Site-directed mutagenesis of streptococcal plasmin receptor protein (Plr) identifies the C-terminal Lys\textsuperscript{334} as essential for plasmin binding, but mutation of the \textit{plr} gene does not reduce plasmin binding to group A streptococci

Scott B. Winram† and Richard Lottenberg

Author for correspondence: Richard Lottenberg. Tel: +1 352 392 3000. Fax: +1 352 392 8530. E-mail: lottenr@medicine.ufl.edu

Division of Hematology/Oncology, Department of Medicine, University of Florida College of Medicine, Box 100277, Gainesville, FL 32610-0277, USA

Plasmin(ogen) binding is a common property of many pathogenic bacteria including group A streptococci. Previous analysis of a putative plasmin receptor protein, Plr, from the group A streptococcal strain 64/14 revealed that it is a glyceraldehyde-3-phosphate dehydrogenase and that the \textit{plr} gene is present on the chromosome as a single copy. This study continues the functional characterization of Plr as a plasmin receptor. Attempts at insertionin inactivation of the \textit{plr} gene suggested that this single-copy gene may be essential for cell viability. Therefore, an alternative strategy was applied to manipulate this gene in vivo. Site-directed mutagenesis of Plr revealed that a C-terminal lysyl residue is required for wild-type levels of plasmin binding. Mutated Plr proteins expressed in \textit{Escherichia coli} demonstrated reduced plasmin-binding activity yet retained glyceraldehyde-3-phosphate dehydrogenase activity. A novel integration vector was constructed to precisely replace the wild-type copy of the \textit{plr} gene with these mutations. Isogenic streptococcal strains expressing altered Plr bound equivalent amounts of plasmin as wild-type streptococci. These data suggest that Plr does not function as a unique plasmin receptor, and underscore the need to identify other plasmin-binding structures on group A streptococci and to assess the importance of the plasminogen system in pathogenesis by inactivation of plasminogen activators and the use of appropriate animal models.

Keywords: streptococci, receptors, plasmin(ogen), glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

INTRODUCTION

Group A streptococci are Gram-positive bacteria capable of causing a variety of diseases in humans, ranging from acute pharyngitis to potentially lethal invasive soft tissue infections and a toxic-shock-like syndrome (Bisno & Stevens, 1996). The mechanisms utilized by group A streptococci to rapidly traverse tissue barriers in the course of invasive infections have yet to be elucidated. The interaction of the bacteria with the host plasmin(ogen) system may be an important contributor to this aspect of streptococcal pathogenesis. Group A streptococci secrete streptokinase which is a potent activator of human plasminogen (Castellino, 1979). Plasmin can degrade a wide range of protein substrates that constitute the extracellular matrix, as well as activating latent proteases (Vassalli 	extit{et al.}, 1991). We have previously shown that group A streptococci, when grown in the presence of human plasma, can activate plasminogen and capture plasmin activity on the bacterial surface (Lottenberg 	extit{et al.}, 1992b). This surface-bound proteolytic activity is no longer inhibitable by physiological plasmin inhibitors such as \textit{a}-2-antiplasmin. To determine the role of bacteria-associated plasmin in the pathogenesis of infections caused by these organisms, the plasmin(ogen)-binding components of the cell surface must first be identified.

†Present address: Department of Infectious Diseases, Children's Hospital and Medical Center, Box 5371/CH-32, Seattle, WA 98105, USA.

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Plr, plasmin receptor protein; S-2251, D-Val-Leu-Lys-p-nitroanilide.
A candidate plasmin receptor of group A streptococcal strain 64/14 was previously purified and the gene encoding this protein was cloned, sequenced and expressed in *Escherichia coli* (Lottenberg *et al.*, 1992a). The predicted amino acid sequence of Prr exhibited identity to that of glyceroldehyde-3-phosphate dehydrogenase (GAPDH) and, moreover, Prr was shown to be an enzymically active GAPDH molecule (Winram & Lottenberg, 1996). Biochemical and functional analyses detected no differences between Prr and cytoplasmic SDH encoded by a single hybridization analyses demonstrated that this protein is an enzymically active GAPDH in strain 64/14. Furthermore, DNA hybridization analyses demonstrated that this protein is encoded by a single *plr* gene on the streptococcal chromosome (Winram & Lottenberg, 1996).

Streptococcal surface dehydrogenase (SDH) from group A streptococci has also been reported (Pancholi & Fischetti, 1992). With the exception of a single amino acid residue, the N-terminal amino acid sequence of SDH was identical to that of Prr. Additionally, amino acid composition profiles of Prr and SDH were similar (Winram & Lottenberg, 1996). These results indicated that Prr and SDH were structurally similar proteins.

In the present investigation, the contribution of Prr to the plasmin-binding phenotype of group A streptococci was examined by applying genetic mutagenesis strategies which circumvented the problems encountered when manipulating an essential gene.

### METHODS

**Bacterial strains and growth conditions.** *E. coli* F′ e14 (McrA') *hsdR514(c−, m−) supE44 supF58 ΔlacZΔM15 galK2 galT22 metB1 trpR551* and *E. coli* DE3 (Novagen) were used for transformation and gene expression. *E. coli* strains harbouring plasmids were grown overnight as shaking cultures at 37 °C in Luria broth. Chloramphenicol, ampicillin, tetracycline and kanamycin were used at 30, 50, 34 and 10 μg ml⁻¹, respectively, where appropriate. Gene expression from pTrc99C derivatives was induced by adding a final concentration of 10 mM IPTG to the Luria broth. Streptococcal strains used in these studies are described in Table 1.

Streptococcal strains were grown overnight as standing cultures in Todd–Hewitt broth containing 0·3% (w/v) yeast extract (THY broth) at 37 °C. Strains containing a kanamycin resistance (QKm') gene cassette (Fellay *et al.*, 1987) were grown in the presence of 300 μg kanamycin ml⁻¹ in THY broth or 500 μg kanamycin ml⁻¹ on THY agar plates.

**Insertional inactivation of the *plr* gene.** DNA manipulations used in construction of plasmids were performed by standard methodology (Sambrook *et al.*, 1989). The 2·3 kb BamHI– HindIII insert of pRL024 (Winram & Lottenberg, 1996) was excised, blunt-ended (by Klenow fill-in) and subcloned into *Pst*I-digested pACYC184 to generate pRL026. The putative promoter of the *plr* gene transcribes in the opposite direction to the Tet' promoter of pRL026. To interrupt expression of Prr, a 2·3 kb *EcoR*I fragment containing the QKm' gene cassette was blunt-ended and subcloned into the unique *Pst*I site of pRL026 located within the *plr* ORF at bp 420 to form plasmid pRL027.

**Site-directed mutagenesis of the *plr* gene.** Site-directed mutagenesis by PCR was performed by synthesizing DNA primers containing restriction enzyme sites for cloning and the complementary sequence to the *plr* gene except for the desired mutation. The plasmid pRL024 was used as the DNA template for PCR. The *plr*-45 gene insert of pRL045 has the terminal codon of the *plr* gene changed from AAA, encoding a lysyl residue, to CTT, encoding a leucine. The base-pair changes are followed by a termination codon, TAA. The forward primer used to generate this mutation was 5′-GTTAATAC-CAATAACTACATGGGGAATTTTTCGAAGTA-CTCAAAG-3′. The amplified product was ligated into the vector pT7Blue (Novagen). The *plr*-45 insert was excised by digestion with *Nco*I and *BamHI* and subcloned into pTrc99c (Amann *et al.*, 1988) to yield pRL045.

The *plr*-63 mutation has two additional codons namely, GGT (glycine) and CTT (leucine), added to the 3′ end of wild-type *plr* gene. The PCR-amplified product was generated by using the same forward primer as used for *plr*-45 and 5′-CGCGGATCTTAAAGACCTTTAGCAATTTTTGCGAAGT-3′ as the reverse primer.

The 5′ and 3′ end junctions of vector and insert were sequenced in all plasmids to verify the presence of the mutation(s).

### Table 1. Group A streptococcal strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
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<tbody>
<tr>
<td>64/14</td>
<td>M-untypable clinical isolate that has been previously mouse-passaged 14 times (Reis <em>et al.</em>, 1984)</td>
</tr>
<tr>
<td>64/14k</td>
<td>A derivative of 64/14, harbouring the QKm' cassette on the chromosome approximately 400 bp downstream of the <em>plr</em> gene at the former <em>BamHI</em> site; the cassette was introduced by electroporation of linear DNA and there are no integrated vector sequences</td>
</tr>
<tr>
<td>64/14k-45</td>
<td>Replacement of the <em>plr</em> gene with <em>plr</em>-45, which has the terminal lysine codon replaced with a leucine codon; the QKm' cassette is located approximately 400 bp downstream of the <em>plr</em> gene at the former <em>BamHI</em> site</td>
</tr>
<tr>
<td>64/14k-63</td>
<td>Replacement of the <em>plr</em> gene in 64/14 with <em>plr</em>-63, which has an additional glycine codon and a leucine codon immediately 3′ to the wild-type terminal lysine codon; the QKm' cassette is located in the same position as in strain 64/14k and strain 64/14k-45</td>
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Construction of the plr gene replacement vector. The construction of plasmid pSWO25 and its derivatives are depicted in Fig. 4. Briefly, the 2.7 kb insert of pRL015 (Lottenberg et al., 1992a) harbouring the plr gene was excised by EcoRI digestion and filled-in with Klenow fragment and dNTPs. This insert was subcloned into a 2.2 kb fragment of the vector pACYC184, which had been digested with AvaiI, blunt-ended and then digested with XmaI. Ligation of this digested vector with the insert generated pSWO25. The plr ORF of pSWO25 was precisely deleted and replaced with Ncol and Smal cloning sites by performing inverse PCR on pSWO25, using the primers 5'-CGGCCGGGTATCCCATGAGTATTTCTCTTCTATGAAAAT-3' and 5'-CGGCCGGGTAGTTATAAGAAAGAGCT-3'. The amplified product was digested with Smal and recircularized to generate pSWO26. The plasmid pSW026 was partially digested with BamHI and the protruding DNA overhangs were blunt-ended. The 2.3 kb ΔKm' cassette was excised from plasmid pRL027 by BamHI digestion and the overhangs were blunt-ended. The ΔKm' cassette fragment was then ligated with the linearized pSW026 to yield pSW027.

The plr-45 insert of pSWO45 was prepared by digesting pRL045 with BamHI, blunt-ending the overhangs and then digesting with Ncol. This insert was cloned into Ncol- and Smal-digested pSW027. The Ncol- and Smal-digested plr-63 insert was cloned into the Ncol and Smal sites of pSW027 to generate plasmid pSW063. The plasmids pSW045 and pSW063 were linearized prior to electroporation by cutting at the unique BamHI site on the vector.

DNA was introduced into strain 64/14 by electroporation following the method of Simon & Ferretti (1991).

**GAPDH purification.** The GAPDH of Bacillus stearothermophilus was isolated from cell lysates of *E. coli* x6060(pBat-gap1) (pBat-gap1 kindly provided by C. Brulant, Vandœuvres-les-Nancy, France; Brulant et al., 1983). The Plr, Plr-45, Plr-63 and *B. stearothermophilus* GAPDH proteins were soluble when expressed in *E. coli* and were purified by NAD+ affinity chromatography (Comer et al., 1975) as described previously (Winram & Lottenberg, 1996).

Protein preparations containing cell-wall-associated components of streptococci were prepared by digestion of the peptidoglycan using mutanolysin as described previously (Winram & Lottenberg, 1996). Purified streptokinase was a gift from Kabikvitrum.

**SDS-PAGE and protein blotting.** SDS-PAGE (10% acrylamide) was used to resolve proteins (Laemmli, 1970). Proteins were identified by staining with Coomassie brilliant blue. Proteins resolved on polyacrylamide gels were prepared for electrotransfer to nitrocellulose membranes by equilibration of the gels in 25 mM Tris/HCl, 0.2 M glycerine, pH 8.0, containing 20% (v/v) methanol. Proteins were electroblotted to nitrocellulose membranes using a Trans-Blot cell (Bio-Rad). Following transfer, membranes were soaked in NET-gel (50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA, pH 7.35) and 0.25% gelatin (VBS-gel). For 125I-labelled plasmin binding experiments, approximately 5000 c.p.m. 125I-labelled plasmin ml⁻¹ NET-gel with nitrocellulose membranes containing proteins of interest for 1 h at room temperature. The 125I-labelled plasmin had a minimum specific activity of 8 x 10⁷ c.p.m. mg⁻¹. Membranes were then washed three times with NET-gel and exposed to autoradiography film overnight at -70 °C.

**GAPDH activity assays of cell lysates.** Bacterial cultures were centrifuged, the bacterial pellets were washed twice with 50 mM potassium phosphate, pH 6.0, and the bacteria finally resuspended in 2 ml phosphate buffer per g bacterial pellet (wet wt). Cells were lysed by two passages through a French pressure cell at 18000 p.s.i. (124 MPa). Unlysed bacteria were removed by centrifugation at 5000 g for 10 min at 4 °C, and insoluble debris was cleared by centrifugation at 33000 g for 30 min at 4 °C. Lysates were assayed for GAPDH activity following the protocol of Ferdinand (1964). Soluble lysate proteins in 50 µl volumes were added to 100 µl 20 mM dl-glyceraldehyde 3-phosphate (Sigma), 100 µl 10 mM NAD+ and 750 µl reaction buffer (40 mM triethanolamine, 50 mM Na₂HPO₄, 5 mM EDTA, pH 8.6). Negative control assays were performed as above but without the addition of dl-glyceraldehyde 3-phosphate. The reduction of NAD+ to NADH was monitored spectrophotometrically at 340 nm; absorbances were recorded at 20 s intervals for 4 min using a Beckman model DU-70 spectrophotometer. Absorbances were converted to nmol NADH using the molar absorption coefficient of 6.22 x 10⁴ mol⁻¹ cm⁻¹ (Horecker & Kornberg, 1948). Protein concentrations were determined using a bicinchoninic acid protein assay (Pierce) to calculate specific activities expressed as nmol NADH produced min⁻¹ (mg extract)⁻¹.

**Plasmin-binding assays using intact bacteria.** Bacteria were pelleted by centrifugation, washed three times with PBS and suspended to 20% (w/v; wet pellet wt) in Veronal-buffered saline (0.28 M NaCl, 10 mM sodium diethyl barbiturate, pH 7.35) and 0.25% gelatin (VBS-gel). For 125I-labelled plasmin binding experiments, approximately 50000 c.p.m. 125I-labelled plasmin in test tubes. The data are presented as the mean percent of counts bound. The percentages were calculated by subtracting the background counts of the ligand alone from the counts bound by the bacteria, divided by the total counts of ligand offered and multiplied by 100.

For assays using unlabelled plasmin, cells were prepared as described for the radiolabelled plasmin assays. Two hundred microlitres of VBS-gel containing 25 µg plasmin ml⁻¹ (~0.6 µM) was added to 200 µl bacteria and incubated at 37 °C for 1 h. Cells were washed as described above. Four hundred microlitres of VBS-gel containing 450 µM S-2251 (p-Val-Leu-Lys-p-nitroanilide; Pharmacia/Hoefer) was used to suspend washed pellets. The substrate S-2251 is cleaved by plasmin on the carboxyl side of the lysine to release p-nitro-
aniline. Samples were incubated for 1 h at 37 °C before the reaction was terminated by the addition of 400 μl 10% (v/v) acetic acid. Bacteria were pelleted by centrifugation and the absorbances of the supernatant fractions were measured with a spectrophotometer at 405 nm. Background activity was determined by adding 100 μl buffer without bacteria. Plasmin activity is represented as the mean of the absorbances minus the background activity.

Assays were also performed to measure the capture of surface plasmin activity by bacteria when grown in the presence of human plasma. One hundred microlitres of an overnight culture was used to inoculate 2 ml THY broth containing 30% (v/v) human plasma (Civitan Regional Blood System, Gainsville, FL) in borosilicate test tubes. Control samples consisted of THY broth without plasma inoculated with strain 64/14 and THY broth with plasma and no bacteria to measure non-specific background activity. All tubes were incubated for 6 h at 37 °C. Bacteria were washed three times and then normalized for growth rate by suspending pellets to an OD 600 of 0.6. The streptococci were then incubated in 400 μl VBS-gel containing 450 μM S-2251 for up to 2 h and the enzymic reaction terminated as described above. Data are presented as the means of the absorbances minus the background activity. Analysis of variance for each set of data was performed as described by Norusis (1990).

RESULTS

The plr gene appears to be essential

A definitive method for examining the role of Plr as a plasmin receptor in streptococci is to generate an isogenic mutant lacking expression of the plr gene. Insertional inactivation of the plr gene with a DNA cassette containing a kanamycin resistance (Km') marker was the first approach taken. Linear pRL027 DNA, which contained the plr ORF interrupted by a ΩKm' cassette, was introduced into strain 64/14 by electroporation (Fig. 1a) and transformants were selected on THY agar plates containing kanamycin. Circular pRL027 was also electroporated into strain 64/14 in separate experiments (Fig. 1b) to verify that strain 64/14 was transformable and that pRL027 could be integrated into the chromosome. In contrast to successful transformation with circular DNA, repeated attempts at integrating the linearized DNA failed to produce viable transformants, even though the plasmid contained over 1 kb of homologous strain 64/14 DNA both 5' and 3' to the ΩKm' cassette. Furthermore, DNA hybridization analysis of chromosomal DNA from an isolate transformed with circular pRL027 using both plr and ΩKm' cassette probes indicated that the plasmid had integrated directly downstream of the plr gene (data not shown). The inability to insertional inactivate plr suggested that this gene may be essential for viability in strain 64/14. Therefore, alternative strategies to generate an isogenic mutant were adopted.

The C-terminal lysine of Plr is required for wild-type levels of plasmin binding

An altered plr gene encoding a plasmin-binding-deficient, but glycolytically active, Plr protein would be a candidate for gene replacement and, thereby, circumvent the problems encountered when attempting to inactivate this essential gene. Accordingly, directed mutagenesis of the recombinant plr gene and characterization of the expressed products were undertaken to identify unique amino acid residues that mediate plasmin binding but are inessential for GAPDH activity. Western blotting techniques were utilized to assess the plasmin-binding activity of purified B. stearothermophilus GAPDH compared to recombinant Plr and purified streptokinase (Fig. 2). Interestingly, the anti-Plr antibody did not react with B. stearothermophilus GAPDH, even though this protein has 53% identity with Plr at the predicted amino acid sequence level.

As expected from previously published studies, streptokinase bound both plasmin and Glu-plasminogen (Broder et al., 1991). In contrast, Plr bound Lys-plasmin but failed to bind significant amounts of Glu-plasminogen. The B. stearothermophilus GAPDH did not bind Lys-plasmin or Glu-plasminogen in this assay, indicating that specific regions are required for binding plasmin which are unique to Plr.

C-terminal lysyl residues in proteins such as α2-antiplasmin have been demonstrated to mediate the binding to plasminogen (Sugiyama et al., 1988). The
Mutational analysis of Plr

Fig. 2. Western blot analysis and plasmin(ogen)-binding of B. stearothermophilus GAPDH compared to streptokinase and Plr. Proteins were separated by electrophoresis on quadruplicate SDS-polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize proteins (a), while the proteins on the other three gels were electroblotted onto nitrocellulose and probed with anti-Plr antibody (b), 125I-labelled plasmin (c), or 125I-labelled Glu-plasminogen (d). In all cases, lane 1 contains streptokinase, lane 2 contains Plr and lane 3 contains recombinant B. stearothermophilus GAPDH.

streptococcal Plr has a lysyl residue at the C-terminus (position 334) (Lottenberg et al., 1992a). To assess the contribution of this residue to the plasmin-binding phenotype of Plr, a plr mutation was generated to yield a gene product, Plr-45, which contained a leucine in place of the C-terminal LYS\textsuperscript{334}. Additionally, a mutant Plr molecule, Plr-63, which possessed the C-terminus of the B. stearothermophilus GAPDH (Branlant et al., 1983) was tested. The plr-63 gene contained codons for glycine and leucine downstream of the plr wild-type terminal lysine codon. The purified proteins were compared to Plr for their ability to bind plasmin (Fig. 3). The reduction of plasmin binding of Plr-45 and Plr-63 relative to wild-type Plr demonstrated the requirement for the presence of an exposed C-terminal lysyl residue for binding. In contrast, substitution of the penultimate lysyl residue at amino acid position 330 of Plr with a leucine had no effect on plasmin binding (data not shown).

A vector was designed so that mutations of plr could replace the wild-type streptococcal plr gene on the chromosome and be expressed utilizing the native promoter. The construction of pSW027 is outlined in Fig. 4. The plr-45 and plr-63 ORFs were cloned into the pSW027 integration plasmid to yield pSW045 and pSW063 (see Methods).

Both Plr-45 and Plr-63 remained soluble when expressed in an E. coli host and were therefore assayed for glycolytic activity. A soluble lysate of E. coli \( \chi \)2602(pSW027) had a GAPDH activity of 1.7 \( \mu \)mol NADH produced min\(^{-1}\) (mg extract\(^{-1}\)) relative to a specific activity of 100.5 \( \mu \)mol NADH min\(^{-1}\) (mg extract\(^{-1}\)) for E. coli \( \chi \)2602(pSW025), which expresses Plr. E. coli \( \chi \)2602(pSW045) and E. coli \( \chi \)2602(pSW063) had specific activities of 72.4 and 26.8 \( \mu \)mol NADH produced min\(^{-1}\) (mg extract\(^{-1}\)), respectively, which indicated that both Plr-45 and Plr-63 retained GAPDH activity. Therefore, the genes encoding mutant Plr products were candidates for the replacement of plr on the streptococcal chromosome.

Introduction of plr mutations into group A streptococci

The linearized plasmids pSW045 and pSW063 were individually introduced into strain 64/14 by electroporation, which resulted in successful mutagenesis of the wild-type plr gene via double crossover events as depicted in Fig. 5(a). Integration of the two different 3' end mutations, plr-45 and plr-63, at the plr locus was verified in isolates by DNA hybridization analysis of chromosomal DNA probed with both the plr gene and \( \Omega \)Km\(^{\prime} \) cassette DNA (shown for plr-63 in Fig. 5b, c). The 5.5 kb fragment shown in lanes 2-5, containing pSW063 integrated into the chromosome, was the predicted size of the wild-type 3.3 kb fragment plus the integrated 2.3 kb \( \Omega \)Km\(^{\prime} \) cassette (Fig. Sb). Additionally, the presence of the \( \Omega \)Km\(^{\prime} \) cassette in these isolates was verified by the reactivity of a 5.5 kb band with a \( \Omega \)Km\(^{\prime} \) cassette probe (Fig. 5c). The introduction of the Smal site at the 3' end of the plr-63 ORF (see Fig. 5a) was verified in the SalI/Smal-digested chromosomal DNA of Fig. 5(c). The 5.5 kb hybridizing fragment in Fig. 5(c) represents incompletely digested DNA. Parallel experiments were performed with chromosomal DNA from isolates transformed with pSW045 (data not shown). The predicted changes in the 3' ends of plr in these strains was confirmed by DNA sequence analysis. The results confirmed the precise introduction of both plr-45 and plr-43 mutations at the plr locus on the chromosome and the presence of the \( \Omega \)Km\(^{\prime} \) cassette downstream of the plr mutations. Bacteria containing the plr-45 and plr-63 mutations were designated strain 64/14k-45 and strain 2029.
Fig. 3. Recombinant C-terminal Plr mutants, Plr-45 and Plr-63, have reduced plasmin-binding activity. Samples were electrophoresed on duplicate reducing SDS-polyacrylamide (10%) gels. One gel was stained with Coomassie brilliant blue to visualize proteins (a). Proteins resolved on the other gel were transferred to a nitrocellulose membrane, blocked and reacted with 125I-labelled plasmin (b). In both cases, lane 1 contains Plr, lane 2 contains Plr-45, which has the C-terminal lysyl residue substituted with a leucine, and lane 3 contains Plr-63, with the C-terminus consisting of residues lysine, glycine and leucine.

64/14k-63, respectively. A control strain, designated 64/14k, was also generated that contains the \(\Omega\)Km\(^r\) cassette downstream of the \(\text{plr}\) gene.

Mutant streptococcal strains bind wild-type levels of plasmin

The 41 kDa protein in mutanolysin extracts prepared from mutant streptococcal strains was assessed for plasmin-binding ability by the ligand blot assay. Plr and the mutated Plr proteins migrated at \(\sim\) 41 kDa on Coomassie brilliant blue stained polyacrylamide gels and corresponded to the immunoreactive proteins identified with anti-Plr antibody in Western blots (Fig. 6a, b). The \(\text{plr}-45\) and \(\text{plr}-63\) mutations introduced into the streptococci yielded expressed products that showed reduced plasmin binding (Fig. 6c), as observed for these recombinant proteins expressed in \(E.\) coli. As expected, mutanolysin-released proteins from these strains did not bind significant amounts of Glu-plasminogen (Fig. 6d).

The isogenic strains 64/14k-45 and 64/14k-63, which expressed plasmin-binding-deficient Plr, were assayed for the ability to bind plasmin to the bacterial surface compared to wild-type strain 64/14 using several in vitro binding assays. Streptococcal strain 64/14k was also included to determine the effects, if any, of the \(\Omega\)Km\(^r\) cassette inserted downstream of the \(\text{plr}\) locus, or the expression of the Km\(^r\) gene product (or both) on plasmin binding. Replicate binding experiments using intact bacteria and \(^{125}\)I-labelled plasmin were performed and yielded consistent results. Data are presented for a representative set of assays (Table 2). Strain 64/14k bound a maximum of 30.5 % of the \(^{125}\)I-labelled-plasmin offered, whereas the isogenic mutant strains with altered \(\text{plr}\) genes bound 27.2 % and 28.9 %, for strain 64/14k-45 and strain 64/14k-63 respectively. These means were compared using analysis of variance (SPSS PC) for each set of data, using an \(\alpha\) value of 0.05. The analyses revealed no statistically significant differences between the means of the control strain, 64/14k, and those of the two isogenic mutant strains. These data indicate that mutations of the \(\text{plr}\) gene, which reduced the plasmin-
Mutational analysis of Plr

**Fig. 5.** Introduction of plr mutations into streptococcal strain 64/14. (a) Linearized plasmids were introduced individually into strain 64/14 and integrated into the streptococcal chromosome via the homologous recombination event depicted here. These events yielded the mutant strains 64/14k-45 and 64/14k-63. (b, c) DNA hybridization analysis of chromosomal DNA from strain 64/14k-63 transformants indicated integration of linear DNA via a double crossover. DNA was either digested with SalI or doubly digested with SalI and Smal as indicated, electrophoresed on 0.7% agarose gels and transferred to nylon membranes. The membranes were then hybridized with a [32P]dCTP-labelled probe consisting of either the plr ORF (b), or the 2.3 kb Km' cassette (c). Membranes were hybridized and washed at room temperature and subjected to autoradiography. Lane 1 contains strain 64/14 chromosomal DNA and lanes 2-5 contain chromosomal DNA from representative 64/14k-63 single-colony isolates.

**Fig. 6.** Plasmin-binding analysis of Plr molecules from strain 64/14 derivatives containing plr mutations. Mutanolysin-extracted proteins were separated by electrophoresis on quadruplicate reducing SDS-polyacrylamide (10%) gels. One gel was stained with Coomassie brilliant blue to visualize proteins (a). Proteins resolved on the other three gels were transferred to nitrocellulose membranes, blocked and reacted with anti-Plr antibody (b), 125I-labelled plasmin (c), or 125I-labelled Glu-plasminogen (d). In all cases, lane 1 contains mutanolysin extract from strain 64/14k, lane 2 contains extract from strain 64/14k-45 and lane 3 contains extract from strain 64/14k-63.

binding activity of purified mutant Plr in vitro, had no effect on the ability of intact bacteria expressing mutant Plr to bind pre-formed plasmin. As expected, there was no statistically significant difference in 125I-labelled Glu-plasminogen binding activity among strain 64/14k and the other isogenic mutant strains. All strains bound less than 4% of the total counts offered.

In addition to the above studies using 125I-labelled plasmin, the streptococcal strains were tested for the ability to bind unlabelled plasmin to control for any effects that radio-iodination of the plasmin may have had on its interaction with the bacteria. This assay required that the cell-bound plasmin retain proteolytic activity, to allow it to cleave the chromogenic substrate.
Table 2. Binding of 125I-labelled plasmin(ogen) to the streptococcal cell surface

Bacteria were incubated with 125I-labelled Lys-plasmin and 125I-labelled Glu-plasminogen. C.p.m. offered: 125I-labelled Lys-plasmin (39460 ± 156 c.p.m. offered; background activity 2553 ± 85); 125I-labelled Glu-plasminogen (37635 ± 1284 c.p.m. offered; background activity 1993 ± 115). n = 2, data are presented as means ± SD.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Bound Lys-plasmin (%)</th>
<th>Bound Glu-plasminogen (%)</th>
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<td>64/14</td>
<td>33.7 ± 2.2</td>
<td>3.4 ± 0.7</td>
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<tr>
<td>64/14k</td>
<td>30.5 ± 1.1</td>
<td>30 ± 0.5</td>
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<tr>
<td>64/14k-45</td>
<td>27.2 ± 0.2</td>
<td>20 ± 1.2</td>
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<tr>
<td>64/14k-63</td>
<td>28.9 ± 0.5</td>
<td>20 ± 0.2</td>
</tr>
<tr>
<td>x2602</td>
<td>8.2 ± 1.8</td>
<td>21 ± 0.2</td>
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</table>

Table 3. Binding of unlabelled plasmin to the streptococcal cell surface

Bacteria were grown in 30% human plasma. The A405 corresponds to the release of p-nitroaniline upon cleavage of the plasmin-specific substrate S-2251. The 'no-cells' row indicates S-2251 hydrolysis in the absence of added bacteria. Samples (n = 3) were normalised for growth by suspending bacteria to an equivalent OD405. Values are shown as means ± SD. The spectrophotometer was zeroed with strain 64/14 grown in THY alone.

<table>
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<th>Strain</th>
<th>S-2251 hydrolysis (A405)</th>
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<tr>
<td>No cells</td>
<td>0.11 ± 0.03</td>
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<td>64/14</td>
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<td>64/14k</td>
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<tr>
<td>64/14k-45</td>
<td>0.44 ± 0.01</td>
</tr>
<tr>
<td>64/14k-63</td>
<td>0.60 ± 0.13</td>
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S-2251. There was greater variability in plasmin binding among isolates as assessed by measuring enzymic activity compared to using 125I-labelled plasmin (data not shown). However, consistent with the binding data obtained using radiolabelled ligand, there appeared to be no reduction in plasmin binding for strains 64/14k-45 and 64/14k-63 containing plr mutations, relative to strain 64/14k.

The isogenic streptococcal strains were also tested using a third technique which was perhaps more representative of the in vivo environment. The assay allows assessment of plasmin-like activity which had been captured by the bacteria during growth in the presence of human plasma (Table 3). Proteolytic activity was again assessed by hydrolysis of S-2251. The means of the absorbance for wild-type strain 64/14, the control strain 64/14k, and the isogenic mutant strains, 64/14k-45 and 64/14k-63, were compared using analysis of variance (SPSS PC) for each set of data, using an α value of 0.05. Consistent with the results of the previous plasmin-binding studies, there were no statistically significant differences among the means of the control strains 64/14k and the two isogenic mutant strains for surface-bound plasmin/plasminogen-activator activity.

**DISCUSSION**

The activation of host plasminogen and plasmin(ogen) binding by group A streptococci has been hypothesized to contribute to the invasive potential of this bacterium (Lottenberg et al., 1994; Boyle & Lottenberg, 1997). To test this hypothesis, bacterial receptors for plasmin(ogen) should be identified. Therefore, these studies were undertaken to examine whether a putative plasmin receptor of group A streptococci, Plr, functions as such in vivo.

There are two forms of plasmin(ogen), which have different binding affinities for potential ligands. The native N-terminal amino acid of secreted plasminogen is glutamic acid (Glu-plasminogen). Plasmin will cleave the peptide bond between lysine and lysine of Glu-plasminogen to form Lys-plasminogen (Castellino, 1995). The conversion of Glu-plasminogen to Lys-plasminogen results in a dramatic conformational change. Many group A streptococcal isolates, including strain 64/14, specifically bind Lys-plasminogen to a greater extent than Glu-plasminogen. Although there are streptococcal species which bind both plasmin and Glu-plasminogen, albeit with different affinities, these strains express specific M-protein serotypes which are reported to be the lower-affinity receptor for Glu-plasminogen (Kuusela et al., 1992). Additionally, a 43 kDa plasmin(ogen)-binding group A streptococcal M-like protein (PAM) has been identified from M-serotypes 33, 41, 52 and 56 (Berge & Sjöbring, 1993; Wistedt et al., 1995). Streptococcal strain 64/14 is an M-untypable strain; however, the DNA sequences of the genes encoding the M-like proteins of this strain have been determined (Boyle et al., 1994) and the gene products do not bind Lys-plasmin or Glu-plasminogen (M. D. P. Boyle, personal communication). Identification of a plasmin receptor(s) of group A streptococci has been one of the primary foci of our laboratory.

Previously, the plasmin-binding protein, subsequently identified as Plr, was isolated from mutanolysin extracts of group A streptococcal strain 64/14 (Broder et al., 1991). Interestingly, Plr is a functional GAPDH molecule and is encoded by a single gene on the streptococcal chromosome (Winram & Lottenberg, 1996). GAPDH is a tetrameric enzyme of the glycolytic pathway responsible for the phosphorylation of glyceraldehyde 3-phosphate to generate 1,3-bisphosphoglycerate (Harris & Waters, 1976). Recently, D'Cotta et al. (1997) provided evidence for surface localization of Plr on group A streptococcus strain 64/14 by immunoelectron microscopy. A biotinylated anti-Plr monoclonal IgM antibody was reacted with strain 64/14 and detected with avidin-conjugated gold particles. An isotypic
matched antibody was used as a negative control. Surface dehydrogenase, also from group A streptococci, is a similar molecule to Plr (Winram & Lottenberg, 1996) and was reported to be localized on the streptococcal surface by immunoblotting using an affinity-purified rabbit polyclonal antibody (Pancholi & Fischetti, 1992). Additionally, Eichenbaum et al. (1996) reported that Plr was released from group A streptococci into culture supernatants under conditions of iron starvation. GAPDH molecules have also been identified on the surface of eukaryotic organisms (Goudot-Crozel et al., 1989; Gil-Navarro et al., 1997; Fernandes et al., 1992).

The plr gene appears to be essential, as has also been postulated for the homologous gene in Streptococcus equisimilis (Gase et al., 1996). Therefore, a series of experiments was performed to generate a mutated gene encoding a non-plasmin-binding mutant Plr molecule which retained GAPDH activity.

The deduced amino acid sequence of Plr revealed the presence of lysyl residues at the C-terminus that we hypothesized could potentially mediate the reversible binding of plasmin observed for intact streptococci and purified Plr (Lottenberg et al., 1992a). The amino acid lysine can efficiently elute plasmin from the surface of group A streptococci. Lysine and lysine analogues also prevent the association of plasmin with the bacterial surface (Broeseker et al., 1988). This inhibition of binding occurred in a concentration-dependent manner that suggested involvement of the high-affinity lysine-binding site of plasmin. Furthermore, the domain of plasmin(ogen) facilitating the interaction is localized to the heavy chain which contains the lysine-binding sites (Broder et al., 1989). Consistent with this hypothesis, the analysis of mutated recombinant Plr proteins revealed that the C-terminal lysine of Plr was necessary for wild-type levels of binding. Interactions of plasmin(ogen) with a C-terminal lysyl residue have previously been reported for cyanogen-bromide-generated fibrinogen fragments (Christensen, 1985), the physiological inhibitor α-2-antiplasmin (Wiman et al., 1979) and for putative eukaryotic plasmin(ogen) receptors (Miles et al., 1991; Hajjar, 1993). In examining plasminogen binding to monocytoid U937 cells, Miles et al. (1991) used a series of synthetic peptides to inhibit Glu-plasminogen from binding to the cell surface. Peptides containing a C-terminal lysyl residue were essentially as effective in the inhibition of plasminogen binding as peptides which contained both a C-terminal and an internal lysine. Peptides which did not contain lysyl residues or only contained an internal lysine would not efficiently inhibit plasminogen binding.

Plr bound plasmin with a greater avidity than Glu-plasminogen, which suggested that the penultimate lysine of Plr may not interact with the low-affinity lysine binding site of plasmin(ogen) as do internal lysyl residues in proteins such as α-2-antiplasmin (Hortin et al., 1989) and the streptococcal plasmin(ogen)-binding group A streptococcal M-like proteins (Wistedt et al., 1995). This was confirmed by construction of a mutated Plr with a leucine substitution of only the penultimate lysyl residue at position 330, which maintained the ability to bind wild-type levels of plasmin (data not shown). Two individual plr mutations, which yielded products possessing GAPDH activity and reduced plasmin-binding activity relative to Plr, were subsequently integrated individually into the streptococcal chromosome. This resulted in isogenic mutant strains that expressed mutated Plr (see Fig. 6). The reduced plasmin-binding phenotype of mutant Plr recovered from mutanolysin extracts of these strains was identical to that of the corresponding purified recombinant proteins expressed in E. coli.

Intact isogenic strains were compared with wild-type streptococcal strain 64/14 for differences in the ability to bind plasmin on the bacterial surface. There were no differences in the ability of mutant strains to bind pre-formed plasmin. An alternative assay requiring that the bacteria, grown in the presence of plasma, generate plasminogen-activator activity and subsequently capture plasmin activity on the surface also demonstrated no difference. Each technique for assessing plasmin binding or capture of plasmin-like activity by streptococci revealed that expression of mutant Plr molecules with alterations of the C-terminal lysine did not affect the ability of the intact streptococci to bind wild-type levels of plasmin.

The interaction of plasmin(ogen) with a wide spectrum of bacteria as well as many eukaryotic cells appears to be through the lysine-binding site of the plasminogen molecule (Broder et al., 1989; Ullberg et al., 1990; Miles et al., 1991; Nakajima et al., 1994). This binding interaction may be mediated through C-terminal lysyl residues on receptor proteins. In certain cases, such as SW1116 cells, the proteolytic action of plasmin or trypsin will uncover lysyl residues that provide additional plasmin(ogen) receptors (Camacho et al., 1989). In contrast, however, there was a 60% decrease in plasmin binding of trypsin-treated strain 64/14 compared to untreated bacteria or bacteria incubated with aprotinin-inactivated trypsin (data not shown). It was, therefore, likely that surface proteins play a significant role in plasmin binding, although non-proteinaceous structures, such as peptidoglycan or lipoteichoic acid, could also contribute to binding. There are potentially multiple plasmin-binding structures on the surface of group A streptococci.

The successful in vivo expression of mutations in the plasmin-binding domain of Plr represented the closest equivalent to a genetic knockout of this essential gene. The findings of this investigation indicate that streptococcal Plr does not function as a unique plasmin receptor. It may be that the plasmin-binding domain of Plr is not accessible to plasmin on the intact bacterium and that other molecules are totally responsible for the plasmin-binding phenotype of group A streptococci. Surface localization studies of mutant Plr in the isogenic strains, such as those performed by D’Costa et al. (1997), could help delineate the precise role of wild-type Plr.
To date, the generation of isogenic mutants of bacteria deficient in plasmin(ogen) binding has not been reported; our studies suggest that the task may be formidable. However, alternative approaches, such as testing strains lacking plasminogen-activator activity in an appropriate animal model and the use of transgenic mice deficient in plasminogen production, are possible and have already provided information pertaining to the pathogenesis of *Yersinia pestis* and *Borrelia burgdorferi* (Sodiende et al., 1992; Coleman et al., 1997).

ACKNOWLEDGEMENTS

This work was done during the tenure of a graduate student fellowship of the American Heart Association, Florida Affiliate (SBW) and supported by research grant HL-41898 from the National Institutes of Health.

REFERENCES


Received 7 October 1997; revised 20 February 1998; accepted 26 March 1998.