Direct Host Plasminogen Binding to Bacterial Surface M-protein in Pattern D Strains of *Streptococcus pyogenes* Is Required for Activation by Its Natural Coinherited SK2b Protein*

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*Background:* Dissemination of Pattern D strains of *S. pyogenes* depends on a functional human fibrinolytic system.

*Results:* hPg binding domains of PAM, when transferred to unrelated M-proteins, up-regulates hPg binding and activation.

*Conclusion:* The nature of the streptokinase and the M-protein influence GAS virulence.

*Significance:* *In vitro* studies indicate pathways for GAS to gain hypervirulence by gene transfer of small functional domains.

Streptokinase (SK), secreted by Group A *Streptococcus* (GAS), is a single-chain ~47-kDa protein containing three consecutive primary sequence regions that comprise its α, β, and γ modules. Phylogenetic analyses of the variable β-domain sequences from different GAS strains suggest that SKs can be arranged into two clusters, SK1 and SK2, with a subdivision of SK2 into SK2a and SK2b. SK2b is secreted by skin-tropic Pattern D M-protein strains that also express plasminogen (human Pg (hPg)) binding Group A streptococcal M-protein (PAM) as its major cell surface M-protein. SK2a-expressing strains are associated with nasopharynx tropicity, and many of these strains express human fibrinogen (hFg) binding Pattern A-C M-proteins, e.g., M1. PAM interacts with hPg directly, whereas M1 binds to hPg indirectly via M1-bound hPg. Subsequently, SK is secreted by GAS and activates hPg to plasmin (hPm), thus generating a proteolytic surface on GAS that enhances its dissemination. Due to these different modes of hPg/hPm recognition by GAS, full characterizations of the mechanisms of activation of hPg by SK2a and SK2b and their roles in GAS virulence are important topics. To more fully examine these subjects, isogenic chimeric SK- and M-protein-containing GAS strains were generated, and the virulence of these chimeric strains was analyzed in mice. We show that SK and M-protein alterations influenced the virulence of GAS and were associated with the different natures of hPg activation and hPm binding. These studies demonstrate that GAS virulence can be explained by disparate hPg activation by SK2a and SK2b coupled with the coherited M-proteins of these strains.

Group A *Streptococcus* (GAS) is a human pathogen affecting >700 million people annually worldwide. Diseases caused by GAS and its sequelae include highly treatable superficial infections, e.g., impetigo and pharyngitis, as well as morbid and mortal infections, e.g., necrotizing fasciitis, glomerulonephritis, and streptococcal toxic shock syndrome (1, 2). To overcome the host immune system, colonize in the host, invade into deep tissues, and eventually cause serious disease, GAS expresses a number of chromosomal virulence factors, e.g., M-proteins, streptolysin, fibronectin-binding proteins, and streptokinase (SK) (1, 3), and bacteriophage-incorporated virulence determinants, e.g., streptococcal pyrogenic exotoxin A (4). An important mechanism that GAS has adapted for dissemination is to interact with the host fibrinolytic system. GAS-secreted SK is a non-canonical activator of the zymogen, human plasminogen (hPg), to form the broad spectrum serine protease, plasmin (hPm). Mechanistically, SK interacts with hPg to form a SK–hPg complex. Within this complex, hPg undergoes a conformational rearrangement and exposes the latent hPm-like active site (5). The SK–hPg complex, with an active site, subsequently recognizes its substrate, hPg, and catalyzes cleavage of the scissile Arg561–Val562 peptide bond in hPg to convert this zymogen to hPm (6). hPm degrades extracellular matrix directly or indirectly, e.g., by activating matrix metalloproteases, which allows GAS to disrupt the subepithelium, and/or subendothelium, migrate into the circulation, and colonize at deep tissue sites (6). Additionally, hPm dissolves the GAS-encapsulating thrombus, thereby resulting in enhanced clearance of fibrin networks and further assisting dissemination of GAS (7). These

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2 The abbreviations used are: GAS, Group A *Streptococcus*; SK, streptokinase; rSK, rat SK; PAM, Group A streptococcal M-protein; rPAM, rat PAM; hPg, human plasminogen; rhPg, recombinant hPg; hPm, human plasmin; hFg, human fibrinogen; AP, antiplasmin; mFg, mouse fibrinogen; mPg, mouse plasminogen; FXa, Factor Xa; Q-RT-PCR, quantitative real time reverse transcription-PCR; SPR, surface plasmon resonance.
findings underscore the role of hPm generation in GAS virulence.

The critical interactions between host protein and bacteria occur within well defined domains of these proteins. For example, hPg/hPm contains at least 6 domains: 5 consecutive ~80 amino acid functionally important kringle domains with various abilities to bind lysine and its analogues and a tryptic-like serine protease domain that is formed in hPm (8–10). Bacterial surface residing M-proteins, which are employed to serotype GAS, have revealed at least ~250 GAS strains. These M-proteins contain N-terminal highly variable A domains, the basis of GAS serotyping (11), less variable B domains, and more conserved C-terminal C and D domains followed by a peptidoglycan domain. Immediately downstream of these regions a sortase-A-recognizable LPXTG module, a membrane-spanning hydrophobic domain, and a very short cytoplasmic domain (12, 13) are present. Several homologous M-related proteins with different functions are present on the GAS surface, with semiconservation at the 3′-ends of the genes. This grouping of emm-type genes, mainly present in the mga regulon, has been classified as Pattern groups A–E based on homologies of their 3′-sequences and the number of these genes in this regulon (14–16).

Attempts to relate the sequence diversity among SKs from different strains of GAS, their hPg activation abilities, and the pattern and nature of the M- and M-like proteins produced to tissue and cell tropism have been described through epidemiologic patient studies (17–20) and in experimental mouse models of GAS infection (7,21). SK consists of three defined domains of approximately equal size, viz. the α, β, and γ domains, with the highest variability in the central β domain (22,23). Based on phylogenetic relationships of β-domains of SKs from different GAS strains, SKs have been grouped in two different clusters, SK1 and SK2, and two SK2 subclusters, SK2a and SK2b (24,25). GAS strains expressing SK2a have been associated with upper respiratory tract infections and express the emm1 gene, encoding (M1)-type M-proteins on their surfaces. On the other hand, GAS strains expressing SK2b have been associated with skin infections and express the pam gene coding for a hPg-binding M-protein, Pam, as its major M-protein (26,27). These Pam-expressing pattern D skin-specialist strains (28) contain a complete mga virulome (16), which maximizes their capabilities to resist the immune response of the host.

A striking difference between SKs of different clusters is that unlike SK1, SK2a and SK2b have a lesser abilities to activate hPg in solution (24,27). To activate hPg and bind to hPg/hPm, it has been postulated that SK2a and SK2b optimally require that a trimolecular complex of SK/hPg/human Fg (hFg) is formed. Herein, the hPg component of the trimolecular complex directly interacts with PAM on SK2b-expressing GAS cells and, in-turn, binds hFg. In SK2b-expressing strains of GAS, hFg in the trimolecular complex binds to M1 or similar M-proteins, e.g. M5 (29). Both mechanisms eventually focus hPg and hPm on GAS cells.

Studies of these types indicate that hPg/hPm interacts with natural SK2b-secreting cells directly, whereas the interaction of hPg/hPm with natural SK2a-secreting cells is indirect. Apart from different modes of interaction with SK, hPm shows differential ability to be inhibited by its major physiological inhibitor, α2-antiplasmin (AP), depending on the manner in which it is bound to proteins on the GAS surface (30). Thus, it was important to determine whether targeted alterations of the SK subtype in the genomes of GAS would affect hPg activation, the result of which would be expected to alter bacterial virulence and may in fact be an evolutionary mechanism for GAS survival. To address this issue, we determined the functional consequences in vitro and in vivo of genetically manipulated M-protein and SK in isogenic AP53 GAS strains. This allows incorporation of evolutionary considerations with mechanisms of GAS virulence. The results of this study are described herein.

**Experimental Procedures**

**Plasma Proteins**—hFg and mFg were purchased from ERL, South Bend, IN. hPg was purified from human plasma by affinity chromatography on Sepharose-lysine resin (31).

**Recombinant Protein Expression**—GAS strain AP53 (from G. Lindahl, Lund University, Lund, Sweden) was used to clone the genes for pam and sk2b. GAS strain SF370 (ATCC 700294) was similarly employed to clone the genes for M1 and sk2a. These strains were cultured on defibrinated sheep blood agar plates or Todd-Hewitt Broth (BD Biosciences, Bacto)/1% yeast extract (THY) and 37 °C and 5% CO₂.

Expression plasmids encoding recombinant proteins were generated as described previously (27). cDNAs for pam, M1, and their chimeric forms, viz. M1(B1B2—a1a2), with translated amino acid residues 94–148 (B1B2 domains) in M1 replaced with residues 55–91 (domains a1a2) in Pam and pam(a1a2→B1B2) with translated residues 55–91 (a1a2 domains) in Pam replaced by translated residues 94–148 (domains B1B2) in M1, were constructed by routine cDNA manipulations of cloned genes for M1 and Pam. The cDNAs for the chimeras were expressed with a His₆ tag at their C termini and a non-native Ala at their N termini. cDNAs of sk2a and sk2b were expressed in plasmids that sequentially contained a glutathione sulfur transferase tag, a His₆ tag, and a Factor Xa (FXa) cleavage site (25,27,32). All cDNA inserts in each of the plasmids were sequenced to assure the integrities of the genes.

The expression plasmids were transformed into Escherichia coli BL21DE3 cells (Invitrogen). Overnight cultures (2 ml) from single colonies were inoculated into 1 liter of lysogeny broth (LB)/40 mg/liter kanamycin and grown at 37 °C in a shaking incubator for 3–4 h until the A₆₀₀ nm reached 0.8–1.0 (late log phase growth stage). Recombinant protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma) for 6 h.

**Protein Purification**—The conditioned cells were harvested by centrifugation. The cell pellet was resuspended in 30 ml of binding buffer (20 mM Tris–HCl, 500 mM NaCl, 20 mM imidazole, pH 7.9) supplemented with EDTA-free protease inhibitor mixture (EMD Biochemical, Billerica, MA). Lysozyme (1 mg/ml) was added, and the suspension was incubated on ice for 30 min and then for another 15 min on a rocking platform at 4 °C. The suspension was next sonicated in the presence of benzylamine (EMD Biochemical), and insoluble debris was removed by...
centrifugation. To specifically isolate the His$_6$ proteins, the samples were applied to a column containing 15 ml of Ni$^{2+}$-NTA-agarose resin (Qiagen, Valencia, CA), and then, after washing, the His$_6$ samples were eluted with 500 mM NaCl, 20 mM Tris-HCl, 500 mM imidazole, pH 7.9. The eluate was concentrated using ultrafiltration columns (10-kDa NMWL (nominal molecular weight limit); Millipore, Billerica, MA). Subsequently, the buffer was exchanged to 100 mM sodium phosphate, pH 7.8, before use.

For rSKs, the eluted proteins were incubated with FXa in cleavage buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM CaCl$_2$, pH 8.0) at a ratio of 1 mg of rSK:1 unit of FXa (Enzyme Research Laboratories, South Bend, IN) overnight at 20 °C. To remove FXa, the protein mixture was incubated with Xarrest-agarose beads and centrifuged, and the supernatant was collected. To remove the FXa-released tags, the protein mixture was reloaded onto a Ni$^{2+}$-NTA column. The flow-through fractions, containing highly purified rSKs, were collected, and the buffer was exchanged with 10 mM sodium phosphate.

**Mass Spectrometry**—The molecular weights of the purified recombinant proteins were measured using a Bruker Autoflex-III MALDI-TOF/TOF mass spectrometer. A saturated solution of sinapinic acid matrix (3,5-dimethoxy-4-hydroxycinnamic acid) was prepared in 50% acetonitrile, 0.1% trifluoroacetic acid. The matrix material (2 µl) was spotted on MALDI plate, and 1 µl of each protein (1 µg) was applied to the matrix and allowed to dry at room temperature. Mass spectra were acquired in the linear positive ion mode.

**Circular Dichroism (CD)**—Far UV CD spectra were collected for rPAM, rM1, and the chimeric proteins, rM1(B1B2→a1a2) and rPAM(a1a2→B1B2). CD spectra were obtained using an Aviv (Lakewood, NJ) model 202 SF spectrometer in a buffer of 35 mM sodium phosphate, 50 mM NaCl, pH 7.4. Spectral data (3 independent scans for each protein) were recorded at 1.0-nm intervals from 200 to 250 nm in a 0.1-cm path length cell at 25 °C. A buffer reference scan was subtracted from each sample scan. The mean residue ellipticities ([θ]) were calculated using [θ] = (θ × MRW)/(I × c), where θ is the CD signal in millidegrees, MRW is the mean residue weight in g/mol, I is the path length in mm, and c is the protein concentration in mg/ml (33).

**Surface Plasma Resonance (SPR)**—SPR experiments were conducted at 25 °C on a BIAcore (Uppsala, Sweden) X100 SPR. Proteins were immobilized to a1a2) for rPAM, rM1, and the chimeric proteins, rM1(B1B2→a1a2) and rPAM(a1a2→B1B2). CD spectra were obtained using an Aviv (Lakewood, NJ) model 202 SF spectrometer in a buffer of 35 mM sodium phosphate, 50 mM NaCl, pH 7.4. Spectral data (3 independent scans for each protein) were recorded at 1.0-nm intervals from 200 to 250 nm in a 0.1-cm path length cell at 25 °C. A buffer reference scan was subtracted from each sample scan. The mean residue ellipticities ([θ]) were calculated using [θ] = (θ × MRW)/(I × c), where θ is the CD signal in millidegrees, MRW is the mean residue weight in g/mol, I is the path length in mm, and c is the protein concentration in mg/ml (33).

**Generating Isogenic GAS Strains**—Allelic replacements in GAS strain AP53 were accomplished by single-double cross-over homologous recombination procedures described earlier (16) using the temperature sensitive plasmid, pHY304 (from M. J. Walker, St. Lucia, Queensland, Australia), to generate the isogenic strains, viz. AP53/ΔSK2b, AP53/SK2b→SK2a and AP53/PAM→M1. Chlamyaphenicol acetyl transferase (cat) and sk2a replaced sk2b in WT-AP53 to generate AP53/ΔSK2b and AP53/SK2b→SK2a, respectively. M1 replaced pam in WT-AP53 to construct AP53/PAM→M1 and M1(B1B2→a1a2) replaced pam in WT-AP53 to generate AP53/PAM→M1(B1B2→a1a2).

**Quantitative Real Time Reverse Transcription-PCR (Q-RT-PCR)**—RNA was isolated from AP53, AP53/Δpam, AP53/ pam→M1, and AP53/M1(B1B2→a1a2) cells as described earlier (16). Q-RT-PCR was performed using synthetic internal primers (forward, 5'-GCTTATTAGAAGACGCAACAGC; reverse, 5'-CAGGGTTGTGGATCTGAT) to amplify a 207-bp region at the 3’-end of the genes that is common to both pam and M1. Q-RT-PCR was performed to quantify expression of pam, M1, and M1(B1B2→a1a2) in the relevant samples by quantifying the fluorescence of SYBR Green. The relative gene expression levels were analyzed by the 2$^{-\Delta\Delta CT}$ method (34), in which CT represents the threshold cycle number of RT-PCR at which the amplified product is first detected. The mean values of triplicate CT values were calculated for the target and reference gene, GAPDH, from both WT and mutant strains. The ΔCT value was determined by (CT of the target gene − CT of the reference gene), and then ΔΔCT was calculated by using (ΔCT of the mutant − ΔCT of the WT gene). The relative change of a gene of interest (%) was calculated from the expression (100 × 2$^{-\Delta\Delta CT}$).

**Western Blotting for SK Expression in GAS Supernatants**—Single colonies from plates of GAS strains incubated overnight at 37 °C were transferred to 2 ml of THY media. The cells were collected from these overnight cultures and washed twice with THY media. A 2-ml aliquot of the culture was inoculated into 35 ml of prewarmed THY media, and the cultures were grown at 37 °C to A$_{600 nm}$ = 0.6 in the presence of 28 µM SpeB inhibitor, E64 (Sigma) to further attenuate SpeB-catalyzed proteolysis of secreted SK (although, as the parent AP53 strain is covS−, thus repressing speB expression, it is unlikely that SpeB-catalyzed SK proteolysis would have occurred). Cultures were centrifuged to remove cells, and the supernatant was passed through a 0.22-µm filter. The supernatant was then concentrated 20× using 10,000 NMWL (nominal molecular weight limit) membrane centrifugation filters (Millipore). An aliquot of 5 µl of supernatants was separated on a 10% SDS-gel, and proteins were transferred to a PVDF membrane at 100V for 90 min. The membrane was then treated with rabbit polyclonal
anti-streptococcal SK (Novus Biologicals, Littleton, CO) followed by anti-rabbit-IgG 2° antibody conjugated to horseradish peroxidase (HRP). The protein was visualized by adding chromogenic HRP-specific substrates.

**hPg Activation Assays in Vitro**—The activation kinetics of Glu-hPg were measured at 37 °C using a Spectramax 96-well plate reader (Molecular Devices, Sunnyvale, CA). rSK2a or rSK2b were used as Glu-hPg activators. The generation of hPm from hPg was measured in 10 mM HEPES/150 mM NaCl, pH 7.4, using the hPm chromogenic substrate, S2251 (H-D-Val-L-Leu-L-Lys-p-nitroanilide). Glu-hPg (200 nM) and S2251 (0.25 mM) with or without 300 nM concentrations of hPg effectors, viz. rPAM, rM1, rM1(B1B2→a1a2), or rPAM(a1a2→B1B2), and/or hFg or mFg (500 nM), were individually included. hPm formation was accelerated by the addition of catalytic levels of rSK (5 nM). Activation of hPg was monitored by continuously measuring the rate of change of the ΔA405 nm. The reactions without rSK were used as negative controls. For quantitative estimates, the ΔA405 nm, which measures the continual appearance of hPm through the hPm-catalyzed cleavage of the substrate, was plotted against time-squares. The rates were normalized to that observed for hPg + rPAM, which was arbitrarily assigned a value of 1.0.

**Plasminogen Activation Assays in Plasma**—Assays were performed ex vivo in plasmas of WT mice and mFg → /− mice (35) that were also depleted of mPg. To obtain this plasma, citrated plasma from Fg → /− mice was incubated with a Sepharose-lysine matrix for 1 h at room temperature to remove the mPg. The sample was then centrifuged to eliminate the resin. The supernatant, which represents mFg/mPg-depleted plasma, was collected. hPm activation assays were performed at 37 °C in the presence of 50 μl of plasma plus or minus added mFg or hFg (1500 nM), Glu-hPg (200 nM), and S2251 (0.25 mM), plus or minus rM1 (300 nM). hPm formation was accelerated by the addition of catalytic levels of rSK2b (5 nM). Activation of hPg was monitored by continuously measuring the rate of change of the ΔA405 nm, and the data were reduced as described in the previous section.

**Survival Analysis Using a Murine Model of GAS Infection**—Single colonies of GAS were picked from freshly streaked blood agar plates, inoculated into 4 ml of THY broth, and grown over-night at 37 °C. A 3-ml aliquot of overnight culture was then collected. hPg activation assays were performed at 37 °C in the presence of 50 μl of plasma plus or minus added mFg or hFg (1500 nM), Glu-hPg (200 nM), and S2251 (0.25 mM), plus or minus rM1 (300 nM). hPm formation was accelerated by the addition of catalytic levels of rSK2b (5 nM). Activation of hPg was monitored by continuously measuring the rate of change of the ΔA405 nm, and the data were reduced as described in the previous section.

**Results**

The importance of PAM, SK, and hPg as the basis for this study is illustrated by mouse post-infection lethality data of (Fig. 1). Here, infection of C57Bl/6 mice with a PAM-containing strain of GAS, viz. AP53, shows rapid and nearly complete (90%) lethality when administered to C57Bl/6 mice that contain the hPg transgene (C57Bl/6(hPg-Tg)). On the other hand, WT-C57Bl/6 mice that contain only native mPg are much less sensitive (100% survival at 10 days) to the effects of AP53 infection (Fig. 1). In similar infections with isogenic strains of GAS, inactivation of the pam gene (strain AP53/ΔPAM) or the sk2b gene (strain AP53/ΔSK) showed attenuated lethality in C57Bl/6(hPg-Tg) mice (50–60% survival; p = 0.005 in each case compared with WT-AP53), thus confirming the importance of PAM and SK2b in this system. It is known that mPg does not interact with PAM due to mutations in the K2-mPg domain (36). Because SK2b requires the PAM-hPg complex for maximal hPg activation activity, we conclude that mPm (mouse plasminogen) is ineffectively formed from nonbound mPg and that these PAM-containing Pattern D GAS strains ineffectively disseminate in WT mice.

**Generation and Characterization of Recombinant Proteins**—To attempt to transfer hPg binding ability from PAM to M1, which is believed to be exist solely in the N terminus a1a2 domains of PAM (37), a chimeric M1, rM1(B1B2→a1a2), was constructed and expressed wherein M1 underwent targeted replacement of its 55-residue hFg-binding B1B2 repeat by the 37-residue hPg-binding a1a2 repeat from PAM (Fig. 2). Similarly, to attempt to transfer hFg binding characteristics from M1 to PAM, a chimeric PAM, viz. rPAM(a1a2→B1B2), was generated and expressed in which the same hPg binding a1a2 repeat of PAM was replaced by the known hFg binding B1B2 repeat from M1 (38) (Fig. 2).
Total DNA sequencing of the constructs was performed to assure the quality of the cDNAs used in the expression plasmids. All cDNAs used possessed the desired sequence. In addition, the expressed and purified proteins were characterized by MALDI to determine their molecular weights. These values were found (calculated; difference between calculated and experimental): 41,226 (41,142; 0.2%) for rPAM; 48,441 (48,270; 0.4%) for rM1; 45,463 (45,396; 0.1%) for rM1(B1B2→a1a2); 43,182 (43,132; 0.1%) for rPAM(a1a2→B1B2). For the purified rSKs, the molecular weights were: 47,207 (47,157; 0.1%) for rSK2a; 47,473 (47,465; 0.05%) for rSK2b.

Possible gross conformational changes in the proteins as a result of their chimeric natures were examined by CD (Fig. 3). From the CD spectra, it appears that rPAM is a fully helical protein, and rM1 has considerably less (~50%) overall helical structure. When the a1a2 domain of PAM is substituted for the B1B2 domain of M1, the helical content of the chimeric rM1 is markedly elevated. The opposite is true for the chimeric rPAM, i.e. replacing a1a2 of PAM with B1B2 of M1 leads to a significant diminishment of the helical content of the chimeric protein. Thus, we conclude that helical distortions in M1 have an influence through their B1B2 domains, a finding that coincides with data from a partial x-ray structure of M1 in which it was shown that the B1B2 region of M1 was splayed and intertwined with B-repeats from the other chain of M1 (39). Replacing B1B2 with a1a2 in M1 leads to a coiled coil of the chimeric protein that contains a substantially higher overall a-helical content equivalent to that of PAM.

**Binding Affinities of rPAM, rM1, and Their Chimeras to Pg and Fg—** The binding of PAM to hPg is central to SK2b-mediated activation of hPg and hFg binding properties between M-proteins. The binding of PAM and rM1(B1B2→a1a2) to hPg was examined by SPR (Fig. 5, A and B). Global fitting of the sensograms obtained for these interactions were accomplished by applying a 1:1 binding model. The deconvoluted data provided an equilibrium binding constant (K_d) of 0.3 nM for the hPg/rM1(B1B2→a1a2) interaction (Fig. 5B), a value comparable with that for the interaction of WT-PAM-AP53 with Glu-hPg, which showed a K_d = 1.0 ± 0.3 nM (Fig. 5A). These results suggest that the a1a2 repeat, placed into a hPg non-binding protein, M1, can direct hPg binding to the chimera. A qualitatively similar result was observed in transferring the a1a2 repeat of AP53 into the M4-related protein, Arp (37). Consistent with this hypothesis, the chimeric protein rPAM(a1a2→B1B2) did not bind hFg, and rM1(B1B2→a1a2) did not bind hFg or mFg due to elimination of a1a2 and B1B2 repeats, respectively (data not shown).

**Stimulation of the SK-mediated Activation of hPg by Chimeric M-proteins—** Although the a1a2 repeat of PAM is essential for binding to hPg, additional exosite interactions have been identified as important for specific rPAM binding to hPg (44). Thus, in our chimeric proteins, when the a1a2 repeat was placed in a background of rM1, exosite interactions outside of the a1a2 repeats that are specific to PAM were eliminated. We first compared rM1(B1B2→a1a2) with rPAM and rM1 for their abilities to stimulate the rSK2b-mediated activation of hPg. The...
data demonstrate that rSK2b does not appreciably activate hPg in solution without or with rM1 (Fig. 6A). Both rPAM and rM1(B1B2→a1a2) stimulate this activation rate by 60–200-fold. The stimulation is greater with rPAM (Fig. 6A), suggesting that other regions of PAM play minor roles in its effectiveness with regard to hPg activation. However, in the presence of excess hFg, the differences in the rates of stimulation between rPAM and rM1(B1B2→a1a2) were much less pronounced (Fig. 6A). Similarly, the rate of hPg activation by SK2b was not stimulated by rM1 but was stimulated to a small degree by rM1 + hFg (Fig. 6A). This latter level of stimulation of rM1 by hFg is only slightly greater than that found with hFg alone (Fig. 6A). Thus, the contribution of hFg and M1 to the rate of stimulation of hPg activation by SK2b was optimal when hPg was bound to a PAM-like M-protein (e.g. rPAM and rM1(B1B2→a1a2)).

We next compared rSK2a and rSK2b with regard to their relative abilities to activate hPg (Fig. 6B). SK2b did not appreciably activate unbound hPg, but SK2a displayed a low level activity with free hPg (Fig. 6B). Furthermore, rM1 or rPAM(a1a2→B1B2) did not significantly stimulate hPg activation with either rSK2a or rSK2b primarily due to the inabilities of these M-proteins to interact with hPg. On the other hand, rPAM greatly stimulated the h Pg activation rates by either rSK2a or rSK2b (Fig. 6A and B), showing that both forms of rSK functioned more effectively with hPg bound to PAM.

Stimulation of the SK-mediated Activation of hPg by r-chimeric M-proteins in Mouse Plasma—To determine whether the in vitro hPg activation assays would be recapitulated in plasma, where the virulence assays are conducted, we first collected mouse plasma, which was devoid of mFg, from mice with a targeted inactivation of the Fg gene (35). We further depleted the mPg by addition of Sepharose-lysine (45). Components were then added back to the plasma as desired. This approach allowed a clearer interpretation of plasma activation assays without interference from mFg and mPg. The former binds weakly to M1, and the latter is not activated by SK but nonetheless has the potential to be inhibitory.

Using mFg/mPg double-deficient plasma, stimulation of hPg activation by SK2b is maximal when hFg is also added to the plasma (Fig. 6C). The addition of mFg in place of hFg is comparatively much less effective in this regard. Furthermore, potential plasma inhibitors of hPm appearance do not shut down this activation process. When M1 is further included in the plasma, activations are similar to those with hFg alone as
seen in Fig. 6A, and the basic conclusions are not affected. Furthermore, mFg is not an effective stimulator of hPg activation (Fig. 6C).

**FIGURE 6. Activation of hPg by SK2B.** SK-mediated hPg activation in vitro was performed in a reaction consisting of 200 nM Glu-hPg, 300 nM rPAM or rM1(B1B2→a1a2), without or with 500 nM hFg or mFg. All factors in reaction were incubated at 37 °C for 10 min in a buffer consisting of 10 mM Heps, 150 mM NaCl, pH 7.4, 250 μM S2251, pH 7.4. The reaction was accelerated by the addition of 5 nM SK, and the activation of hPg was monitored by measuring the absorbance at 405 nm due to the hydrolysis of S2251 catalyzed by the generated hPm. Initial rates of hPg activation were calculated from a plot of A405 nm versus time. A, all initial activation rates by SK2b were normalized to the rate determined with rPAM (relative activation rate = 1.0). B, comparisons of activation rates of hPg under identical conditions, except for the hPg activator being SK2a or SK2b. C, activation of hPg by SK2b in mouse plasma. Mouse plasma, totally deficient in mFg and mPg, was reconstituted with human and mouse components at their same plasma concentrations as in the original plasma, and hPg activation by SK2b was measured. The activation rates were normalized to those obtained from plasma containing hPg/hFg, which was set at 100%. Whereas hFg stimulated activation of hPg, mFg was ineffective in this regard.

**FIGURE 7. Characterization of the chimeric AP53 strains.** A, Q-RT-PCR of the transcription of the chimeric M-proteins using primers that amplify the same regions of M1 and PAM. The data show that both M1 and M1(B1B2→a1a2) in the isogenic AP53 lines are expressed to ~65% of pam expression in WT-AP53 cells, whereas as expected the isogenic AP53/Δpam does not show PAM expression (Fig. 7A). With regard to SK secretion, Western blot analysis of the culture supernatants of the GAS strains show very comparable secretion of SK2b in WT-AP53 and SK2a in isogenic AP53/SK2b→SK2a cells, whereas no SK expression is found in isogenic AP53/ΔSK cells (Fig. 7B). The small molecular weight differences between SK2a and SK2b are as expected and confirm the SK2 subcluster class (32).

Subsequent to the in vitro characterization of these newly modified GAS strains, their virulence was examined utilizing a C57BL6 mouse model that expressed the hPg transgene (C57BL6/hPg-Tg) (7). SK and M-proteins (PAM and M1) were suggested to be important virulence factors of GAS (29). This is supported by our data that demonstrated that deletion of SK2b in AP53/Δsk2b and, independently, PAM in AP53/Δpam, display reduced virulence compared with WT-AP53 (Fig. 1).

A coinheritance of the SK subtype and M-proteins has been proposed, as strains carrying SK2b express an M-like protein, PAM, and those secreting SK2a expressed M1 and related M-proteins (32). To further understand the functional relationships between SK subtypes and the associated M protein, GAS strains AP53/pam→M1 and AP53/pam→M1(B1B2→a1a2) were examined in this survival model. In strain AP53/pam→
M1, PAM has been replaced by M1 from a SK cluster 2a strain, whereas similar to the recombinant proteins, AP53/pam→M1(B1B2→α1α2) expresses a modified M1m, which has the hPg binding α1α2 of PAM in place of the hFg binding B1B2 repeat of M1. These isogenic strains enabled us to study the function of M proteins and their domains in non-native host strains in combination with SK of the host strain. It is confirmed in our studies that hPg is a necessary feature for high virulence in native PAM-producing strains (Fig. 1). Despite the hPg binding ability of rPAM→M1(B1B2→α1α2), the isogenic GAS strain, AP53/pam→M1(B1B2→α1α2), is not as virulent as WT-AP53 (Fig. 8), suggesting that other regions of PAM function in the killing mechanisms and/or that in live bacteria conformational variances in this chimeric M-protein affect the full participation of the hPg system in bacterial killing. Furthermore, the role N-terminal residues unique to PAM that surround α1α2 and that were not transferred to rM1(B1B2→α1α2) cannot be disregarded as being important in virulence. Our results imply that SK2b strains are adapted to bind hPg directly for an enhanced virulence, whereas cluster 2a strains may have other virulence factors, such as Sda1 (e.g. GAS strain 5448, serotype M1T1), to compensate for the virulence due to the absence of direct hPg binding. Thus, virulence is conserved among GAS strains by different mechanisms.

It is also seen from the survival data (Fig. 1) that secreted SK is a necessary virulence factor. There is a 50% survival rate of C57Bl/6(hPg-Tg) mice upon inactivation of the SK2b gene in GAS AP53. Whether these bacteria produce SK2b or SK2a in a PAM producing strain, virulence is very strong and equivalent (Fig. 8) and relies on SK production.

Discussion

GAS is a human pathogen consisting of >250 serotypes, which are characterized based on N-terminal polymorphisms in their surface M or M-like proteins. This large genetic diversity of GAS poses difficulty in vaccine development, and GAS continues to be a potent pathogen. Apart from M-polymorphisms, variability in sk alleles across GAS isolates has also been reported. Based on phylogenetic analysis of β-domain amino acid sequences of SK from several GAS isolates, GAS strains are classified into cluster 1 and cluster 2 strains, and cluster 2 is subdivided into subclusters 2a and 2b (24, 32).

A long-sought objective in GAS research is to attempt to correlate the genetic diversity of GAS with its tissue tropism and virulence (26). In this regard the majority of the available literature suggests that GAS strains secreting SK2b contain the Pattern D emm cluster, express PAM-like proteins on their cell surfaces, and are normally found in skin infections (24, 32). On the other hand, GAS strains expressing SK2a are normally Pattern D cluster Pattern A-C, are isolated from nasopharynx infections, and express a wide variety of M-proteins on their cell surfaces, e.g. M1 (24, 32). Whereas PAM is a direct functional hPg/hPm-binding protein (46), which the relevant GAS employs to generate surface protease (hPm) activity from hPg, M1 has accomplished a similar goal by indirectly interacting with hPg/hPm via M-protein bound hFg (47).

The binding of hPg to PAM is associated with the N-terminal variable α1α2 domains of PAM and the lysine binding site of the K2 domain of hPg (46). The major binding site of hFg to M1 is located on the FD domain of hFg and the variable N-terminal B1B2 domains of M1 (48, 49). Although two different M proteins of GAS interact with different components of the host fibrinolytic system, the interaction of GAS and hPg/hPm, directly or indirectly, is critical for optimal GAS virulence. One important feature of SK2b is that this protein activates hPg when directly bound to PAM-like proteins to a far greater extent than when hPg is free in solution. On the other hand, SK2a is more promiscuous in that this form of SK not only activates GAS-bound hPg but can weakly activate hPg in solution. In contrast, GAS strains expressing cluster 1 SKs do not contain M-proteins that interact directly with hPg or, in many cases, hFg. Consequently, SK1 readily activates hPg in solution (27). As might be expected, these particular SK1 expressing strains possess variable tissue and cell tropism in experimental settings. This is not particularly puzzling as hPm formed in blood in vivo would be rapidly inactivated by the circulating natural hPm inhibitor, α2-antiplasmin, whereas receptor- or surface-bound hPm is more resistant to inactivation from α2-antiplasmin (50, 51).

It is critical to consider that importing direct hPg binding ability into M1 via the α1α2 domains is not necessarily reflected in stimulation of hPg activation. For example, hPg binds equally well to PAM and the construct, rM1(B1B2→α1α2), but the hPg/PAM complex is much more rapidly activated by SK2b than is the hPg/rM1(B1B2→α1α2) complex in the absence of hFg. Obviously, the strength of binding of hPg does not necessarily suggest that binding orientation is optimal for SK2b-catalyzed activation unless optimal activation effectors are present (in this case, hFg). However, hPm binding should be equivalent to that of hPg, as the α1α2-binding hPg-K2 domain is intact in hPm and hPm, and this would allow a proteolytic surface to nonetheless develop under appropriate circumstances when hPm is

FIGURE 8. Kaplan-Meier plots of the survival of C57BL6/hPg(Tg) mice after infection. Aliquots of cells containing 1.5 × 10^5 cfu of various GAS lines were injected subcutaneously into C57Bl/6 mice (dotted black line) or C57Bl/6 mice containing the hPg-transgene (all other curves) and monitored for survival for 10 days. The GAS cell lines used were: WT-AP53 (n = 20), AP53/PAM→M1 (n = 10), and AP53/PAM→M1(B1B2→α1α2) (n = 10). The controls with WT-AP53 administered to WT-C57Bl/6 mice (dotted black line) and WT-AP53 with C57Bl/6(hPg-Tg) mice (red line) are also shown. p < 0.01 for the pairwise comparisons between WT-AP53 (n = 20) and other cell lines, indicating significant differences. Student’s t test was used for pairwise comparisons.
formed. Because the isogenic strains, AP53/ΔPAM and AP53/PAM→M1, exhibit weak virulence compared with WT-AP53 in an animal model that requires hPg, it appears clear that insufficient hPm is being generated by SK2b in these strains, likely due to a lack of the a1a2-mediated hPg binding site. This is also suggested by the in vitro assays. Furthermore, because all of these AP53/covS− strains display a low SpeB phenotype, it is highly unlikely that degradation of virulence factors by this secreted protease affects these conclusions.

The question of molecular coinheritance relationships between the nature of the SK subcluster, the particular M-protein produced, the emm cluster pattern, and tissue tropicity, has been generally addressed in several previous publications (17, 52, 53) without firm conclusions. Part of the confounding issues are that GAS strains that have been studied are clonal patient isolates and have already interacted with the host, perhaps altering important bacterial genes. For example, many clones of deep tissue isolates, especially from strain 5448, have ceased to produce the critical transcriptional regulatory gene, covS, and consequently SpeB, which has a large inverse relationship with virulence (54).

Finally, the work presented here, employing two GAS strains, one exclusively producing hPg-binding PAM (AP53) and the other producing hFg-binding M1 (SF370), are prototypical strains that bind hPg/hPm via different molecular routes. AP53 encodes PAM and SK2b and SF370 produces M1 and SK2a, thus allowing relationships to be studied between different hPg binding modalities of GAS and SK subtypes. Due to the very low levels of direct hFg-binding proteins in AP53, e.g. serum opacity factor, AP53 was used as a convenient background strain to perform in vivo gene deletions/replacements and domain substitutions with coding sequences for SK and from M1. AP53 was used as a convenient background strain to perform in vivo gene deletions/replacements and domain substitutions with coding sequences for SK and from M1. We conclude that the a1a2 region PAM is necessary to allow hPg to directly interact with GAS, and SK2b is only produced in natural PAM-containing strains. In these strains (e.g. AP53) SK2b-mediated activation of hPg is greatly enhanced via PAM binding and further enhanced by binding of host hFg. The hPm produced remains bound to the cells, and the strain is highly virulent. Replacement of PAM by M1 in a SK2b-secreting system does not lead to highly stimulated hPm activation, even in the presence of hPg, and the chimeric strain is hypovirulent. In all cases a complementary consideration is that the most virulent GAS strains have undergone a genetic switch from CovS+ to CovS− at some stage of dissemination, thereby eliminating a secreted protease, SpeB, which preserved the surface expression of M-proteins and other virulence determinants. Thus, hypervirulent strains of GAS strategically employ several host systems, including the fibrinolytic system as well as switches in its own gene expression for maximal effect to circumvent host resistance pathways to dissemination.

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