Site-directed mutagenesis of streptococcal plasmin receptor protein (Plr) identifies the C-terminal Lys³³⁴ as essential for plasmin binding, but mutation of the *plr* gene does not reduce plasmin binding to group A streptococci

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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 *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 insertional inactivation of the *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 *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 *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 *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 *et al.*, 1992b). This surface-bound proteolytic activity is no longer inhibitable by physiological plasmin inhibitors such as α -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.

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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 Plr exhibited identity to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and, moreover, Plr was shown to be an enzymically active GAPDH molecule (Winram & Lottenberg, 1996). Biochemical and functional analyses detected no differences between Plr and cytoplasmic 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 Plr. Additionally, amino acid composition profiles of Plr and SDH were similar (Winram & Lottenberg, 1996). These results indicated that Plr and SDH were structurally similar proteins.

In the present investigation, the contribution of Plr 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* χ 2602 [F⁻ e14⁻ (McrA⁻) *hsdR514*(r⁻, m⁺) *supE44* *supF58* Δ (*lacIZY*)6 *galK2* *galT22* *metB1* *trpR55*] 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 μ g 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 (Ω Km^r) gene cassette (Fellay *et al.*, 1987) were grown in the presence of 300 μ g kanamycin ml⁻¹ in THY broth or 500 μ g 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 *Bam*HI–*Hind*III insert of pRL024 (Winram & Lottenberg, 1996) was excised, blunt-ended (by Klenow fill-in) and subcloned into *Pvu*II-digested pACYC184 to generate pRL026. The putative promoter of the *plr* gene transcribes in the opposite direction to the Tet^r promoter of pRL026. To interrupt expression of Plr, a 2.3 kb *Eco*RI fragment containing the Ω Km^r gene cassette was blunt-ended and subcloned into the unique *Pvu*II 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-CAATAACTACCATGGGCC-3' and the reverse primer was 5'-CGCGGATCCTTAAAGAGCAATTTTTGCGAAGTA-CTCAAG-3'. The amplified product was ligated into the vector pT7Blue (Novagen). The *plr*-45 insert was excised by digestion with *Nco*I and *Bam*HI 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'-CGCC-CGGGTTAAAGACCTTTAGCAATTTTTGCGAAGT-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

Strain	Description
64/14	M-untypable clinical isolate that has been previously mouse-passaged 14 times (Reis <i>et al.</i> , 1984)
64/14k	A derivative of 64/14, harbouring the Ω Km ^r cassette on the chromosome approximately 400 bp downstream of the <i>plr</i> gene at the former <i>Bam</i> HI site; the cassette was introduced by electroporation of linear DNA and there are no integrated vector sequences
64/14k-45	Replacement of the <i>plr</i> gene with <i>plr</i> -45, which has the terminal lysine codon replaced with a leucine codon; the Ω Km ^r cassette is located approximately 400 bp downstream of the <i>plr</i> gene at the former <i>Bam</i> HI site
64/14k-63	Replacement of the <i>plr</i> gene in 64/14 with <i>plr</i> -63, which has an additional glycine codon and a leucine codon immediately 3' to the wild-type terminal lysine codon; the Ω Km ^r cassette is located in the same position as in strain 64/14k and strain 64/14k-45

Construction of the *plr* gene replacement vector. The construction of plasmid pSW025 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 *AvaI*, blunt-ended and then digested with *XmnI*. Ligation of this digested vector with the insert generated pSW025. The *plr* ORF of pSW025 was precisely deleted and replaced with *NcoI* and *SmaI* cloning sites by performing inverse PCR on pSW025, using the primers 5'-CGCCCGGGTATCCATGGATGAT-TTCCTCCTTATGAAAAT-3' and 5'-CGCCCGGGTTAG-TTATAACGAAAGAGAGCT-3'. The amplified product was digested with *SmaI* and recircularized to generate pSW026. The plasmid pSW026 was partially digested with *BamHI* and the protruding DNA overhangs were blunt-ended. The 2.3 kb Ω Km^r cassette was excised from plasmid pRL027 by *BamHI* digestion and the overhangs were blunt-ended. The Ω Km^r cassette fragment was then ligated with the linearized pSW026 to yield pSW027.

The *plr*-45 insert of pSW045 was prepared by digesting pRL045 with *BamHI*, blunt-ending the overhangs and then digesting with *NcoI*. This insert was cloned into *NcoI*- and *SmaI*-digested pSW027. The *NcoI*- and *SmaI*-digested *plr*-63 insert was cloned into the *NcoI* and *SmaI* 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* χ 6060(pBst-gap1) (pBst-gap1 kindly provided by C. Branlant, Vandoeuvre-les-Nancy, France; Branlant *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 Kabivitrum.

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 electro-transfer to nitrocellulose membranes by equilibration of the gels in 25 mM Tris/HCl, 0.2 M glycine, 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, 0.05%, v/v, Triton X-100 and 0.25%, w/v, gelatin) prior to incubation with either primary antibody or ¹²⁵I-labelled plasmin.

Polyclonal mouse anti-Plr antibody (Broder *et al.*, 1991) was used as primary antibody, followed with goat anti-mouse IgG (Organon Teknika) as secondary antibody, which was detected with ¹²⁵I-labelled protein G (Sigma). Human Glu-plasminogen (American Diagnostica) was converted to plasmin using urokinase (Sigma) as described previously (Broder *et al.*, 1991), immediately prior to incubation with blots. Plasmin ligand blots were performed by incubating

approximately 50000 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 8×10^{11} c.p.m. mg⁻¹. Membranes were then washed three times with NET-gel and exposed to autoradiography film overnight at -70 °C.

Protein G and plasminogen were radiolabelled with ¹²⁵INa (Amersham) by a lactoperoxidase reaction using enzymobeads (Bio-Rad); labelled proteins were separated from free label by gel filtration using a PD-10 column (Pharmacia Biotech).

GAPDH activity assays of cell lysates. Bacterial cultures were centrifuged, the bacterial pellets 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 μ l volumes were added to 100 μ l 20 mM DL-glyceraldehyde 3-phosphate (Sigma), 100 μ l 10 mM NAD⁺ and 750 μ l 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 μ mol NADH using the molar absorption coefficient of 6.22×10^3 l mol⁻¹ cm⁻¹ (Horecker & Kornberg, 1948). Protein concentrations were determined using a bicinchoninic acid protein assay (Pierce) to calculate specific activities expressed as μ mol 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 100 μ l was added to 100 μ l of the cell suspensions in borosilicate test tubes. Samples were mixed and incubated at 37 °C for 1 h. Two millilitres of VBS-gel was added to each tube and the cells were then pelleted by centrifugation at 2000 g for 5 min, the supernatant fraction aspirated, and the pellet resuspended in 2 ml VBS-gel. This washing procedure was repeated twice more before the bacterial pellets were assayed in a Beckman Gamma 4000 gamma counter. Background counts were determined by adding 100 μ l buffer without bacteria to 100 μ l ¹²⁵I-labelled plasmin in test tubes. The data are presented as the mean percentage 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 μ g plasmin ml⁻¹ (~ 0.6 μ M) was added to 200 μ l bacteria and incubated at 37 °C for 1 h. Cells were washed as described above. Four hundred microlitres of VBS-gel containing 450 μ M S-2251 (D-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 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, Gainesville, 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 OD₆₀₀ 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*^r) marker was the first approach taken. Linear pRL027 DNA, which contained the *plr* ORF interrupted by a Ω *Km*^r 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 Ω *Km*^r cassette. Furthermore, DNA hybridization analysis of chromosomal DNA from an isolate transformed with circular pRL027 using both *plr* and Ω *Km*^r cassette probes indicated that the plasmid had integrated directly downstream of the *plr* gene (data not shown). The inability to insertionally 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, cir-

Fig. 1. Potential recombination events resulting from electroporation of plasmid pRL027 into strain 64/14. (a) Electroporation of linearized pRL027 would potentially yield a double crossover event by homologous recombination, indicated by the arrows, resulting in inactivation of the chromosomal *plr* gene. (b) Circular pRL027 is integrated via a single crossover event occurring downstream of the *plr* gene on the chromosome and therefore a full-length copy of the *plr* gene is retained.

cumvent 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. stearo-thermophilus* GAPDH compared to recombinant Plr and purified streptokinase (Fig. 2). Interestingly, the anti-Plr antibody did not react with *B. stearo-thermophilus* 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. stearo-thermophilus* 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 α -2-antiplasmin have been demonstrated to mediate the binding to plasmin(ogen) (Sugiyama *et al.*, 1988). The

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), 125 I-labelled plasmin (c), or 125 I-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 Lys³³⁴. 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* χ 2602(pSW027) had a GAPDH activity of $1.7 \mu\text{mol NADH produced min}^{-1} (\text{mg extract})^{-1}$ relative to a specific activity of $100.5 \mu\text{mol NADH min}^{-1} (\text{mg extract})^{-1}$ for *E. coli* χ 2602(pSW025), which expresses Plr. *E. coli* χ 2602(pSW045) and *E. coli* χ 2602(pSW063) had specific activities of 72.4 and 26.8 $\mu\text{mol NADH produced min}^{-1} (\text{mg extract})^{-1}$, 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 ΩKm^r 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 ΩKm^r cassette (Fig. 5b). Additionally, the presence of the ΩKm^r cassette in these isolates was verified by the reactivity of a 5.5 kb band with a ΩKm^r cassette probe (Fig. 5c). The introduction of the *Sma*I site at the 3' end of the *plr-63* ORF (see Fig. 5a) was verified in the *Sal*I/*Sma*I-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-63* mutations at the *plr* locus on the chromosome and the presence of the ΩKm^r cassette downstream of the *plr* mutations. Bacteria containing the *plr-45* and *plr-63* mutations were designated strain 64/14k-45 and strain

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 ^{125}I -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 ΩKm^r cassette downstream of the *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 ~ 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 *plr-45* and *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 ΩKm^r cassette inserted downstream of the *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

Fig. 4. Plasmid construction for the replacement of the wild-type *plr* gene with *plr-45* and *plr-63* mutations in strain 64/14. The *EcoRI* fragment of pRL015 containing the *plr* gene and streptococcal flanking DNA sequences was subcloned into pACYC184 to yield plasmid pSW025. Inverse PCR was used to delete the *plr* ORF, leaving flanking regions intact in pSW026. Partial *Bam*HI digestion of pSW026 allowed ligation of the ΩKm^r cassette into the streptococcal homologous sequences resulting in plasmid pSW027. The *plr-45* and *plr-63* ORFs were subcloned into pSW027 to yield plasmids pSW045 and pSW063, respectively (see Methods for details). Abbreviations: E, *EcoRI*; B, *Bam*HI; N, *Nco*I; Sm, *Sma*I; X, *Xmn*I; A, *Ava*I; P, putative promoter elements; RBS, ribosome-binding site; Km^r , kanamycin resistance gene; cm^r , chloramphenicol resistance gene; tet^r , tetracycline resistance gene.

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 *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 α 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 *plr* gene, which reduced the plasmin-

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 *Sal*I or doubly digested with *Sal*I and *Sma*I 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 Ω Km^r 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 ^{125}I -labelled plasmin(ogen) to the streptococcal cell surface

Bacteria were incubated with ^{125}I -labelled Lys-plasmin and ^{125}I -labelled Glu-plasminogen. C.p.m. offered: ^{125}I -labelled Lys-plasmin (39460 ± 156 c.p.m. offered; background activity 2553 ± 85); ^{125}I -labelled Glu-plasminogen (37635 ± 1284 c.p.m. offered; background activity 1993 ± 113). $n = 2$, data are presented as means $\pm$ SD.

Strain	Bound Lys-plasmin (%)	Bound Glu-plasminogen (%)
64/14	33.7 ± 2.2	3.4 ± 0.7
64/14k	30.5 ± 1.1	3.0 ± 0.5
64/14k-45	27.2 ± 0.2	2.0 ± 1.2
64/14k-63	28.9 ± 0.5	2.0 ± 0.2
$\chi 2602$	8.2 ± 1.8	2.1 ± 0.2

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

Bacteria were grown in 30% human plasma. The A_{405} 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 normalized for growth by suspending bacteria to an equivalent OD_{600} . Values are shown as means $\pm$ SD. The spectrophotometer was zeroed with strain 64/14 grown in THY alone.

Strain	S-2251 hydrolysis (A_{405})
No cells	0.11 ± 0.03
64/14	0.52 ± 0.03
64/14k	0.51 ± 0.07
64/14k-45	0.44 ± 0.01
64/14k-63	0.60 ± 0.13

S-2251. There was greater variability in plasmin binding among isolates as assessed by measuring enzymic activity compared to using ^{125}I -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 lysine⁷⁷ and lysine⁷⁸ of Glu-plasmin(ogen) to form Lys-plasmin(ogen) (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-plasmin(ogen) 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 & Sjöbrink, 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'Costa *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).

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