Molecular Co-operation between Protein PAM and Streptokinase for Plasmin Acquisition by Streptococcus pyogenes

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Bacterial surface-associated plasmin formation is believed to contribute to invasion, although the underlying molecular mechanisms are poorly understood. To define the components necessary for plasmin generation on group A streptococci we used strain AP53 which exposes an M-like protein (“PAM”) that contains a plasminogen-binding sequence with two 13-amino acid residues long tandem repeats (a1 and a2). Utilizing an Escherichia coli-streptococcal shuttle vector, we replaced a 29-residue long sequence segment of Arp4, an M-like protein that does not bind plasminogen, with a single (a1) or the combined a1a2 repeats of PAM. When expressed in the coli, the purified chimeric Arp/PAM proteins both bound plasminogen, as well as plasmin, and when used to transform group A streptococcal strains lacking the plasminogen-binding ability, transformants with the Arp/PAM constructs efficiently bound plasminogen. Moreover, when grown in the presence of plasminogen, both Arp/PAM- and PAM-expressing streptococci acquired surface-bound plasmin. In contrast, plasminogen activation failed to occur on PAM- and Arp/PAM-expressing streptococci carrying an inactivated streptokinase gene: this block was overcome by exogenous streptokinase. Together, these results provide evidence for an unusual co-operation between a surface-bound protein, PAM, and a secreted protein, streptokinase, resulting in bacterial acquisition of a host protease that is likely to spur parasite invasion of host tissues.

Plasminogen is a single chain 92-kDa glycoprotein present in plasma (~200 mg/liter) and in extracellular fluids. The protein contains five characteristic kringle domains that mediate interactions with multiple ligands, such as fibrinogen, α2-antiplasmin (α2AP),1 and cellular plasminogen receptors (1–3). In vivo, two specific activators, the tissue-type plasminogen activator and urokinase, convert the single-chain zymogen to a two-chain enzyme, plasmin, by cleavage at a single site (4). Plasmin efficiently degrades fibrin and probably also participates in the breakdown of soft tissue glycoproteins (5). The plasma concentration of active plasmin is normally low, due to its rapid inactivation by proteinase inhibitors, primarily α2AP. Under pathophysiological conditions some pathogens, including group A, C, and G streptococci and Staphylococcus aureus, secrete highly specific and potent plasminogen activators such as streptokinase and staphylokinase, respectively (1, 2). Other bacteria, including Escherichia coli and Yersinia pestis, express surface-bound plasminogen activators (6, 7). Evidence suggest that plasmin generated by bacterial plasminogen activators contributes to virulence, possibly by facilitating bacterial penetration through host barriers (7).

It has been known for some time that many virulent bacterial species express surface proteins capable of binding plasmin(o)gen without causing its activation (8). Some of these can also contribute to bacterial dissemination (9). For Streptococcus pyogenes (group A streptococci), a major causative agent of both mucosal and skin infection in humans, four major types of surface proteins have been forwarded as candidate plasmin(o)-binding proteins. Of these the dehydrogenase-like Plr protein (10–12) binds primarily plasmin, whereas a 45-kDa protein (13) also binds plasminogen. Some members of the M protein family, a well known class of surface-exposed streptococcal virulence determinants (14), have been shown to capture plasmin(o)gen indirectly, through fibrinogen (15, 16). Finally, a set of group A streptococcal strains, associated with skin infection (17–19), expose M-like proteins that bind plasminogen as well as plasmin, directly and with high affinity (20, 21). For one of these proteins, PAM (plasminogen-binding group A streptococcal M-like protein) from type 53 group A streptococci, a plasminogen-binding sequence characterized by two 13-amino acid residues long repeats (a1 and a2), has been identified in its surface-exposed variable NH2-terminal domain. Similar motifs are also present in M-like proteins from the other “skin” strains (21).

Here we have set out to study the molecular requirements for the binding and activation of plasminogen by PAM-expressing bacteria. We demonstrate a joint role for the plasminogen-binding motif in PAM and streptokinase in the acquisition and exposure of plasmin at the surface of group A streptococci.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions**—Streptococcal strains, their M or M-like proteins and known ligands for these proteins are listed in Table I. AP4, a type M4 group A streptococcal strain expressing Arp4 (22) and the PAM-expressing type 53 group A streptococcal strain AP53 were obtained from the Institute of Hygiene and Epidemiology, Prague, Czech Republic. JRS4, an M6 protein-expressing group A streptococcal strain (23, 24), and JRS145 (25), an M6-deficient derivative of JRS4, were from Dr. June Scott, Emory University, Atlanta, GA. JRS145(arp4), a JRS145 strain transformed with the shuttle plasmid pJRS264 (26) that encodes the arp4 gene of AP4, was a kind gift from Dr. Gunnar Lindahl. Streptococci were grown in Todd-Hewitt broth (Difco, Detroit, MI), or in Todd-Hewitt broth supplemented with 30% (v/v) fresh human heparinized plasma, in 5% CO2 at 37 °C for 16 h.

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1 The abbreviations used are: α2AP, α2-antiplasmin; Arp4, IgA-binding group A streptococcal M-like protein; IgA, immunoglobulin A; PAM, plasminogen-binding group A streptococcal M-like protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; ska, streptokinase gene.

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Streptococci transformed with plasmids carrying resistance markers for antibiotics were grown in medium supplemented with 15 mg liter⁻¹, 200 mg liter⁻¹, or 70 mg liter⁻¹ erythromycin, kanamycin, or spectinomycin, respectively. The streptococci were harvested by centrifugation, washed twice in 0.15 M NaCl, 0.03 M phosphate, pH 7.2 (PBS), and were finally resuspended in PBS. Transformed *E. coli* LE392 was grown in LB medium supplemented with erythromycin (750 mg liter⁻¹), kanamycin (200 mg liter⁻¹), or spectinomycin (20 mg liter⁻¹) depending on the selection marker of the plasmid.

### Binding of Radiolabeled Ligands to Streptococci

**Binding of [³²P]-labeled plasminogen, IgA, or fibrinogen to bacteria** was measured in a total volume of 250 µl of PBS containing 0.1% (v/v) Tween 20. Following incubation for 60 min at 20 °C, 2 ml of PBS containing 0.1% Tween 20 was added, and the bacteria were centrifuged at 4,000 × g for 5 min. The supernatant was discarded and the radioactivity associated with the pellet was measured in a γ-counter. The number of plasmidogen-binding proteins expressed on streptococci was determined from experiments in which [³²P]-labeled plasminogen (0.02 nM) was allowed to compete with various concentrations of unlabeled plasminogen (50-0.025 nM) for the binding to streptococci (1 × 10⁸ bacteria ml⁻¹). The number of receptors was then estimated from the intercept with the abscissa of a curve generated by plotting bound (abscissa) versus bound/free (ordinate) plasmogen.

### Plasma Absorption Experiments

**Heat-killed streptococci** (10¹⁰ cells) were incubated with 1.0 ml of fresh human plasma for 60 min at 20 °C. Bacterial cells were pelleted and washed three times with PBS. To elute absorbed proteins, the bacteria were incubated with 0.1 M glycine, pH 2.0, for 10 min at 20 °C. The bacteria were removed by centrifugation (4,000 × g, 5 min), and the proteins of the supernatant were analyzed by SDS-PAGE and immunoblotting (see below).

### Plasminogen Activation Assays

**Recombinant** with *E. coli* LE392 transformed with plasmids carrying resistance markers for antibiotics were grown in medium supplemented with 15 mg liter⁻¹, 200 mg liter⁻¹, or 70 mg liter⁻¹ erythromycin, kanamycin, or spectinomycin, respectively. The streptococci were harvested by centrifugation, washed twice in 0.15 M NaCl, 0.03 M phosphate, pH 7.2 (PBS), and were finally resuspended in PBS. Transformed *E. coli* LE392 was grown in LB medium supplemented with erythromycin (750 mg liter⁻¹), kanamycin (200 mg liter⁻¹), or spectinomycin (20 mg liter⁻¹) depending on the selection marker of the plasmid.

### Table I

**Description of streptococcal strains, their M- and M-like proteins and known ligands**

| Strain     | Description                        | M/M-like protein | Ligand(s)            |
|------------|------------------------------------|------------------|----------------------|
| AP4        | Type M4 group A streptococcal strain | Ap4              | IgA (37)             |
| AP53       | Type M53 group A streptococcal strain | Pam              | Plasminogen (20)     |
| JRS4       | Type M6 group A streptococcal strain | M6               | Fibrinogen (23, 24)  |
| JRS145     | Isogenic M-mutant of JRS4           | Nil              | Nil (25)             |
| JRS145(Arp)| JRS145 carrying plasmid pJRS264    | Arp4             | IgA (22, 26)         |
| JRS145(Arp/a1)| JRS145, modified plasmid pJRS264 | Arp(PAM)         | Plasminogen          |
| JRS145(Arp/a2)| JRS145, modified plasmid pJRS264 | Arp(PAM)         | Plasminogen          |
| JRS4(Arp/a2)| JRS4, modified plasmid pJRS264     | Arp6, M6, PAM    | Plasminogen and fibrinogen |

### Inactivation of the Streptokinase Gene (skα)—Chromosomal DNA from AP53 or JRS4 streptococci was used as templates for PCR to amplify a 1180-base pair skα fragment with primers SK3 (5'-GAGCTC GACACCTGCATCCTGGAAATATGGTC-3') and SK4 (5'-GACGGATCC TCTCTTCGTCTTG-3'), or to amplify a 950-base pair skα fragment with primers SK5 (5'-GACGGATCCCTCTTGAGAAATTTACCTG-3') and SK7 (5'-GACGGATCCCTCTTTTCTTCTTCTTCTCTG-3'); the relative locations of the SK primers in the *skα* sequence are indicated in Fig. 1C. The 1180- and 950-base pair fragments were digested with *SalI/BamHI* and *BamHI/SacI*, respectively, and were then ligated with *SalI/-*, *SacI/-* digested pUC18 DNA. Following transformation and amplification in *E. coli*, the resultant plasmid DNA was linearized with *BamHI* and ligated with the *BamHI/-* digested kanamycin-resistance cassette tKm-2 (25). Plasmid DNA was prepared from a kanamycin-resistant transformant and digested with *SalI* and *SacI*. The resulting *SalI/SacI* fragment, containing tKm-2 flanked by *skα* fragments, was blunted and ligated with the *SalI/-* and *BamHI/-* digested plasmid pJRS233. Plasmid pJRS233 contains an erythromycin resistance marker (*erm*), a temperature-insensitive *E. coli* replicon, and a promiscuous replicon that is sensitive to temperatures above 35 °C in both *E. coli* and *S. pyogenes* (27). Following transformation and subsequent propagation in *E. coli* at 37 °C, the resulting plasmid (pJRS233-kana-*skα*) was used to electroporate *S. pyogenes*. Kanamycin-resistant and erythromycin-sensitive isolates were selected following sequential culturing of the transformed bacteria at 30 and 37 °C, respectively. Integration of tKm-2 into the *skα* genes of AP53 and JRS145, respectively, was monitored by PCR: the oligonucleotides SK9 (5'-GACGTC-9'-GACGGATC), located on opposite strands immediately outside the *skα* fragment defined by SK3 and SK6, were used in combination with the *Km*-2-derived outwardly directed primers KM1 (5'-CTCTTCATGTCGCTGG-3') and KM2 (5'-ATCCGGAACTGCATACCTG-3') (Fig. 1C). PCR-positive clones failed to produce active streptokinase as determined by analysis of growth supernatants and streptococcal whole lysates by the S-2251 assay. No difference in the growth rates was observed between the *skα* positive and the *skα* negative streptococcal isolates.

### Recombinant DNA Techniques

**Standard ligation and DNA isolation procedures** were employed (28). Ligase, T4 DNA polymerase, and restriction enzymes were from Promega (Madison, WI). PCR was performed (29) using AmpliTaq (Perkin-Elmer, Wilton, CT). The sequences of the SK primers were derived from the published DNA sequence (30, 31). Chimeric *Arp/PAM* genes were sequenced using the dideoxy nucleotide chain termination method (32). Electroporation of *E. coli* and streptococci was performed as described (25).

### Protein Analysis and Preparation of Affinity Matrices

**Recombinant** and *E. coli* LE392 transformed with the corresponding genes cloned in pJRS264 or derivatives thereof. *PAM* and *Arp/PAM* hybrid proteins were purified on plasmidogen agarose, whereas Ap4 was purified on an agarose column coupled with human IgA. Proteins bound to the affinity columns were eluted using 0.1 M glycine-HCl, pH 2.0, and the effluent was dialyzed against PBS. Plasminogen was purified on a column with lysine-Sepharose 4B (Pharmacia, Uppsala, Sweden). Proteins bound to the column were eluted with 0.2 M ethanolic acid. Fractrons containing plasminogen were pooled and dialyzed against PBS. Plasminogen was purified by incubation of 0.8 mg of plasminogen with 50 µg of tissue-type plasminogen activator at 37 °C, for 45 min. Activation was interrupted by incubation at 20 °C (10 min) with a 100-fold molar excess of aprotinin. The efficiency of plasminogen activation was monitored by SDS-PAGE (33) and im...
binding to AP53 streptococci expressing the PAM protein on their surface. To this end the bacteria were grown in medium containing 30% human plasma. Following centrifugation, the plasma proteins bound to the bacterial surface were eluted, separated by SDS-PAGE, and visualized by immunoblotting using a 125I-labeled specific antibody recognizing both human plasmin and plasminogen (Fig. 1, panel A, top). The plasmin/plasminogen ratio increased with time of incubation (Fig. 1A). Using a chromogenic substrate we were able to demonstrate that at least part of the bacteria-associated plasmin was present in its active form (Fig. 1A, bottom). These results indicate that efficient plasminogen-binding and activation mechanism(s), which are not blocked by the proteinase inhibitors present in human plasma, must exist at the streptococcal surface.

The a1-repeat of PAM Is Sufficient to Endow Group A Streptococci with Plasminogen Binding—Initially we focused on the role of PAM for the streptococcal capture of plasminogen. However, previous experiments with recombinant proteins and synthetic peptides (21) have suggested that a major plasminogen-binding site is located in a 30-amino acid residue long segment containing a sequence with two characteristic tandem repeats (a-repeats) in the NH2-terminal variable region of PAM. To test the notion that a-repeats confer plasminogen binding properties, we used another M-like protein, Arp4, that lacks a plasminogen-binding site, but exposes a well defined IgA-binding site (37), as a recipient for the a-repeats of PAM. We first replaced 29 residues in the IgA-binding region of Arp4 (positions 29–57 of the Arp4 sequence) by a segment of 36 residues that comprises only a1 (positions 62 to 74). We then inserted PAM sequence: DAELQRLKNERHE) or both a-repeats (Arp/Arp4, inserted PAM sequence: LKDDVEKLTADEALQRLKNERHEEAEELRLKSERHD). When making the constructs, breaking of the heptad repeat patterns in Arp4 and PAM (39) was avoided. The arrowsheads point to the IgA- and plasminogen-binding sites. "C" denotes conserved repeated segments that are present in many M or M-like proteins. The bars representing the sequence segments of the M proteins are not drawn to scale.

RESULTS

Activation of Plasminogen Bound to the Surface of S. pyogenes—We followed the time course of plasminogen/plasmin binding to AP53 streptococci expressing the PAM protein on their surface. To this end the bacteria were grown in medium containing 30% human plasma. Following centrifugation, the plasma proteins bound to the bacterial surface were eluted, separated by SDS-PAGE, and visualized by immunoblotting using a 125I-labeled specific antibody recognizing both human plasmin and plasminogen (Fig. 1, panel A, top). The plasmin/plasminogen ratio increased with time of incubation (Fig. 1A). Using a chromogenic substrate we were able to demonstrate that at least part of the bacteria-associated plasmin was present in its active form (Fig. 1A, bottom). These results indicate that efficient plasminogen-binding and activation mechanism(s), which are not blocked by the proteinase inhibitors present in human plasma, must exist at the streptococcal surface.

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unlabeled plasminogen inhibited the binding of $^{125}$I-plasminogen to Arp/a1a2 and Arp/a1 that had been immobilized in microtiter plates (Fig. 4A). Similarly, unlabeled plasmin inhibited the binding of radioabeled plasmin to the two chimeric proteins (Fig. 4B). The affinity constants ($K_a$) for the interactions between plasminogen and plasmin and the two chimeras, were calculated by Scatchard plots from the data obtained in the competitive binding experiments. For the interaction with plasminogen, the $K_a$ values were calculated to be $6 \times 10^8$ M$^{-1}$ for both chimeras (Fig. 4A, insets), a value close to that ($8 \times 10^8$ M$^{-1}$) previously determined for the interaction between radiolabeled plasminogen and immobilized PAM. The corresponding $K_a$ values for the interactions between plasmin and the chimeric proteins (Fig. 4B, insets) were 1 order of magnitude lower ($3 \times 10^7$ M$^{-1}$ and $6 \times 10^7$ M$^{-1}$, for Arp/a1a2 and Arp/a1, respectively). As was the case for the interaction between $^{125}$I-plasminogen and PAM (20), the binding of plasminogen to the Arp/PAM constructs could be inhibited by the lysine analogue e-aminocaproic acid at low concentrations, indicating that the interaction involves lysine-binding site(s) in plasminogen (data not shown). Hence, the results show that the a-repeat motif(s) can confer the plasminogen-binding properties of PAM to a plasminogen non-binding member of the M protein family and that the a1-repeat contains the major plasminogen-binding site of PAM.

The plasmids encoding Arp/a1a2 and Arp/a1 were then used to transform the group A streptococcal strain JRS145, an isogenic M-protein-deficient (emm$^{-}$) derivative of the M6-expressing strain JRS4. Using radiolabeled ligands, streptococci expressing the Arp/PAM constructs were shown to bind plasminogen but failed to bind IgA (Fig. 5). Furthermore, heat-killed Arp/a1a2- and Arp/a1-expressing streptococci absorbed plasminogen from plasma, whereas the Arp4-expressing strain JRS145 did not (Fig. 6). The finding that JRS145(Arp/a1) showed a reduced capacity to bind $^{125}$I-plasminogen compared with JRS145(Arp/a1a2), although the recombinant chimeric proteins have the similar affinity for thezymogen, is likely to be attributable to different surface expression levels. Thus, estimation of the numbers of receptors from Scatchard analysis of experiments where $^{125}$I-plasminogen and different concentrations of plasminogen were allowed to compete for the binding to JRS145 transformed with the two PAM/Arp hybrid genes, showed that JRS145(Arp/a1a2) expressed 30 times as many chimeric molecules as compared with JRS145(Arp/a1) (data not shown). Because of this difference, JRS145(Arp/a1a2) was used in further experiments.

We also transformed strain JRS4, the parent strain of JRS145 that expresses the fibrinogen-binding M6 protein, with the plasmid encoding the hybrid Arp/A1a2 protein, and thereby converted the phenotype of JRS4 from plasminogen non-binding to plasminogen binding while retaining its fibrinogen binding capacity (Fig. 7, inset). When grown in medium containing plasma, significant surface acquisition of plasmin was observed with transformed JRS4, but not with the wild-type strain (Fig. 7).

Co-operation between Streptokinase and the a-repeats of PAM in the Generation of Streptococcal-associated Plasmin—Our initial experiments with the AP53 strain demonstrated a conversion of plasminogen to plasmin after >30 min of incubation of the bacteria with plasma-containing medium. Because of the presence of a large molar excess of proteinase inhibitors in plasma which likely inactivate any exogenous plasminogen activators, we speculated that activators of bacterial origin, such as streptokinase, could be involved in plasmin generation. Indeed, streptokinase added to plasminogen-loaded heat-inactivated AP53 cells converted plasminogen to plasmin in a dose-dependent manner (data not shown). To rigorously test the hypothesis that streptokinase contributes to the activation of surface-associated streptococcal plasminogen, we inactivated the streptokinase gene (ska) of strain AP53 by homologous recombination (Fig. 1C). ska$^{-}$ AP53 streptococci bound $^{125}$I-plasminogen at a level (65% of the total radioactivity) comparable to the parent strain (63%), and demonstrated a plasma absorption pattern similar to that of the original isolate (data not shown). However, little if any conversion of plasminogen was observed when the ska$^{-}$ variant was incubated with human plasma (Fig. 1B). The inability of ska$^{-}$ AP53 streptococci to acquire surface-associated plasmin could be reversed by addition of streptokinase (data not shown).

The notion that streptokinase might be critically involved in plasminogen activation at the streptococcal surface was further investigated in a derivative of the M protein-deficient group A streptococcal strain JRS145 also deleted in the streptokinase gene. As expected, this strain failed to bind plasminogen at all (data not shown). Transformation with the plasmid encoding Arp/a1a2 conferred plasminogen-binding properties to JRS145(emm$^{-}$/ska$^{-}$). However, little if any activation of plasminogen was observed by immunoblotting and by the amidase activity assay (Fig. 8). In contrast, expression of Arp/a1a2 in ska$^{-}$ JRS145 bacteria allowed acquisition of plasminogen from plasma-containing growth medium as well as time-dependent conversion of plasminogen to plasmin (Fig. 8). As with the AP53 ska$^{-}$ strain, the inability of streptokinase-deficient JRS145(Arp/a1a2) streptococci to capture plasmin could be restored following addition of streptokinase. In accord with these observations we found that heat-killed JRS145(Arp/a1a2) bacteria, unable to secrete streptokinase, bound plasminogen but failed to acquire significant amounts of plasmin (data not shown). Hence, the co-operation of two bacterial effectors, the surface-bound acceptor protein PAM and the secretory activator protein streptokinase, is mandatory for generation of human plasmin at group A streptococcal surfaces.
DISCUSSION

Group A streptococci elaborate at least two classes of plasminogen-binding proteins, i.e., secretory proteins such as streptokinase and streptococcal cysteine proteinase (38), and surface attached proteins such as PAM (20), Plr (10), and a 45-kDa protein (13). Moreover, group A streptococci can capture plasminogen indirectly through fibrinogen which in turn binds to M-like proteins (15). Such a multiplicity of interactions with the key player of the fibrinolytic pathway suggests that the plasminogen-binding property carries a selective advantage to streptococci, motivating an analysis of the relative importance and biological role of the different mechanisms.

FIG. 4. Inhibition of the binding of radiolabeled plasminogen or plasmin to immobilized Arp/PAM chimeric proteins. The interactions were inhibited with various concentrations of unlabeled plasminogen (A), or plasmin (B), at the indicated concentrations. The affinity constants ($K_i$) for the interactions between radiolabeled plasminogen (A, inset), or plasmin (B, inset), and the immobilized Arp/a1 or Arp/a1a2 proteins were calculated from Scatchard plots of data obtained when an equilibrium had been reached. The data represent the mean values of three experiments with duplicate samples.

FIG. 5. Binding of radiolabeled protein ligands to S. pyogenes expressing chimeric Arp/PAM proteins. The binding of $^{125}$I-labeled purified plasminogen or IgA to emm$^-$ strain JRS145 (negative control; □), to Arp4-expressing strain JRS145(Arp) (○), to strains JRS145(Arp/a1) (△) and JRS145(Arp/a1a2) (◇) and JRS145(Arp/a1) (□) that express Arp/PAM chimeric proteins, and to the PAM-expressing strain AP53 (positive control; ●) was measured. The data represent mean values from three separate experiments with duplicate samples each.

FIG. 6. Plasma absorption patterns of Arp/PAM-expressing S. pyogenes. Heat-killed group A streptococci were incubated with 1.0 ml of human plasma for 30 min at 20 °C. Following washing, the absorbed proteins were eluted with 0.1 M glycine-HCl, pH 2.0. The acid-stripped bacteria were pelleted, and 20 μl of each supernatant containing 20–40 μg of total protein was analyzed by SDS-PAGE under reducing conditions; for control, 2 μg of purified human plasminogen was included (left panel). An electroblotted replica of the gel was probed with $^{125}$I-antiplasminogen (right panel). Elutes from the following strains were analyzed: lane 1, JRS145(Arp/a1); lane 2, JRS145(Arp/a1a2); lane 3, AP53; lane 4, JRS145(Arp); lane 5, JRS145; lane 6, plasminogen.

In the present work we have focused on the role of protein PAM, a member of the streptococcal M family of surface proteins. To assess the contribution of the PAM α-repeats for in vitro plasmin acquisition by group A streptococci, we grafted them on the plasminogen non-binding protein Arp4, another member of the M protein family. We chose Arp4 as the “recipient” molecule since this protein and PAM are similar in many ways. Thus, both proteins are anchored to the bacterial cell wall through their highly conserved COOH-terminal segments, they contain three conserved C-repeats in the COOH-terminal half of the protein, and have surface-exposed variable NH$_2$-terminal regions. Moreover, both PAM and Arp can be predicted to form α-helical coiled coil dimers as a consequence of their high contents of characteristic heptad repeats (39). Finally, the binding patterns of PAM and Arp resemble one another (i) both proteins bind C4bp with high affinity through a site in the NH$_2$-terminal hypervariable domain (40); (ii) they do not bind fibrinogen; (iii) a unique binding property (i.e., plasminogen-binding for PAM and IgA-binding for Arp4) is for both proteins located in the central region of their NH$_2$-terminal domains. The experiments showed that replacement of the IgA-binding site of Arp by the α-repeats of PAM was necessary and sufficient to endow group A streptococci expressing two (JRS145) or three (JRS4) of the other putative plasminogen(s)-
The binding to PAM is mediated through the lysine-binding site(s) of a plasminogen fragment comprising its first three kringle domains (20). The principal physiological regulator of plasmin, α₂AP, binds to related site(s) (41). Given the high affinity for the interaction with plasmin(ogen), this protease is likely to be protected from regulation by α₂AP when bound by PAM-expressing streptococci. Our finding that absorption and activation of plasminogen on PAM- and Arp/PAM-expressing streptococci can take place in plasma supports such a notion. Since the binding is mediated through lysine-binding site(s), the catalytic domain of plasminogen remains accessible by the major streptococcal plasminogen activator, streptokinase. Indeed, streptokinase binds to PAM-expressing bacteria or to PAM-coated polyacrylamide beads only when they have been pre-absorbed with plasminogen.² This suggests that streptokinase might bind and activate PAM-bound plasminogen although our present experiments do not entirely rule out the possibility that a cycle of dissociation from and association with PAM is required to allow plasminogen activation in solution. Unlike plasmin in solution, neither the plasminogen-streptokinase complex nor the plasmin-streptokinase complex can be inactivated by α₂AP (42). Therefore the finding that PAM- and Arp/PAM-expressing bacteria acquire active plasmin even in the presence of plasma points to the possibility that bacteria gain an unregulatable plasminogen activator due to the deposition of plasmin(ogen)-streptokinase complexes on the streptococcal surfaces.

The key role of plasmin is to degrade fibrin (43, 44). However, due to its broad specificity, plasmin can serve a host of other functions, e.g., cleavage of connective tissue proteins and basement membrane components, and activation of latent metalloproteinases. Cells that are able to generate plasmin on their surface may therefore use it to degrade physiological barriers other than fibrin. Indeed, plasmin-induced matrix degradation has been shown to facilitate the tissue penetration of tumor cells exhibiting an increased surface-associated plasminogen activation (45, 46). The present results as well as data published by other investigators suggest that bacteria and certain mammalian cells may use similar mechanisms to cause pericellular proteolysis. Recent results using in vivo models suggest that surface-bound plasmin(ogen) can contribute to bacterial invasion. Thus, expression of a surface-bound plasminogen activator vastly increased the virulence of Y. pestis by facilitating their dissemination from the site of infection in the skin of mice (7). Moreover, plasminogen boosted spirochtemia in mice infected with the plasminogen-binding bacterial species Borrelia burgdorferi (9). It is conceivable that plasmin bound to the streptococcal surface may also function as a means to break tissue barriers, thereby promoting the spreading of these bacteria. The results presented here emphasize the unique property of some group A streptococci to expose a surface protein that binds plasminogen with high affinity, and at the same time to secrete a highly potent plasminogen activator that likely activates the surface-bound plasminogen. The joint role for protein PAM and streptokinase in the acquisition of surface-bound plasmin by group A streptococci associated with skin infections is yet another facet of the complex interplay that governs host-parasite relationships.

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