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The plasmin-binding protein Plr of group A streptococci is identified as glyceraldehyde-3-phosphate dehydrogenase

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Group A streptococci bind the serine protease plasmin with high affinity. Previously, a 41 kDa protein was identified as a candidate plasmin receptor protein (Plr) from group A streptococcal strain 64/14. The *plr* gene encoding Plr was cloned and the deduced amino acid sequence of Plr had significant similarity to glyceraldehyde-3-phosphate dehydrogenases (GAPDHs). In this study we have isolated cytoplasmic GAPDH of streptococcal strain 64/14. This enzyme was examined, on both structural and functional levels, for its relatedness to the Plr of strain 64/14 purified from mutanolysin extract and to recombinant Plr. We report here that no differences were detected between streptococcal Plr and cytoplasmic GAPDH on the basis of antibody reactivity, plasmin-binding activity, GAPDH activity, N-terminal amino acid sequence, peptide map analysis by V8 protease digestion and amino acid composition analysis. Furthermore, the *plr* gene appears to be present as a single copy in group A streptococci.

Keywords: glyceraldehyde-3-phosphate dehydrogenase, streptococci, plasmin

INTRODUCTION

Group A streptococci are Gram-positive bacteria capable of causing a variety of illnesses in humans, including highly invasive soft tissue infections (Stevens, 1992). The mechanisms utilized by the streptococci to rapidly traverse host tissue barriers have not yet been elucidated. One potential means is by interaction with the host plasminogen system (Lottenberg *et al.*, 1994). Plasminogen is converted to the active serine protease, plasmin, by either host or bacterial plasminogen activators such as urokinase and streptokinase, respectively. Our laboratory has demonstrated that group A streptococci can bind the enzyme plasmin with high affinity (Broeseker *et al.*, 1988). Once bound, the protease remains proteolytically active and can no longer be down-regulated by physiological plasmin inhibitors such as α-2-antiplasmin (Lottenberg *et al.*, 1987). Plasmin can degrade a wide range of substrates that comprise tissue planes and the extracellular matrix either directly or indirectly through activation of latent metalloproteases (Vassalli *et al.*, 1991). An unregulated protease bound to the streptococcal surface may provide a mechanism for these bacteria to degrade tissue barriers during invasive infections.

There is a wide range of both Gram-positive and Gram-negative bacteria that have been reported to bind plasmin(ogen) including group A, C and G streptococci, *Staphylococcus aureus*, *Proteus mirabilis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Escherichia coli* and *Borrelia burgdorferi* (Ullberg *et al.*, 1989, 1990, 1992; Kuusela & Saksela, 1990; Fuchs *et al.*, 1994). To determine the role of this phenotype in the pathogenesis of infections caused by these organisms, the plasmin(ogen)-binding components of the cell surface must first be identified. Several of the prokaryotic surface receptor proteins for plasmin(ogen) which have been reported thus far are flagella and several types of fimbriae of *E. coli* (Parkkinen & Korhonen, 1989; Lahteenmaki *et al.*, 1993), the OspA protein of *B. burgdorferi* (Fuchs *et al.*, 1994) and certain M-related proteins of group A, C and G streptococci (Berge & Sjobring, 1993; Ben Nasar *et al.*, 1994). In addition, a plasmin-binding protein (Plr) has been isolated from mutanolysin extracts of the group A streptococcal strain 64/14 by our laboratory (Broder *et al.*, 1991). The *plr* gene encoding this protein has been cloned, sequenced and

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Abbreviations: (c)GAPDH, (cytoplasmic) glyceraldehyde-3-phosphate dehydrogenase; GAP, glyceraldehyde 3-phosphate; (s,r)Plr, (streptococcal, recombinant) plasmin receptor protein.

expressed in *E. coli* (Lottenberg *et al.*, 1992). The predicted amino acid sequence of Plr exhibits 55% identity with *Bacillus subtilis* GAPDH.

The isolation and characterization of cytoplasmic glyceraldehyde-3-phosphate dehydrogenase (cGAPDH) from group A streptococci have not been reported. We wanted to address whether group A streptococci expressed more than one isoform of GAPDH, perhaps utilizing one isoform for glycolytic functions and another for receptor functions such as plasmin binding. Alternatively, the streptococci might possess a single GAPDH which could possibly perform functions in addition to glycolysis. In this study, we have examined the relatedness of streptococcal cytoplasmic GAPDH to Plr. Additionally, there might be a single gene or multiple genes from which GAPDH(s) could be expressed and therefore the gene copy number of *plr* was determined for strain 64/14 and for a series of group A streptococcal clinical isolates.

METHODS

Bacterial strains and growth conditions. Group A streptococcal strain 64/14 is an M-untypable clinical isolate that had been mouse-passaged 14 times previously (Reis *et al.*, 1984). Other group A streptococcal strains used in this study were clinical isolates described previously (Wang *et al.*, 1994). Streptococci were grown as standing cultures to stationary phase at 37 °C in Todd–Hewitt broth containing 0·3% yeast extract. *E. coli* strain χ6060 [F′(*traD36 proAB lacI*^{q} Δ*lacZM15*)::Tn*5* (Km^{r}) *araD139* Δ(*ara leu*) *7697* Δ*lacX74* Δ*phoA20 galE galK recA1 rpsE argE* (Am) *rpoB thi*] was used for transformation and gene expression (Lottenberg *et al.*, 1992). *E. coli* strain χ6060 bearing plasmid pRL024 was grown as a shaking culture to stationary phase at 37 °C in Luria broth with 30 μg chloramphenicol ml^{-1}.

Plasmids. The plasmid pRL015 contains a 2·7 kb DNA fragment isolated from group A streptococcal strain 64/14, which includes *plr* and its flanking chromosomal sequences (see Fig. 5), ligated into the *Eco*RI restriction site of the low-copy-number cloning vector pYA2204 (Galan *et al.*, 1988). A 2·3 kb *Bam*HI–*Hin*dIII fragment from the 2·7 kb insert of plasmid pRL015 was subcloned into the *Bam*HI–*Hin*dIII restriction sites of the medium-copy-number vector pACYC184 to generate plasmid pRL024.

Protein purification. Streptococcal Plr (sPlr) was isolated from a mutanolysin extract of strain 64/14 prepared by a modification of a previously described protocol (Broder *et al.*, 1991). Bacteria were pelleted by centrifugation, washed 3 times with PBS, pH 7·4, and resuspended in PBS containing 30% (w/v) raffinose to prevent cell lysis, 1 mM PMSF and 1 mM TLCK. Mutanolysin (Sigma) was added at a concentration of 180 U (g wet wt)$^{-1}$ of bacterial pellet to degrade the peptidoglycan cell wall. The suspension was mixed gently for 2 h at 37 °C to release cell-wall-associated components. Protoplasts were pelleted by centrifugation and the supernatant fraction was filtered through a 0·2 μm filter. Ammonium sulfate was added to the filtrate to 60% saturation and stirred slowly overnight at 4 °C. Precipitated proteins were removed by centrifugation at 15000 ***g*** for 10 min at 4 °C. The supernatant fraction was dialysed extensively in PBS and contained primarily sPlr.

Recombinant Plr (rPlr) was separated from the majority of *E. coli* proteins as described previously (Lottenberg *et al.*, 1992). Briefly, ammonium sulfate was added to the supernatant fraction of a French pressure cell lysate of *E. coli* strain χ6060(pRL024) to 55% saturation and stirred gently overnight at 4 °C. Precipitated proteins were pelleted by centrifugation at 15000 ***g*** for 10 min at 4 °C. rPlr remained predominantly in the supernatant fraction.

Streptococcal cytoplasmic GAPDH (cGAPDH) was purified from strain 64/14 protoplasts generated by mutanolysin treatment as described above. Protoplasts were washed three times with 10 mM potassium phosphate buffer, pH 6·8, and lysed by two passages through a French pressure cell. Unlysed protoplasts were removed by centrifugation at 8000 ***g*** for 10 min and the resulting supernatant fraction was subjected to ultracentrifugation at 30000 ***g*** for 30 min to remove the remaining insoluble material (referred to in the text as the insoluble fraction). cGAPDH was purified from the soluble cytoplasmic fraction by NAD^+ affinity chromatography (Comer *et al.*, 1975). NAD^+-agarose (Sigma) was hydrated in 10 mM potassium phosphate buffer, pH 6·8, washed extensively and loaded into a chromatography column. Protein extracts were incubated in the gel bed for 1·5 h at room temperature by end-over-end rotation. The gel bed was washed extensively with phosphate buffer to remove unbound proteins. Bound proteins were eluted from the NAD^+-agarose using 10 mM phosphate buffer, pH 6·8, containing 10 mM NAD^+ (Sigma).

The streptococcal 41 kDa protein used for amino acid sequencing and amino acid composition analysis was isolated from a whole cell extract of strain 64/14. Bacteria were incubated with mutanolysin and the mixture passed through a French pressure cell twice. Insoluble material was pelleted by centrifugation at 15000 ***g*** for 10 min at 4 °C. The supernatant fraction was loaded onto a NAD^+ affinity chromatography column (as described above) to isolate the 41 kDa protein.

SDS-PAGE and protein blotting. SDS-PAGE on 10% polyacrylamide gels 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, pH 8·0, containing 0·2 M glycine with 20% (v/v) methanol. Proteins were electrotransferred from gels to nitrocellulose membranes using a Trans-Blot cell (Bio-Rad). Membranes were soaked in NET-gel (50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA, 0·05% Triton X-100, 0·25% gelatin) prior to incubation with either primary antibody or ^{125}I-labelled plasmin.

Polyclonal mouse anti-sPlr antibody (Broder *et al.*, 1991) was used as primary antibody (Ab), followed with goat anti-mouse IgG (Organon Teknika) as secondary Ab which was detected with ^{125}I-labelled protein G (Sigma). Human glu-plasminogen (American Diagnostica) was converted to plasmin using urokinase (Sigma) as the plasminogen activator (Broder *et al.*, 1991) immediately prior to incubation with blots. Plasmin ligand blots were performed by incubating approximately 50000 c.p.m. ^{125}I-labelled plasmin (ml NET-gel)$^{-1}$ with nitrocellulose membranes containing proteins of interest for 1 h at room temperature. 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 Na^{125}I (Amersham) by a lactoperoxidase reaction using Enzymobeads (Bio-Rad) and labelled proteins were separated from free label by gel filtration using a PD-10 column (Pharmacia).

Peptide map analysis. Peptide maps were generated for sPlr,

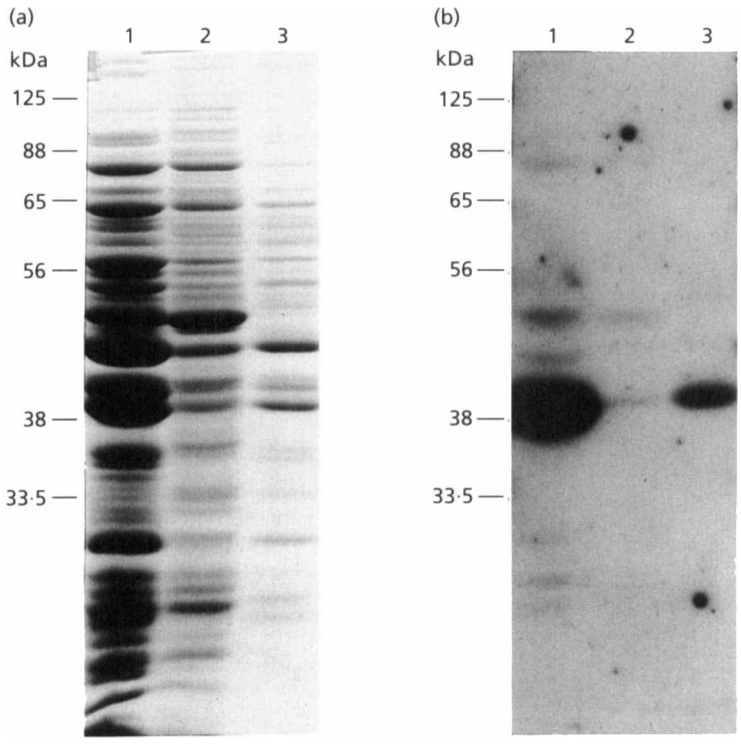

Fig. 1. Anti-sPlr antibody reactivity with strain 64/14 lysate fractions. Samples were subjected to SDS-PAGE on duplicate reducing 10% polyacrylamide gels. One gel was stained with Coomassie Brilliant Blue to visualize proteins (a) and the other gel was used to electroblot proteins to nitrocellulose membranes which were then reacted with mouse anti-sPlr polyclonal antibody raised against mutanolysin-extracted sPlr and goat anti-mouse IgG (b). ^{125}I-labelled protein G was used to detect bound antibody. The autoradiograph is shown in (b). Lanes: 1, soluble cytoplasmic fraction; 2, insoluble fraction; 3, mutanolysin-extracted proteins.

cGAPDH and rPlr with V8 protease following the method of Cleveland *et al.* (1977). Purified proteins were separated by SDS-PAGE on a 10% polyacrylamide gel. Protein bands were visualized by staining with Coomassie Brilliant Blue and the 41 kDa proteins excised from the gel. Gel slices were incubated for 30 min in buffer (0·125 M Tris/HCl, 0·1% SDS, 1 mM EDTA, pH 6·8). Gel slices were loaded into the wells of a SDS-polyacrylamide gel (4% stacking gel and a 15% separating gel) and overlaid with buffer containing 20% (v/v) glycerol and bromophenol blue. Five micrograms (15 U mg^{-1}) of V8 protease (Calbiochem–Novabiochem) in 10% glycerol was added to each well. Samples were concentrated in the stacking gel by electrophoresis and the proteolytic digestion was allowed to proceed for 3 h at room temperature before resuming electrophoresis. Following electrophoresis, proteins were visualized by staining with Coomassie Brilliant Blue.

Amino acid sequencing. The N-terminal amino acid sequence of sPlr has been determined previously (Lottenberg *et al.*, 1992). The 41 kDa protein isolated from the whole cell extract and rPlr were separated by electrophoresis on a SDS-polyacrylamide gel and electrotransferred onto Immobilon PVDF membrane (Millipore) in the presence of 10 mM MES, pH 6·0, in 20% methanol. The 41 kDa protein band from each preparation was excised and then subjected to microsequencing using automated Edman chemistry at the Interdisciplinary Center for Biomedical Research (ICBR), Protein Chemistry Core Laboratory, University of Florida, Gainesville, USA.

Amino acid composition. The amino acid composition was determined for the streptococcal 41 kDa protein and rPlr. Proteins were separated by electrophoresis on SDS-polyacrylamide gel and then electrotransferred onto Immobilon PVDF membrane. The 41 kDa proteins were visualized with Coomassie Brilliant Blue and excised from the membrane. Amino acid composition analysis was performed at the ICBR Protein Chemistry Core Laboratory. Proteins were hydrolysed in 6 M HCl with 1% (v/v) phenol for 22 h at 110 °C and amino acid composition was determined using the sodium buffer system with a Beckman System 6300 high performance analyser.

GAPDH assays. Purified proteins were assayed for GAPDH activity following the protocol of Ferdinand (1964). Purified proteins (50 µl) were added to 100 µl 20 mM DL-glyceraldehyde 3-phosphate (DL-GAP), 100 µl 10 mM NAD^+ and 750 µl reaction buffer (40 mM triethanolamine, 50 mM Na_2HPO_4, 5 mM EDTA, pH 8·6). Negative control assays were performed as above without the addition of DL-GAP. The reduction of NAD^+ to NADH was monitored spectrophotometrically at A_{340} at 20 s intervals for 4 min using a Beckman model DU-70 spectrophotometer. Absorbances were converted to µmol NADH using a molar absorption coefficient of $6{\cdot}22 \times 10^{-3}$ (Horecker & Kornberg, 1948). Protein concentrations were determined using a bicinchoninic acid protein assay (Pierce) to calculate specific activities expressed as µmol NADH min^{-1} mg^{-1}.

DNA hybridization. DNA hybridization was performed by the method of Southern (Sambrook *et al.*, 1989). Chromosomal DNA was isolated by the method outlined by Caparon & Scott (1991) and some preparations were further purified in CsCl gradients. Five micrograms of genomic DNA was used for restriction enzyme digestion. For strain 64/14, digested DNA was separated by electrophoresis in duplicate 0·7% (w/v) agarose gels and transferred to nylon membranes by a capillary blot procedure outlined in the manufacturer's instructions (Gene Screen Plus, Dupont). The DNA probe consisted of the 1 kb *plr* ORF which was amplified by PCR using plasmid pRL024 as DNA template. The probe was labelled with [^{32}P]dCTP (Amersham) using a random priming kit (USB). The probe was then incubated with the membranes in the presence of 10% (w/v) dextran sulfate, 50% (v/v) formamide and 0·5% SDS for 18 h at either room temperature (low stringency) or 55 °C (high stringency). Membrane washes were consistent with

hybridization temperatures of room temperature or 55 °C. Hybridizing bands were visualized by autoradiography. Southern blots of the 19 other streptococcal isolates were performed by the same method but with a hybridization temperature of 42 °C and the washes were performed at 65 °C.

RESULTS

cGAPDH of group A streptococci binds plasmin and is antigenically related to sPlr and rPlr

We first examined cellular fractions of streptococcal strain 64/14 proteins with anti-sPlr polyclonal antibody to identify proteins immunologically related to Plr. The antibody detected sPlr in the mutanolysin extract and an immunologically reactive protein band also migrating at 41 kDa in the cytoplasmic fraction as shown in Fig. 1. No other reactive proteins were detected in either the soluble cytoplasmic fraction or the mutanolysin extract. Additionally, the antibody did not detect any reactive proteins in the insoluble fraction (Fig. 1a and b, lane 2).

To determine if the anti-sPlr immunoreactive protein in the cytoplasmic fraction was a streptococcal GAPDH and to examine the relatedness of this protein to sPlr and rPlr, these proteins were purified and resolved on triplicate SDS-PAGE. One gel was stained with Coomassie Brilliant Blue to visualize proteins (Fig. 2a) and revealed that the predominant band migrated at 41 kDa for each preparation. The other two gels were electrotransferred to nitrocellulose and probed with either ^{125}I-labelled plasmin or polyclonal anti-sPlr antibody. As shown in Fig. 2b, all three 41 kDa proteins reacted with anti-sPlr antibody. Additionally, each of the proteins bound radiolabelled plasmin (Fig. 2c), demonstrating a functional similarity among them.

The proteins sPlr, cGAPDH and rPlr are similar

The three protein samples were then examined at the primary amino acid level to determine the extent of structural relatedness. To determine if similarities among sPlr, cGAPDH and rPlr extended to amino acid residue position, peptide maps were generated by V8 protease. V8 protease cleaves peptide bonds on the carboxylic side of aspartate and glutamate. Digested proteins were separated by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue. sPlr, cGAPDH and rPlr yielded identical size peptides (Fig. 3), revealing conservation of aspartate and glutamate residues throughout the sequence of the three proteins, as well as approximately the same number of amino acids between them.

N-terminal amino acid sequencing of rPlr and the streptococcal 41 kDa protein (from a whole-cell preparation) was performed to establish if similarities extended to the conservation of amino acid sequence. rPlr and the streptococcal 41 kDa protein revealed the identical N termini to that of the previously determined sequence of

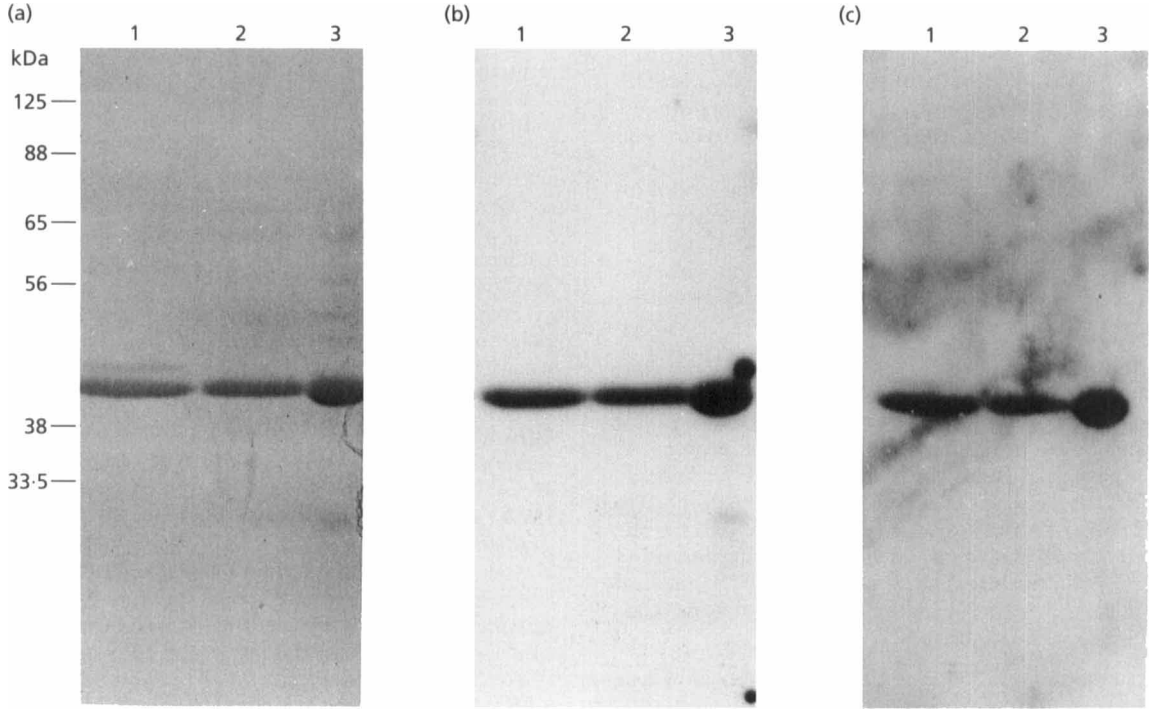

Fig. 2. Anti-sPlr antibody reactivity and plasmin-binding ability of purified sPlr, cGAPDH and rPlr. Samples were subjected to SDS-PAGE on triplicate reducing 10% polyacrylamide gels. One gel was stained with Coomassie Brilliant Blue to visualize proteins (a). The other two gels were electroblotted onto nitrocellulose membranes and the electroblots reacted with either mouse anti-sPlr antibody, goat anti-mouse IgG and ^{125}I-labelled protein G (b) or with ^{125}I-labelled plasmin (c). Panels (b) and (c) are autoradiographs. Lanes: 1, sPlr; 2, cGAPDH; 3, rPlr.

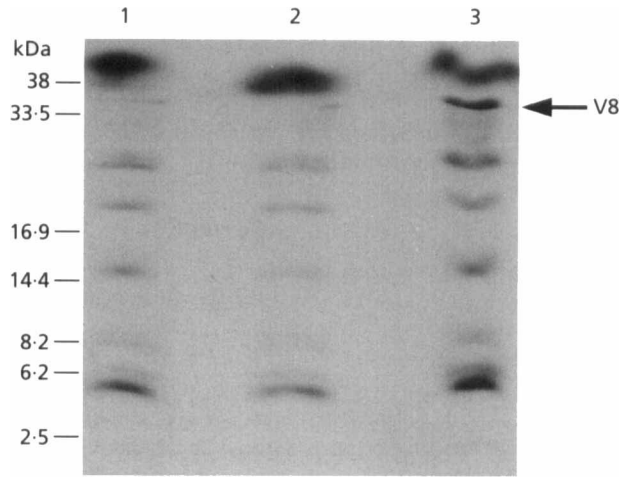

Fig. 3. Peptide map analysis of purified sPlr, cGAPDH and rPlr generated by staphylococcal V8 protease digestion. Purified proteins were prepared as described in Methods and digested with V8 protease in the gel. Peptides were separated by SDS-PAGE on a reducing 15% polyacrylamide gel and revealed with Coomassie Brilliant Blue staining. The V8 protease is indicated. Lanes: 1, sPlr; 2, cGAPDH; 3, rPlr.

sPlr. Unambiguous sequence was obtained for all samples. The amino acid sequence of the streptococcal 41 kDa protein was: VVKVGINGFGRIGRLAFRRI. Valine was also the N-terminal amino acid for sPlr, whereas the recombinant protein contained an approximately 50:50 mixture of protein with and without the N-terminal methionine. Although the N termini were identical, it was possible that amino acid differences existed elsewhere in the proteins.

In addition to the N-terminal identity of the three samples, amino acid composition analysis of rPlr and the streptococcal 41 kDa protein revealed no significant differences in overall amino acid composition between samples. This further indicated the relatedness of the proteins at the amino acid level (see Table 1). However, amino acid composition analysis may not be sensitive enough to detect small quantitative differences for some amino acids. In contrast to the experimentally derived data, the deduced amino acid sequence of Plr indicates the presence of seven methionines in the mature protein (not including the N-terminal methionine). Four of these seven residues have been confirmed in our laboratory by cyanogen bromide fragmentation of Plr (Lottenberg *et al.*, 1992).

The proteins sPlr, cGAPDH and rPlr share functional properties

No differences were detected among the sPlr, cGAPDH and rPlr utilizing the techniques described above, indicating that they are structurally similar proteins. The comparative analysis was accordingly extended to functional properties of GAPDH to further examine the 41 kDa proteins. Streptococcal cGAPDH was purified by NAD^+ affinity chromatography from a cytoplasmic ex-

Table 1. Amino acid composition comparisons of rPlr, streptococcal 41 kDa protein and streptococcal surface dehydrogenase

Amino acid	Number of amino acid residues			
	rPlr	Streptococcal 41 kDa protein	Plr based on predicted amino acid sequence	Streptococcal surface dehydrogenase*
Ala	25·3	29·6	33	38·1
Arg	19·8	27·2	13	15·5
Gly	35·5	38·7	33	36·6
His	8·0	7·8	8	7·2
Ile	19·4	16·6	21	22·4
Leu	24·5	24·4	22	23·4
Lys	19·7	19·8	20	21·4
Met	3·8	2·1	7	1·8
Phe	12·7	12·0	12	13·8
Ser	20·7	21·1	15	16·8
Val	25·3	24·1	35	36·5
Asn/Asp	49·0	39·2	42	43·3
Gln/Glu	30·5	32·6	27	29·9
Pro	5·5	8·9	10	13·6
Thr	27·7	22·8	26	27·0
Tyr	7·7	8·3	7	9·1

* Pancholi & Fischetti (1992).

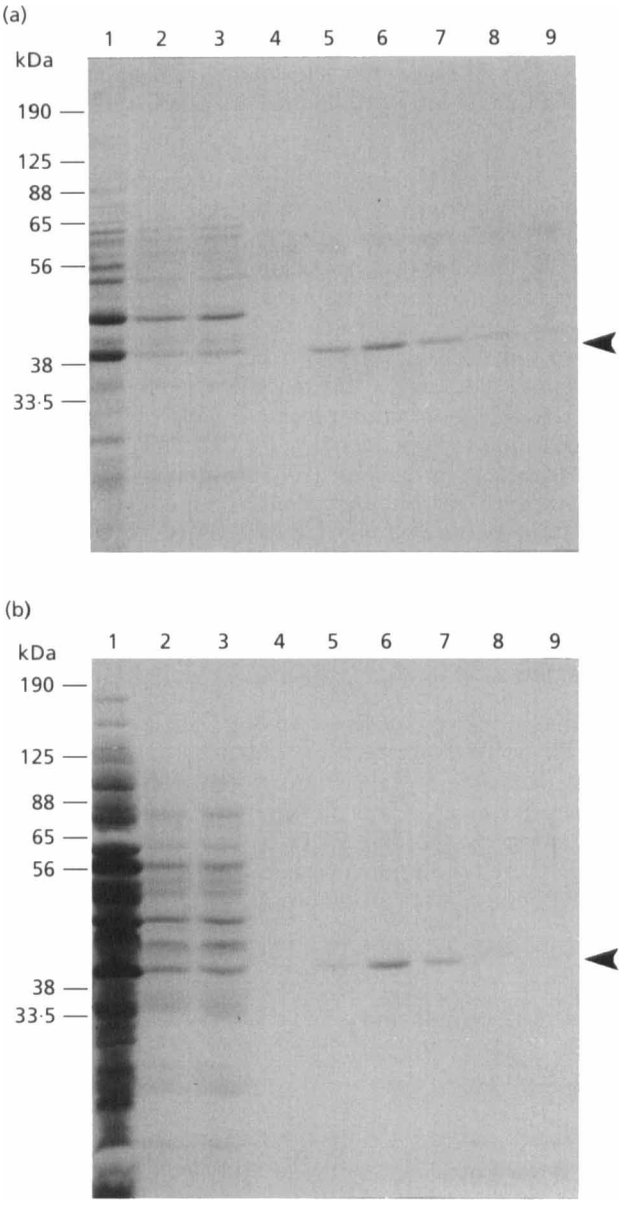

Fig. 4. NAD$^+$ affinity chromatography purification of sPlr and cGAPDH. Mutanolysin-extracted proteins from strain 64/14 (a) or soluble cytoplasmic material from strain 64/14 (b) were applied to NAD$^+$-agarose affinity columns and bound proteins eluted from the column with 10 mM NAD$^+$. Proteins were resolved by SDS-PAGE on 10% polyacrylamide gels and stained with Coomassie Brilliant Blue. Lanes: 1, starting material applied to the column; 2 and 3, wash fractions; 4–9, fractions eluted by the addition of 10 mM NAD$^+$ to the wash buffer. The NAD$^+$-eluted fractions from both columns demonstrated the presence of a 41 kDa protein (arrowheads).

tract (Fig. 4b, lane 1) prepared from strain 64/14. In this experiment, a 41 kDa protein was the predominant protein in the NAD$^+$ eluted fractions (Fig. 4b, lanes 4–9). The identical protocol used to isolate cGAPDH was also utilized to demonstrate that sPlr was a NAD$^+$-binding protein. A mutanolysin extract of strain 64/14 was applied to the affinity column and sPlr was the predominant protein eluted with 10 mM NAD$^+$ (as shown in Fig. 4a). Similarly, rPlr was affinity-purified from a soluble lysate of *E. coli* strain χ6060(pRL024) (data not shown). Therefore sPlr, cGAPDH and rPlr share the GAPDH characteristic of having functional NAD$^+$-binding domains.

The three purified 41 kDa proteins were assayed for GAPDH activity to verify that they were functional enzymes. sPlr, cGAPDH and rPlr yielded specific activities of 63, 153 and 206 μmol NADH min^{-1} mg^{-1}, respectively. There was never any detectable spontaneous conversion of NAD$^+$ to NADH without the addition of DL-GAP to the reaction mixtures. Therefore, the three protein preparations demonstrated functional GAPDH enzymic activity and have specific activities within the range reported for other GAPDHs of prokaryotic origin (Branlant *et al.*, 1983). The lower specific activity of the sPlr preparation may be due to the degree of purity of the preparation. Based on both the structural and functional data, it appears that cGAPDH and sPlr are the same primary protein in strain 64/14 and that this protein is identical to rPlr.

The genome of group A streptococci contains a single copy of the *plr* gene

Organisms which express only a single GAPDH may possess more than one *gap* gene. Therefore, DNA hybridization analysis by the method of Southern was performed on strain 64/14 chromosomal DNA using a probe consisting of only the 1 kb *plr* ORF to examine whether the genome contained a single or multiple copies of the *plr* gene. To identify possible related genes or pseudogenes, both hybridization with the *plr* probe and washes of the membrane were done under conditions of low stringency at room temperature to allow for maximum mismatch detection using this technique (Fig. 5a). Conditions of high stringency at 55 °C were used for comparison (Fig. 5b). In lanes containing chromosomal DNA digested with restriction enzymes which cut once within the *plr* gene (*Eco*RV, *Pvu*II and *Xmn*I), the probe hybridized with the same two fragments, respectively, at both room temperature and 55 °C. The probe hybridized with a single fragment in lanes containing DNA digested with restriction enzymes which cut outside the *plr* gene (*Bam*HI, *Eco*RI, *Hin*dIII and *Sal*I). These results are consistent with a single copy of the *plr* gene. The *Bam*HI/*Sal*I double digest yielded a single 2·2 kb fragment that is too small to harbour more than one copy of *plr* (see restriction map in Fig. 5). Therefore, *plr* is a single copy gene in group A strain 64/14.

To confirm that a single copy of the *plr* gene is typical for group A streptococci, a series of 19 other group A streptococcal strains isolated from both throat and blood cultures (Wang *et al.*, 1994) was examined by DNA hybridization. Chromosomal DNA was digested with *Bam*HI, *Sal*I and both enzymes. The hybridization pattern using the *plr* probe for all 19 strains was identical to that

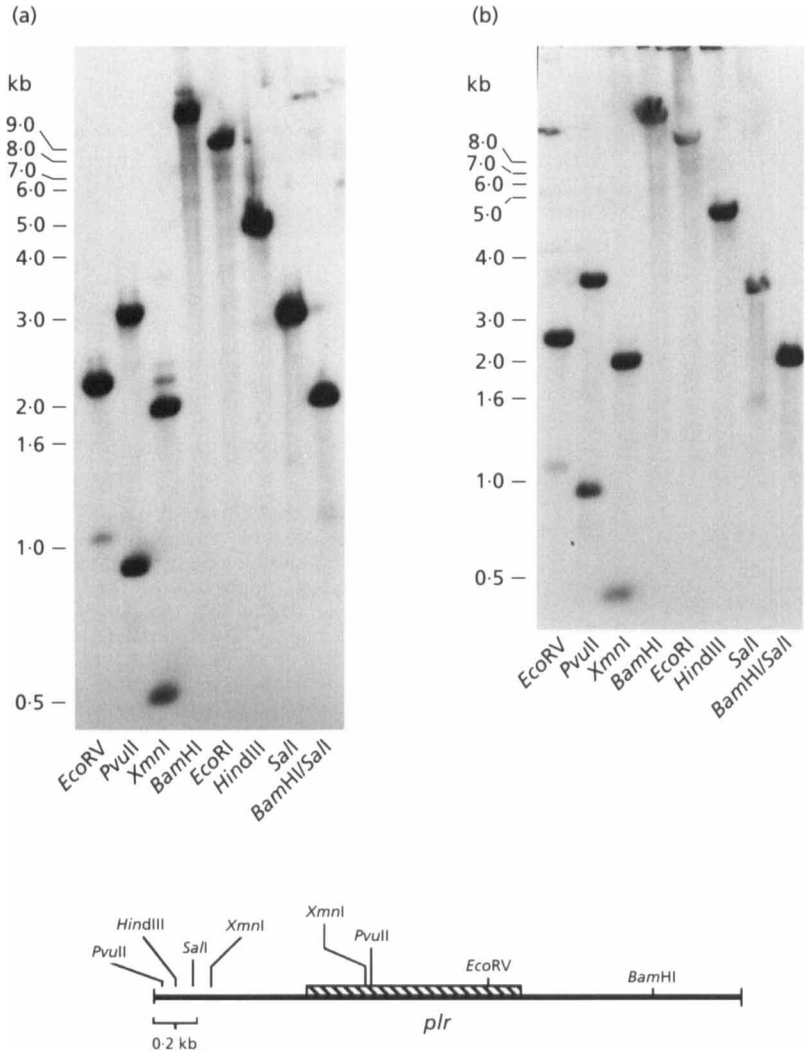

Fig. 5. DNA hybridization analysis of chromosomal DNA from strain 64/14 to determine the gene copy number of *plr*. DNA was digested with the restriction enzymes indicated. The digests were subjected to electrophoresis on duplicate 0·7% agarose gels and the restriction fragments transferred to nylon membranes. The membranes were then reacted with a [^{32}P]dCTP-labelled probe consisting of the *plr* ORF (amplified by PCR), washed and subjected to autoradiography. Autoradiograph (a) shows the low stringency hybridization performed at room temperature. Autoradiograph (b) shows the high stringency hybridization performed at 55 °C. The restriction enzyme cleavage map indicates relevant restriction enzyme sites located on the 2·7 kb DNA fragment cloned from strain 64/14 which harbours the *plr* gene and flanking regions.

of strain 64/14 as shown for the four representative strains SHS-7, SHS-9, SHS-17 and 230041 in Fig. 6. The *Bam*HI and *Sal*I digests yielded a > 10·0 kb and a 3·0 kb fragment, respectively, and the *Bam*HI/*Sal*I double digests yielded a single 2·2 kb fragment for all isolates. These results indicate that all strains tested contain a single copy of the *plr* gene and suggest that this may be typical for group A streptococci.

DISCUSSION

In this study, the relationship between sPlr and cGAPDH from streptococcal strain 64/14 was examined. GAPDH is a tetrameric enzyme of the glycolytic pathway responsible for the phosphorylation of GAP to generate 1,3-bisphosphoglycerate (Harris & Waters, 1976). NAD^+ is bound to GAPDH at a specific site and serves as an electron acceptor for the substrate during the reaction. By using NAD^+ affinity chromatography, we were able to isolate streptococcal cGAPDH from a cytoplasmic extract of strain 64/14. This 41 kDa protein was compared with both Plr isolated from mutanolysin extracts of group A strain 64/14 (sPlr) and purified recombinant Plr (rPlr). Polyclonal antibody raised against sPlr also recognized both rPlr and the cGAPDH on Western blot analysis. Although anti-Plr polyclonal antibody recognizes only a single protein in group A streptococcal lysates, GAPDHs from a single organism may differ significantly in amino acid composition and contain different antigenic epitopes. For example, Sakai *et al.* (1990) demonstrated that *Trichoderma koningii* expresses two divergent GAPDH isozymes, GAPDH I and GAPDH II. However, no differences in the primary protein structure or function of sPlr, cGAPDH and rPlr were detected.

Prokaryotic and eukaryotic organisms may possess single or multiple GAPDH genes. The DNA hybridization results suggest that group A streptococci may typically possess a single gene encoding Plr. However, the possibility remains that a second, highly divergent *gap* gene may be present on the streptococcal chromosome but was not detectable by DNA hybridization analysis using the *plr* probe. This situation would be analogous to *Saccharomyces cerevisiae* or *E. coli* which have three and two GAPDH genes, respectively (Holland, 1983; Alefounder & Perham, 1989).

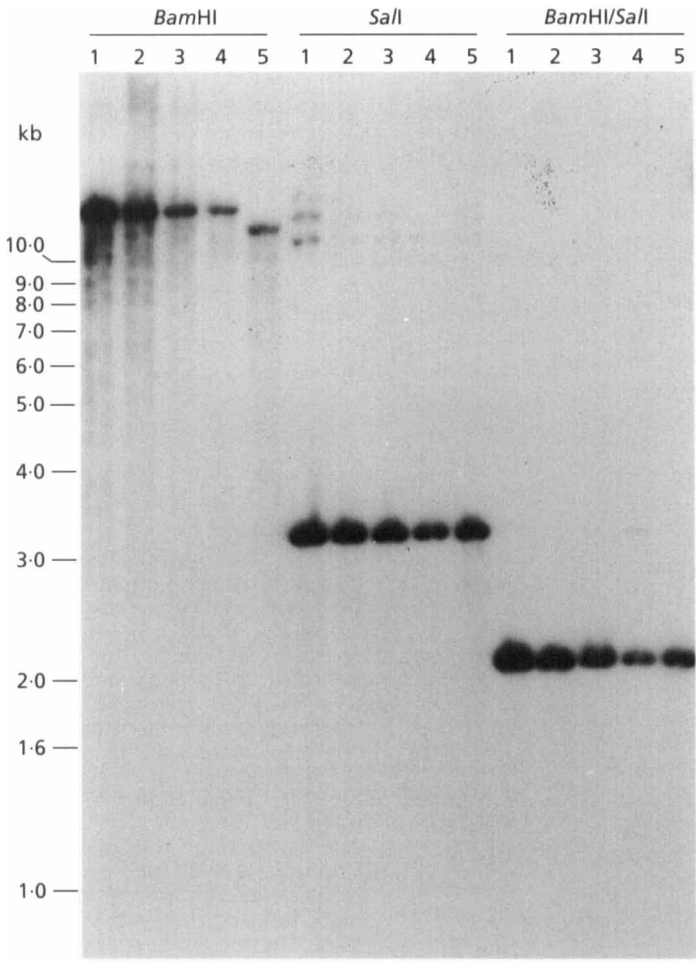

Fig. 6. DNA hybridization analysis of chromosomal DNA to determine the gene copy number of *plr* in group A streptococcal clinical isolates. Strain 64/14 and four representative strains of the 19 isolates tested are shown. Chromosomal DNA was digested with the restriction enzymes *Bam*HI, *Sal*I or both enzymes. The digests were subjected to electrophoresis on a 0·7 % agarose gel and the restriction fragments transferred to a nylon membrane. The membrane was then reacted with a [^{32}P]dCTP-labelled probe consisting of the *plr* ORF amplified by PCR. Following overnight hybridization at 42 °C, the membranes were washed and hybridization bands visualized by autoradiography. Lanes: 1, strain 64/14; 2, strain SHS-7; 3, strain SHS-9; 4, strain SHS-17; 5, strain 230041.

There are organisms which utilize one GAPDH for cytoplasmic functions while another GAPDH is targeted for specific organelles such as glycosomes or mitochondria (Michels *et al.*, 1991). The protozoans *Trypanosoma brucei* and *Leishmania mexicana* have one GAPDH isozyme located in a glycosome and a second GAPDH isozyme residing solely in the cytoplasm. However, in the present study there was no experimental evidence for either a second GAPDH product in strain 64/14 or more than one homologous gene encoding Plr. Plr was not detected by Western blot analysis in the insoluble fraction of a strain 64/14 lysate (Fig. 1b, lane 2), indicating that Plr may not be associated with the bacterial membrane. In addition, the DNA sequence of the *plr* gene does not encode the putative Gram-positive membrane anchor motif nor does it reveal a protein secretion signal sequence (Lottenberg *et al.*, 1992).

The muramidase mutanolysin, which degrades peptidoglycan, has been used previously for solubilization of well characterized surface components of group A streptococci such as IgG-binding proteins (Yarnall & Boyle, 1986). However, examination of these preparations for the presence cytoplasmic proteins has not previously been reported for group A streptococci. Mutanolysin extracts and the corresponding cytoplasmic proteins of strain 64/14 were assayed for phosphoglycerate kinase (PGK) activity and revealed the presence of enzymic activity in both fractions. The PGK-specific activities in the cytoplasmic lysates were generally four- to sixfold higher than the corresponding mutanolysin preparation (data not shown). This leaves open the possibility that the protein extracts generated with mutanolysin contain some cytoplasmic proteins. However, the unambiguous N-terminal amino acid sequence, the identical peptide maps and the identification of a single *plr* gene indicated that there was only a single Plr protein encoded by a single gene.

GAPDHs have previously been reported to be localized on the surface of other organisms. Goudot-Crozel *et al.* (1989) identified GAPDH localized on the surface of *Schistosoma mansoni*. In addition, Fernandes *et al.* (1992) described an accumulation of a 37 kDa protein in the cell wall of the yeast *Kluyveromyces marxianus* during heat-stress-induced flocculation. The N-terminal amino acid sequences of V8-protease-generated fragments from the 37 kDa protein revealed over 80 % identity to one of the GAPDHs from *Saccharomyces cerevisiae*. Interestingly, the cell-wall-associated GAPDH homologue is glycosylated, whereas the cytosolic form is not. Although post-translational modifications occur less frequently in prokaryotes, analysis for potential post-translational modifications of Plr/GAPDH has not yet been performed

by our laboratory, but could yield clues regarding putative secondary functions and/or localization of Plr/GAPDH.

Recently Pancholi & Fischetti (1992) reported a putative surface GAPDH molecule, streptococcal surface dehydrogenase, from group A streptococci. Using methodology similar to that utilized for the isolation of Plr, streptococcal surface dehydrogenase was purified from cell wall extracts of group A streptococci that were prepared using a phage lysin enzyme. Streptococcal surface dehydrogenase was reported to bind to fibronectin, lysozyme, actin and myosin in a ligand blot assay and to have ADP-ribosylation activity *in vitro* (Pancholi & Fischetti, 1992, 1993). The N-terminal amino acid sequence of streptococcal surface dehydrogenase is 97·4% identical to the N-terminal amino acid sequence of Plr. In our studies, NAD^+ affinity chromatography was performed to purify a 41 kDa protein from a whole-cell preparation of strain 64/14. This 41 kDa protein, as well as rPlr, were subjected to acid hydrolysis to discern the amino acid composition of the proteins. The differences in amino acid composition between our 41 kDa protein and rPlr were no greater than differences between the deduced amino acid sequence of rPlr and the experimentally determined amino acid composition of rPlr. Furthermore, these differences are no greater than those of the reported amino acid composition of streptococcal surface dehydrogenase compared to that of the 41 kDa protein and rPlr (see Table 1). It appears that Plr and streptococcal surface dehydrogenase may be structurally similar proteins. Plr was the only GAPDH enzyme detected in our studies and this appears to be typical for group A streptococci. It is not uncommon for small amino acid changes in GAPDH to occur among different strains of the same species as has been previously shown for both *E. coli* and *Salmonella* strains (Nelson *et al.*, 1991).

Based on the data in this study, the plasmin-binding protein, Plr, of streptococcal strain 64/14 is identical to GAPDH isolated from the streptococcal cytoplasm. Furthermore, the *plr* gene is present as a single copy in the clinical group A streptococcal strains tested. These analyses have yielded novel data regarding group A streptococcal GAPDH. This information is being applied towards introducing mutations of the *plr* gene into strain 64/14 so that the role of Plr as a plasmin receptor can be addressed.

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