2 and NS88.2prpRC, the NS88.2prp mutant exhibited significantly reduced ability to bind human plasminogen and accumulate cell surface plasmin activity during growth in human plasma. Utilising a humanised plasminogen mouse model of invasive infection, we demonstrate that the capacity to bind plasminogen and accumulate surface plasmin activity plays an essential role in GAS virulence.

Keywords

Plasmin, Group A streptococcus, innate immunity, CMMB

Disciplines

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M protein mediated plasminogen binding is essential for the virulence of an invasive *Streptococcus pyogenes* isolate

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Abbreviations: GAS, group A streptococcus; PAM, plasminogen binding group A streptococcal M protein; Prp, PAM related protein; GAPDH, glyceraldehyde phosphate dehydrogenase; THB, Todd-Hewitt Broth; SOF, serum opacity factor; HBA, horse blood agar

ABSTRACT

The human protease plasmin plays a crucial role in the capacity of the group A streptococcus (*Streptococus pyogenes*; GAS) to initiate invasive disease. The GAS strain NS88.2 was isolated from a case of bacteremia from the Northern Territory of Australia, a region with high rates of GAS invasive disease. Mutagenesis of the NS88.2 plasminogen binding M protein Prp was undertaken to examine the contribution of plasminogen binding and cell surface plasmin acquisition to virulence. The isogenic mutant NS88.2*prp* was engineered whereby four amino acid residues critical for plasminogen binding were converted to alanine codons in the GAS genome sequence. The mutated residues were reverse complemented to the wildtype sequence to construct GAS strain NS88.2*prpRC*. In comparison to NS88.2 and NS88.2*prpRC*, the NS88.2*prp* mutant exhibited significantly reduced ability to bind human plasminogen and accumulate cell surface plasmin activity during growth in human plasma. Utilising a humanised plasminogen mouse model of invasive infection, we demonstrate that the capacity to bind plasminogen and accumulate surface plasmin activity plays an essential role in GAS virulence.

Key words: Plasmin, Group A streptococcus, innate immunity

INTRODUCTION

Streptococcus pyogenes (group A streptococcus, GAS) is the major etiological agent of a variety of human infections. These include simple pharyngitis (> 600 million cases per year) and impetigo (> 100 million cases per year), but also life-threatening invasive infections such as necrotizing fasciitis, bacteremia, and toxic shock syndrome (> 600,000 cases and 163,000 deaths per year) (1). Over the past two decades there have been numerous reports of a resurgence in GAS invasive disease of increasing severity in Western countries (2, 3). Additionally, in developing nations, the epidemiology of GAS disease is less well-described, and GAS infection remains endemic in many areas (4). In the Northern Territory of Australia, the incidence of invasive GAS disease in indigenous communities is extremely high, with rates of bacteremia reported to be 5 times that seen in the non-indigenous population (4). Similar to the epidemiology seen in developing countries, a diversity of *emm* types are found to be associated with both invasive and endemic infections in the Northern Territory, and invasive disease is generally secondary to endemic skin infections (1, 4-6).

A mounting body of clinical, epidemiological and experimental evidence suggests an important role for plasminogen activation in GAS virulence (7, 8). Plasminogen is a single chain glycoprotein found in plasma and extracellular fluids at concentrations of approximately 2 μM (9). Cleavage of plasminogen at a single site (Arg^{560}-Val^{561}) by specific plasminogen activators results in the formation of the two-chain plasmin molecule, which contains a serine protease active site in the C-terminal region (10). Human plasminogen can also be activated to plasmin by the GAS protein streptokinase, as part of a highly species specific plasminogen/streptokinase activator

complex (11). Plasmin has the ability to degrade fibrin clots, connective tissue and the extracellular matrix (9, 10). Thus activation of this proteolytic system by GAS may have significant pathological consequences for the host. A subset of GAS strains express M proteins that bind plasminogen and plasmin directly and with high affinity. The first of these M proteins to be identified was PAM, initially identified in M53 serotype GAS (12). Plasminogen-binding motifs in M proteins have been identified in other GAS serotypes associated with both invasive and non-invasive disease (13). Plasminogen binding M proteins have been well characterised in vitro (12, 14-20), however the direct contribution of these proteins to GAS virulence has not been defined. Whilst the deletion of the PAM gene results in a loss of virulence (7, 14), this may be due to a loss of other functions such as protection from phagocytosis or binding of other host molecules attributed to M proteins (21).

The PAM related protein Prp is associated with an *S. pyogenes* strain isolated from a severe invasive infection in the Northern Territory. Prp has been shown to interact with plasminogen via the same mechanism, and with a similar affinity to other plasminogen binding M proteins. It has also been demonstrated that the plasminogen binding ability of Prp can be abrogated without resulting in a loss of protein structure (18). Thus, Prp provides an ideal candidate for examining the contribution of plasminogen binding M proteins to streptococcal virulence. Here we report the construction of an isogenic GAS Prp mutant (NS88.*2prp*) which is attenuated for plasminogen binding and surface plasmin accumulation. Reverse complementation of the *prp* gene to wildtype (NS88.2*prpRC*) restored both cell surface plasminogen binding and activation. NS88.2, NS88.2*prp* and NS88.2*prpRC* expressed equivalent levels of hyaluronic acid capsule, streptokinase, α-enolase and GAPDH, and showed

no difference in the ability to bind fibrinogen, survive in whole blood or interact with human polymorphonuclear leukocytes. The virulence of the isogenic mutant NS88.2*prp* was, however, significantly attenuated in a humanised plasminogen mouse model of invasive infection, underpinning the central role of plasminogen binding and surface plasmin accumulation in GAS virulence.

MATERIALS AND METHODS

Bacterial strains and culture methods

GAS strains were routinely cultured in Todd-Hewitt Broth containing 1% yeast (THBY), or grown on horse blood agar plates (HBA), at 37˚C. GAS strain NS88.2 has been described previously (McKay et al. 2004). Allelic exchange was used to create NS88.2*prp* via precise replacement of the wildtype *prp* gene in GAS strain NS88.2 with the previously described $prp_{A96A101A107A108}$ gene, which encodes a protein that is completely attenuated for plasminogen binding (18). The mutation was subsequently reverse complemented by the replacement of $prp_{A96A101A107A108}$ with the wildtype *prp* gene to create NS88.2*prpRC*. *Escherichia coli* MC1061 was propagated using Luria Bertani (LB) broth or agar. For selection of the plasmid pHY304 in either GAS or *E. coli*, erythromycin was used at a concentration of either 4 µg/ml (GAS) or 500 µg/ml (*E .coli*) respectively, and bacteria cultured at 30ºC.

Construction of *prp* mutant GAS strains

GAS mutants were constructed essentially as described previously (22, 23). Both the wildtype *prp* gene and the $prp_{A96A101A107A108}$ gene were previously cloned into the expression vector pGEX2T (18). In order to facilitate allelic replacement, these genes were subcloned into the temperature sensitive vector pHY304, which contains the

gene for erythromycin resistance, using *Bam*HI/*Eco*RI restriction enzyme digestion and ligation with T4 DNA ligase. Plasmids were transformed into *E.coli* MC1061 using standard electroporation procedures, and the recombinant plasmids screened by DNA sequence analysis using primers PAMN1 and PAMN2 (12) to confirm the presence of either the mutated or wildtype gene. The resulting plasmids (pHY*prp* and pHY*prpRC*) were transformed into NS88.2, or NS88.2*prp* respectively, by electroporation (24). Integration into the chromosome was achieved by 2 h incubation at the permissive temperature for plasmid replication (30˚C). Following subculture at 37˚C, single crossover insertions were selected using erythromycin resistance screening. Chromosomal DNA was also screened for the presence of the erythromycin resistance gene by PCR using primers pHYermF (5'-GAAGGAGTGATTACATGAAC-3') and pHYermR (5'-CATAGAATTATTTCCTCCCG-3'), to avoid the selection of spontaneously resistant mutants. Serial passage at 30˚C and the removal of antibiotic selection was used to achieve double crossover. The resulting isogenic mutants NS88.2*prp* and NS88.2*prpRC* containing the $prp_{A96A101A107A108}$ and *prp* genes respectively, were confirmed by DNA sequence analysis using primers M1 and M2 (12). The growth rates of the wildtype and isogenic mutant strains in THBY were found to be identical (data not shown).

Vir typing

The Vir regulon was amplified from GAS chromosomal DNA, and the resulting amplification product subjected to restriction enzyme digestion using *Hae*III as described previously (5).

Capsule assay

Overnight GAS cultures were used to inoculate fresh THBY, and grown to an OD_{600} of 0.5-0.6. Capsule was extracted and assayed using the Stains-All method as described previously (25).

Determination of M protein, streptokinase, GAPDH and α-enolase expression

Mutanolysin extracts and GAS culture supernatants from overnight cultures were prepared as described previously (26). Following SDS-PAGE of the protein samples according to the method of Laemmli (27), proteins were transferred to nitrocellulose membrane using a Bio-Rad Trans-Blot apparatus (Bio-Rad, USA). For the determination of M protein expression, mutanolysin extracts were subjected to western blot analysis using rabbit polyclonal anti-sera raised against PAM. Specific rabbit anti-sera were also used to examine expression of the plasminogen receptors GAPDH and α-enolase. TCA precipitated proteins from overnight GAS culture supernatants were assayed for the presence of streptokinase using rabbit streptokinase anti-serum. The anti-sera used and the conditions for western blotting have been described in detail elsewhere (19, 26).

Cell surface plasmin acquisition

Cell surface plasmin activation assays were conducted following incubation of GAS in human plasma, essentially as described previously (26). Briefly, overnight GAS cultures were adjusted to OD_{600} 0.5 and incubated with human plasma for 3 h at 37ºC. Following washing twice with PBS /0.01% gelatin/0.01 M EDTA, bacteria were resuspended in PBS/ 0.01% gelatin, and the plasmin activity of this resuspension determined using the chromogenic substrate Spectrozyme®PL.

Binding of human plasminogen and fibrinogen

Bacteria were collected from overnight GAS cultures by centrifugation, and resuspended to 10% transmission at OD_{600} in PBS/ 0.01% TWEEN-20. A 250 µl sample of this cell suspension was used for ^{125}I-plasminogen and ^{125}I-fibrinogen binding analysis as described previously (13). For analysis of plasminogen and fibrinogen capture from human plasma, 400 µl of bacterial cell suspension was pelleted by centrifugation, and resuspended in 100 µl of human plasma. Following a 1 h incubation at 37˚C, bacteria were washed 3 times with 1 ml PBS and bound proteins eluted by incubation in 50 µl 100 mM Glycine-HCl (pH 2.0) for 15 min at room temperature. Following centrifugation, the supernatant was collected and 4 µl of 1.5 M Tris-HCl added to 40 µl of protein solution. Following SDS-PAGE and western transfer as described above, membranes were probed with polyclonal goat anti-human plasminogen, or polyclonal rabbit anti-human fibrinogen antibodies diluted 1:3000. Goat anti-rabbit or rabbit anti-goat horse radish peroxidase conjugated secondary antibodies diluted 1:3000 were used for detection of primary antibody binding. Membranes were developed by enhanced chemiluminescence.

Growth of GAS in whole blood

Survival of GAS strains in whole blood was determined using the Lancefield method (28). Fold increase in colony forming units (cfu) was determined by dividing the number of cfu present after 3 h incubation in heparanized human blood, by the number of cfu present in the original inoculum, as determined by plating on HBA and colony counting. Assays were performed in triplicate, using two different blood donors.

Polymorphonuclear leukocyte phagocytosis assay

Polymorphonuclear leukocytes (2.5×10^5 cells) were isolated from fresh human blood, and incubated with 2.5×10^6 Alexa-488 labelled GAS in the presence of non-immune human serum as described previously (29, 30). Cells were analysed using flow cytometry and the percentage of fluorescent polymorphonuclear leukocytes used as a measure of phagocytosis.

Humanised plasminogen mouse model of invasive disease

Transgenic humanised plasminogen *AlPLG1* mice heterozygous for the human plasminogen transgene (7) were backcrossed with C57BL/J6 mice. Groups of 10 *AlPLG1* mice were infected subcutaneously with 100 µl each of NS88.2, NS88.2*prp* or NS88.2*prpRC*, and survival monitored over a 10 day period. The number of cfu used for infection was determined by serial dilution of the inoculum, plating on HBA, and colony counting following overnight incubation at 37˚C.

Statistical Analyses

Differences in the survival of humanised plasminogen transgenic mice infected with GAS strains were determined by log-rank test. All other data were analysed via one way analysis of variance with Dunnett's multiple comparison test. Data sets were considered significantly different at $P < 0.05$. All analysis was perfomed using GraphPad Prism version 4.00.

Ethics Approval

Ethics permission was obtained from the University of Wollongong ethics committee prior to the commencement of animal experiments. Volunteers provided informed consent before blood samples were obtained.

RESULTS

Characterisation of GAS strains NS88.2, NS88.2*prp* and NS88.2*prpRC*

The *mga* regulon of NS88.2, a pattern D GAS strain (13), encodes multiple emm and emm-like genes (31). As the C-terminal of these gene products are highly conserved between different *emm* and *emm*-like genes, it was important to ensure the integrity of NS88.2*prp* (*prp* gene replaced with $prp_{A96A101A107A108}$ gene) and NS88.2*prpRC* ($prp_{A96A101A107A108}$ gene replaced with *prp* gene), and that illegitimate gene rearrangements had not occurred. The *mga* regulon from NS88.2, NS88.2*prp* and NS88.2*prpRC* was PCR amplified and a restriction profile generated using *Hae*III (Fig. 1 *A*). Amplification of the *mga* regulon from each strain resulted in amplicons of comparative size (~ 7 kb) and the restriction pattern generated for each strain was identical, suggesting that integration of modified *prp* genes into the *mga* regulon of NS88.2 did not result in unwanted chromosomal rearrangements. The integrity of the recombined *prp* genes in the chromosomes of NS88.2*prp* and NS88.2*prpRC* was confirmed by DNA sequence analysis (results not shown).

To further characterise these strains, the expression of Prp by NS88.2, NS88.2*prp* and NS88.2*prpRC* was examined. Western blot analysis of mutanolysin cell wall extracts was used to confirm equivalent expression levels of M protein by GAS strains NS88.2, NS88.2*prp* and NS88.2*prpRC* (Fig. 1 *B*). As expected, mutagenised Prp

expressed by NS88.2*prp* displays a modified electrophoretic mobility upon SDS-PAGE (18). No significant difference in the expression of hyaluronic acid capsule by NS88.2, NS88.2*prp* and NS88.2*prpRC* was evident ($P > 0.05$; Fig. 1 *C*). Furthermore, each of these strains expressed equivalent amounts of the plasminogen activator streptokinase (Fig. 1 *D*), and the other known plasminogen receptors GAPDH and α-enolase (results not shown). Thus, the techniques used for the construction of the isogenic NS88.2*prp* and NS88.2*prpRC* strains did not result in changes to the expression of other virulence factors involved in the interaction of GAS with human plasminogen.

Interaction of NS88.2, NS88.2*prp* and NS88.2*prpRC* with innate immune effectors

A significant factor in GAS virulence is the ability of strains to evade phagocytosis by host immune cells, and the interaction of GAS with fibrinogen has been shown to provide protection against phagocytosis by polymorphonuclear leukocytes (32, 33). Additionally, some GAS strains are able to interact with the plasminogen activation system indirectly via fibrinogen binding M proteins (34). As such, the interaction of wildtype and mutant strains with fibrinogen, whole human blood and human polymorphonuclear leukocytes was investigated.

Incubation of GAS strains in human plasma, and subsequent analysis of the eluted proteins indicated that NS88.2 wildtype and mutant strains of GAS are able to acquire fibrinogen from human plasma (Fig. 2 *A*). Furthermore, in a quantitative analysis of the binding of ^{125}I-fibrinogen (Fig. 2 *B*), NS88.2, NS88.2*prp* and NS88.2*prpRC* bound equivalent levels of fibrinogen ($P > 0.05$). Therefore, it can be assumed that

fibrinogen mediated interactions with both host immune cells and the plasminogen activation system will be maintained.

This observation is supported by data from whole blood assays indicating no significant difference in the ability of NS88.2, NS88.2 *prp* and NS88.*2prpRC* to replicate in whole blood from two separate blood donors ($P > 0.05$; Table 1). Importantly, all strains showed a greater than 32-fold increase in population, which is regarded as the threshold for an intact antiphagocytic capacity (35). Furthermore, in three independent experiments, no significant difference was seen between the uptake of GAS strains NS88.2, NS88.2*prp* or NS88.2*prpRC* by polymorphonuclear leukocytes (Fig. 3 $P > 0.05$). Thus, it appears that the expression of the mutated *prp* gene by NS88.2*prp* does not alter the antiphagocytic properties of NS88.2.

Plasminogen binding analysis

GAS have been found to interact directly with plasminogen via multiple receptors. Of these receptors, the plasminogen binding M protein has been shown to have the highest affinity for the circulating form of plasminogen. Additonally, there appears to be a direct correlation between the presence of a plasminogen binding M protein gene in the GAS chromosome, and the ability of isolates to bind plasminogen (13). However, to date, the overall contribution of plasminogen binding M protein to the acquisition of plasminogen by GAS has not been established. Data presented in Figure 4 clearly indicates that the abrogation of plasminogen binding by the M protein Prp significantly decreases the ability of GAS strain NS88.2 to acquire plasminogen, both from human plasma (Fig. 4 *A*), and in direct plasminogen binding assays (Fig. 4 *B*). The plasminogen binding ability of NS88.2*prp* was restored by

reverse complementation with the wildtype *prp* gene. In addition, the expression of the mutant Prp protein by NS88.2 completely prevented the acquisition of cell surface plasmin activity (Fig. 4 *C*). Thus, it appears that for GAS strains which express plasminogen binding M proteins, the ability to bind and activate plasminogen at the cell surface is almost exclusively dependant on the expression of these M proteins.

Virulence in the humanised plasminogen mouse infection model

A mounting body of evidence suggests that the ability to localise plasminogen at the cell surface is critical for the virulence of certain GAS isolates. The role of M-protein dependant plasminogen acquisition in virulence was investigated using a humanised plasminogen transgenic mouse infection model. Following subcutaneous injection of *AlPLG1* mice with either NS88.2, NS88.2*prp* or NS88.2*prpRC*, survival was monitored over a ten day period. Whilst the wildtype strain was highly virulent, with 80% of infected mice dead by day 5, NS88.2*prp* was significantly attenuated for virulence in this model of infection (10% mortality ; $P < 0.05$; Fig. *5*). Replacement of the wildtype gene resulted in restoration of virulence equivalent to that of the wildtype (90% mortality; $P > 0.05$).

DISCUSSION

The human plasminogen system has been shown to significantly contribute to GAS virulence. The introduction of the human plasminogen transgene into C57B/J6 mice resulted in reduced survival of transgenic mice, in comparison to non-transgenic littermate control mice, upon GAS subcutaneous infection (7). In the same study, deletion of the GAS plasminogen activator streptokinase brought about a significant reduction in virulence of isogenic GAS mutants, in comparison to the wildtype parental strain (7) . Here, we have demonstrated that the capacity of the plasminogen binding M protein Prp to bind plasminogen to the GAS cell surface is also a requirement for full GAS virulence. Whilst displaying intact M protein-mediated innate immune defense and streptokinase expression equivalent to wildtype, the isogenic mutant NS88.2*prp* is reduced in capacity to cause lethal infection in the humanised plasminogen mouse model of invasive infection. These observations indicate the capacity to accumulate human plasmin activity at the bacterial surface is an important additional step in the transition of GAS from asymptomatic or benign infection to life-threatening invasive disease.

Isolated from a bacteremic infection in the Northern Territory, GAS strain NS88.2 has been shown to bind high levels of plasminogen when compared to other GAS isolates from this region (13). This strain expresses the PAM related protein Prp, which functions as a high affinity plasminogen receptor (18). In order to investigate the contribution of M protein to plasminogen binding and cell surface plasmin acquisition, mutagenesis of the NS88.2 M protein Prp was undertaken. The isogenic mutant NS88.2*prp* was engineered whereby two lysine residues, an arginine and a histidine residue critical for plasminogen binding by Prp, were converted to alanine

codons in the GAS genome sequence. Expression of virulence factors α-enolase, GAPDH and streptokinase, which have been shown to interact with plasminogen (34, 36-38), were unaffected in NS88.2*prp*. The ability of this isogenic mutant to interact with fibrinogen, resist phagocytosis by polymorphonuclear leukocytes and grow in whole blood was unchanged compared to the wildtype parental strain. However, replacement of the wild type *prp* gene in NS88.2 with a *prp* gene encoding a protein attenuated for plasminogen binding, significantly reduced the ability of this strain to accumulate both plasminogen and plasmin at the cell surface.

The lack of either plasminogen acquisition or activation in human serum by NS88.*2prp* suggests that for GAS expressing plasminogen binding M proteins (PAM-positive GAS), α-enolase and GAPDH which likely interact with kringle domains 4 and 5 of plasminogen (11, 39), do not significantly contribute to plasminogen binding and activation. Plasminogen binding M proteins interact with kringle domain 2 of plasminogen and are specifically co-inherited with the sub-cluster 2b allele of streptokinase (8). These observations suggest that, for PAM-positive *S. pyogenes* strains, the specific interaction between human plasminogen kringle 2 domain, sub-cluster 2b streptokinase and plasminogen binding M proteins provides selection pressure for the co-inheritance of these GAS virulence factors (8, 11). A number of PAM-negative GAS strains, including GAS serotype M1T1, also demonstrate human plasminogen dependent virulence (7, 26, 40) We therefore hypothesise that PAM-positive and PAM-negative GAS strains employ differing strategies for surface plasminogen acquisition and activation. For PAM-negative isolates, the formation of a trimolecular complex of streptokinase, plasminogen and fibrinogen, bound to the GAS cell surface via fibrinogen receptors or the plasminogen receptors α-enolase and

GAPDH may represent an alternative human plasminogen-dependent virulence pathway employed by this strain set (11, 13, 34, 41).

Previous studies investigating the role of PAM in GAS disease have focused on strain ALAB49. This isolate was associated with uncomplicated impetigo infection (7, 14). Studies using the hu-skin-SCID mouse model for streptococcal impetigo found that a PAM negative isogenic deletion mutant of GAS strain ALAB49 displayed partial attenuation for virulence when compared to the wild type (14). However, a recently published animal study of GAS infection showed that in mice expressing the human plasminogen transgene, infection with the PAM positive GAS strain AP53 resulted in 80% mortality whereas an isogenic mutant of AP53, where the PAM gene was replaced by an antibiotic resistance cassette, exhibited only minimal virulence (7). While this observation supports the findings of the present study, the creation of isogenic PAM deletion mutants may also reduce resistance to phagocytosis (14). Hence, the contribution of plasminogen binding, resistance to phagocytosis (42), and other potential polar effects cannot be accounted for. The GAS isogenic mutant strains NS88.2*prp* and NS88.2*prpRC* used in this study were precisely constructed, were found to have comparable levels of interaction with polymorphonuclear leukocytes and showed equivalent levels of growth in human blood.

Our results suggest that the plasminogen binding M protein Prp is a critical requirement for virulence in GAS strain NS88.2. Nonetheless, PAM-negative GAS are capable of virulent infection. This apparent dichotemy is not unprecedented. In two seperate studies, isogenic mutagenesis of the fibronectin and fibrinogen binding protein serum opacity factor (SOF) resulted in a loss of GAS virulence in an animal model of infection (43, 44). Yet, *sof* is associated with only 40-50% of GAS isolates

(45, 46). Similarly, targeted mutagenesis of the fibronectin binding protein FbaA, which is associated with approximately 70 – 85% of GAS isolates (47-49), resulted in a significant increase in survival when compared to wildtype GAS in a murine model of skin infection (47). These examples provide further evidence for the concept of divergent sets of genotypically distinct GAS isolates exhibiting different virulence strategies in the face of numerous host defenses.

The requirement for GAS surface plasminogen binding and activation for lethal murine infection described in this work, suggests an important role for the human plasminogen activation system in the transition from benign to invasive disease. Rather than employing a strategy whereby diffuse activation of plasminogen by streptokinase occurs at or near the site of infection, the formation of active plasmin on the *S. pyogenes* surface may allow the bacterium to specifically penetrate innate immune barriers leading to the breakout of human plasmin decorated GAS cells systemically. An obvious innate immune defense which would be susceptible to this form of bacterial offensive strategy would be the fibrinogen layer deposited around the site of local GAS infection by the host (7).

Recent data indicates that over 660,000 cases of invasive GAS infection occur worldwide each year. Of these, almost one quarter are fatal (1). In order to establish invasive infection, *S. pyogenes* must overcome barriers posed by the host innate immune response. Our data support the contention that the human plasminogen activation system plays a central role in this process. The expression of functional Prp at the GAS cell surface is essential for the virulence of strain NS88.2, suggesting that other plasminogen binding M proteins of both GAS (13, 16, 50, 51), and different

streptococcal species (52), may play an important role in bacterial dissemination within the host.

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FIGURE LEGENDS

Figure 1. Characterisation of GAS strains NS88.2, NS88.2*prp* and NS88.2*prpRC*. *A*) Amplification of the *mga* regulon of NS88.2 (lane 1), NS88.2*prp* (lane 2) and NS88.2*prpRC* (lane 3), and *Hae*III digestion of *mga* amplification products for NS88.2 (lane 4), NS88.2*prp* (lane 5) and NS88.2*prpRC* (lane 6). Molecular size markers are given in kilobase pairs (kbp). *B*) Western blot analysis of mutanolysin cell wall extracts from NS88.2 (lane 1), NS88.2*prp* (lane 2) and NS88.2*prpRC* (lane 3) using rabbit polyclonal anti-PAM antibodies. Bold arrowhead indicates Prp. Molecular mass markers are given in kilodaltons (kDa). *C*) NS88.2, NS88.2*prp* and NS88.2*prpRC* produce equivalent levels of hyaluronic acid capsule (mean +/- SD, n = 3; $P > 0.05$). *D*) Western blot analysis of TCA precipitated GAS supernatants from NS88.2 (lane 1), NS88.2*prp* (lane 2) and NS88.2*prpRC* (lane 3), using rabbit polyclonal anti-streptokinase antibodies. Bold arrowhead indicates streptokinase. Molecular weight markers are given in kilodaltons

Figure 2. Interaction of NS88.2, NS88.2*prp* and NS88.2*prpRC* with human fibrinogen. *A*) Western blot analysis of eluted cell surface proteins following incubation in human plasma, using anti-human fibrinogen polyclonal antibodies. Lane 1, purified fibrinogen; lane 2, NS88.2; lane 3, NS88.2*prp*; lane 4, NS88.*2prpRC*. *B*) Binding of ^{125}I-fibrinogen by GAS strains NS88.2, NS88.2*prp* and NS88.2*prpRC* (mean +/- SD, n = 6 ; $P > 0.05$).

Figure 3. Flow cytometric analysis of the uptake of NS88.2, NS88.2*prp* and NS88.2*prpRC* by human polymorphonuclear leukocytes. Panels *A*-*C* show

representative data from a single experiment. Data has been gated to exclude autofluorescence. Uptake by polymorphonuclear leukocytes, as indicated by the percentage of flourescent polymorphonuclear leukocytes, of GAS strains *A*) NS88.2, *B*) NS88.2*prp* and *C*) NS88.2*prpRC*. *D*) Combined data from 3 separate experiments (mean +/- SD, $n = 9$; $P > 0.05$).

Figure 4. Interaction of NS88.2, NS88.2*prp* and NS88.2*prpRC* with human plasminogen. *A* Western blot analysis of eluted cell surface proteins following incubation in human plasma, using anti-human plasminogen polyclonal antibodies. Lane 1, purified plasminogen; lane 2, NS88.2; lane 3, NS88.2*prp*; lane 4 NS88.*2prpRC*). *B*) Binding of ^{125}I-plasminogen by GAS strains NS88.2, NS88.2*prp* and NS88.*2prpRC* (mean +/- SD, $n = 6$; $P < 0.05$). *C*) Acquisition of cell surface plasmin activity in human plasma by GAS strains NS88.2, NS88.2*prp* and NS88.*2prpRC* (mean +/- SD, $n = 9$; $P < 0.05$).

Figure 5. Virulence of NS88.2, NS88.2*prp* and NS88.2*prpRC*. Cohorts of 10 humanised plasminogen transgenic mice were subcutaneously infected with 1.13×10^7 cfu NS88.2 (■), 1.3×10^7 cfu NS88.2*prp* (▲) or 2.6×10^7 cfu NS88.2*prpRC* (▼). Survival was measured over a 10 day period. NS88.2*prp* was significantly attenuated for virulence when compared to both NS88.2 and NS88.2*prpRC* ($P < 0.05$).

Table 1. Fold increase in bacterial colonies compared to inoculum following growth in whole human blood (mean +/- SD, $n = 3$; $P > 0.05$).

	Growth of NS88.2[a, b]	**Growth of NS88.2*prp* [a, b]**	**Growth of NS88.2*prpRC* [a, b]**
Donor 1	272.3 +/- 32.3	179.3 +/- 46.1	244.3 +/- 59.2
Donor 2	174.3 +/- 67.7	217.7 +/- 95	347.7 +/- 68.3

[a] Increase in the number of GAS following 3 h incubation in whole blood, determined by dividing the number of cfu in blood following incubation, by the number of cfu in the original inoculum.

[b] The inoculum in cfu used for experiments usin blood from donor 1 and 2 respectively was as follows: NS88.2, 485 and 417; NS88.2*prp*, 673 and 493; NS88.2*prpRC*, 603 and 577.

Figure 2

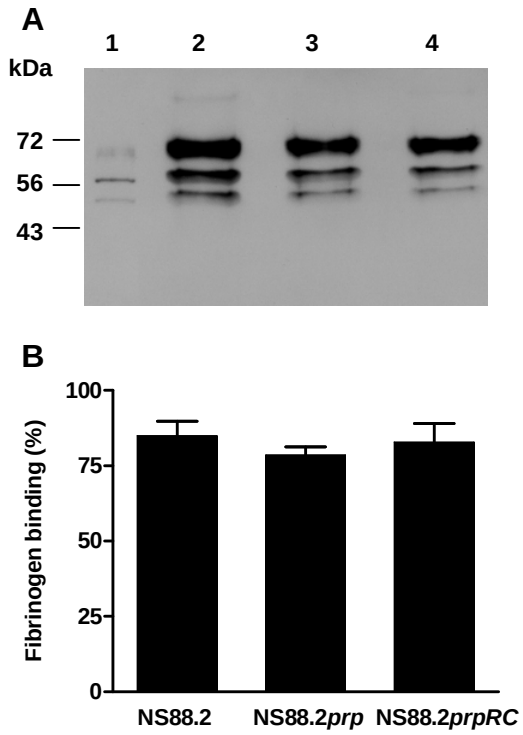
A
1
2
3
4
kDa
72
56
43
B
Fibrinogen binding (%)
100
75
50
25
0
NS88.2
NS88.2prp
NS88.2prpRC

Figure 3

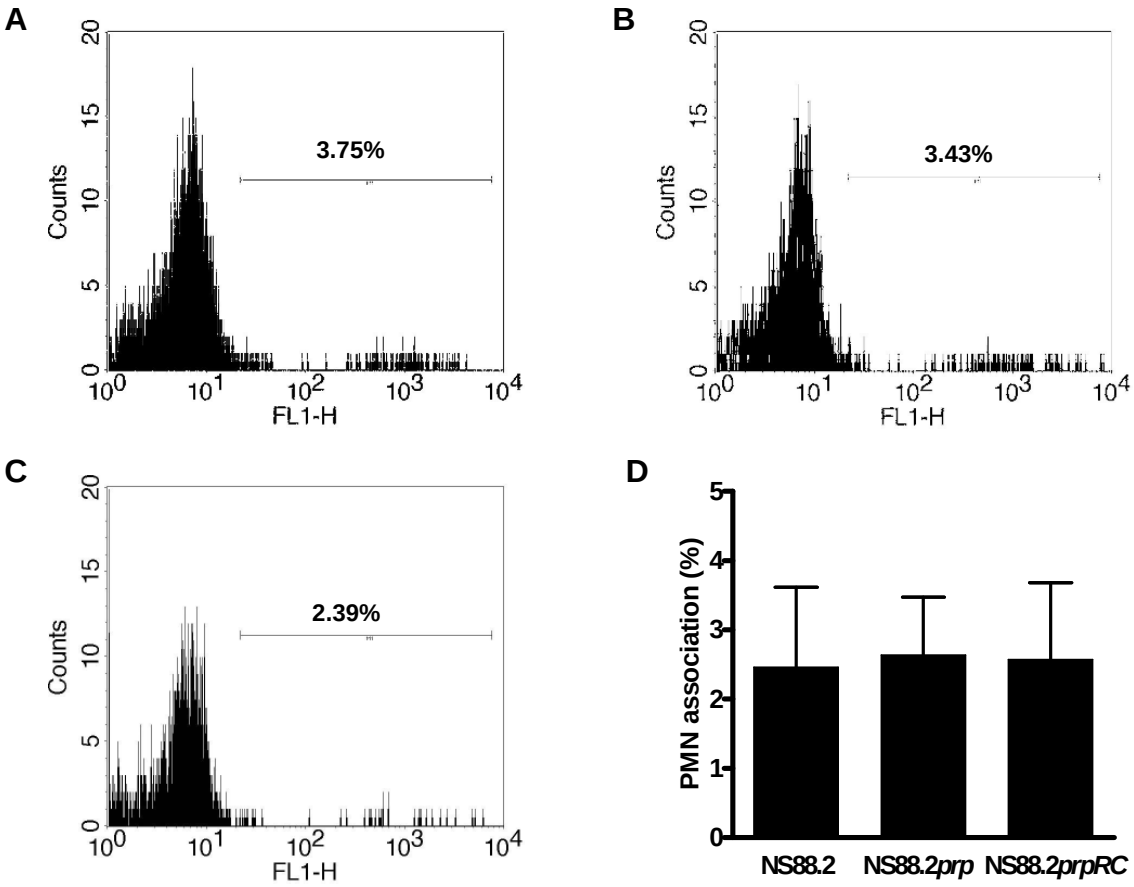
A
3.75%
Counts
FL1-H
B
3.43%
Counts
FL1-H
C
2.39%
Counts
FL1-H
D
PMN association (%)
NS88.2
NS88.2prp
NS88.2prpRC

Figure 4

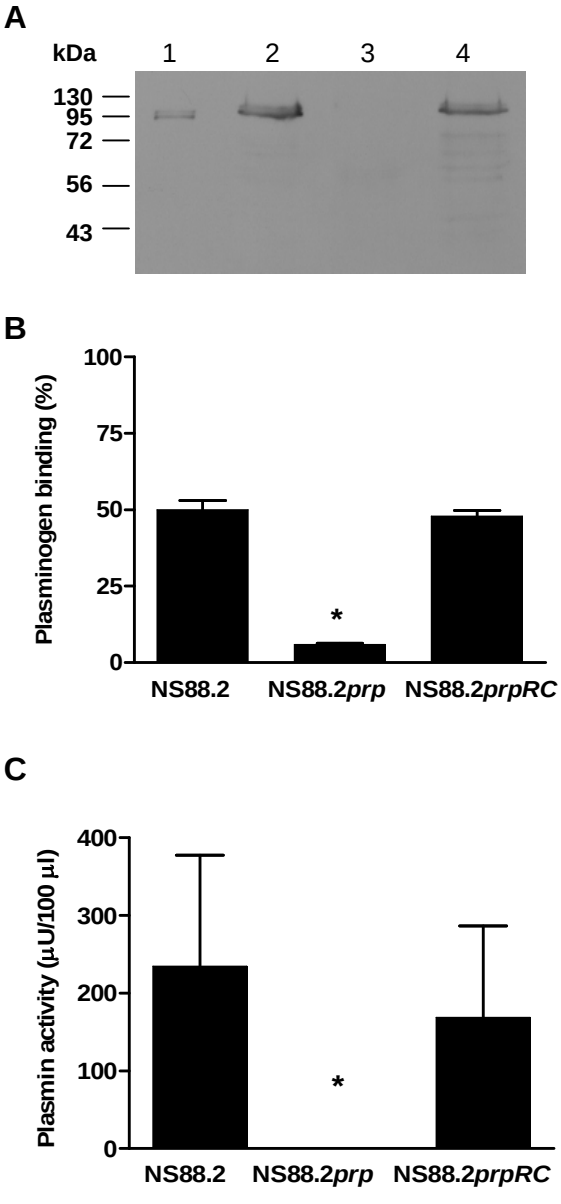
A
kDa
1
2
3
4
130
95
72
56
43
B
Plasminogen binding (%)
100
75
50
25
0
*
NS88.2
NS88.2prp
NS88.2prpRC
C
Plasmin activity (µU/100 µl)
400
300
200
100
0
*
NS88.2
NS88.2prp
NS88.2prpRC

Figure 5