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Identification of a Specific Receptor for Plasmin on a Group A Streptococcus

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Certain group A streptococci demonstrate surface receptors that bind selectively to the key fibrinolytic enzyme, plasmin. These bacteria show no reactivity with the zymogen protein plasminogen or with other serine class proteases, such as trypsin or urokinase. Bacterium-bound plasmin retains its ability to cleave synthetic substrates and its ability to hydrolyze a fibrin clot. The bacterium-bound plasmin is not effectively regulated by its physiological regulator, α_2-plasmin inhibitor. This study is the first report of a bacterium-associated receptor for plasmin.

Many group A streptococcal infections are characterized by tissue invasion. A variety of characteristics of the microorganism contribute to its ability to break down natural tissue barriers and to avoid elimination by the host immune response. Certain surface proteins or secreted products associated with streptococci have been identified that enable the organism to elude the immune system, and proteins and toxins produced by the bacteria are known to contribute to tissue damage (6, 8, 17). In addition, receptors of the streptococcal surface that bind specific human proteins with a high affinity have also been described (20, 22). In this report, we describe the presence of a specific receptor on certain group A streptococci that bind the key enzyme in the fibrinolytic system, plasmin. Plasmin has been demonstrated to retain its enzymatic activity when bound to bacteria and might be able to provide localized proteolytic capability to the streptococcus, which might contribute to its tissue-invasive properties.

MATERIALS AND METHODS

Human plasminogen. Human plasminogen was prepared from human plasma by chromatography on lysine Sepharose and molecular sieving chromatography on Sephadex G-100 (13).

Enzymes, inhibitors, and other reagents. The enzymes urokinase and trypsin were obtained from Sigma Chemical Co., St. Louis, Mo. Aprotinin was obtained as Trasylol from Mobay Pharmaceuticals, New York, N.Y. Phe-Pro-Arg-chloromethylketone (PPACK) was obtained from Calbiochem-Behring, San Diego, Calif. *p*-Nitrophenyl *p*-guanidinobenzoate hydrochloride (pNpGB) was obtained from Sigma. Human α_2-plasmin inhibitor (α_2-PI) was obtained from American Diagnostica Inc., Greenwich, Conn. H-D-Val-Leu-Lys-paranitroanilide (S-2251) was obtained from Helena Laboratories, Beaumont, Tex.

Radioiodination of proteins. Human plasminogen, urokinase, and trypsin were iodinated by a mild lactoperoxidase method by using Enzymobeads (Bio-Rad Laboratories, Richmond, Calif.) (16). The labeled proteins were separated from free iodine by passage over a G-25 column (PD-10; Pharmacia Fine Chemicals, Piscataway, N.J.) and collected in 0.15 M Veronal-buffered saline, pH 7.35, containing 0.001 M Mg^{2+}, 0.00015 M Ca^{2+}, and 0.1% gelatin (VBS-gel). The labeled proteins were stored in portions containing 0.02% sodium azide at −20°C. Labeled portions were used once and discarded.

Generation of plasmin. Plasmin was generated from either radiolabeled or unlabeled plasminogen by reaction with urokinase. Urokinase (3 μl; Sigma; 20 U/ml) was added to a 400-μl solution of 1 μM plasminogen containing 0.04 M lysine. The mixture was incubated in a 37°C water bath for 45 min unless stated otherwise. The efficiency of plasmin generation was monitored by measuring the conversion of the single-chain plasminogen molecule (M_r, 90,000) into heavy chains (M_r, 60,000) and light chains (M_r, 25,000) as determined by the migration of radiolabeled proteins, after reduction, on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels. The migration of labeled proteins was determined by autoradiographic exposure of dried gels to Kodak XAR 5 film (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens at −70°C for 20 h.

Determination of binding of radiolabeled proteins to bacteria. The beta-hemolytic group A streptococcus strains were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) overnight at 37°C as stationary cultures (21). The bacteria were harvested by centrifugation and suspended in VBS-gel. The optical density at 550 nm was determined to standardize the concentration of organisms used in subsequent tests. An optical density of 0.3 at 550 nm corresponded to approximately 2×10^9 organisms per ml (21). A standard number of bacteria (approximately 10^9 organisms) were incubated with labeled proteins (approximately 30,000 cpm per tube) in a total volume of 40 μl of VBS-gel for 1 h at 3°C. The bacteria were pelleted by centrifugation at 1,000 × *g* for 10 min and washed twice with 2 ml of VBS-gel. The radioactivity associated with the bacteria was determined in a Beckman 5500 Autogamma counter (Beckman Instruments, Inc., Fullerton, Calif.). All estimates were done in duplicate.

Fibrin plate assay. Fibrin plates were prepared by using 5-cm-diameter disposable petri dishes. Fibrinogen (0.1%; 10 ml) in phosphate-buffered saline was clotted with 0.2 ml of bovine thrombin (10 National Institutes of Health U/ml) in 0.5 M $CaCl_2$. Plasmin (20 pmol) was bound to 100 μl of a 10% (wt/vol) solution of the group A streptococci, strain 64/14, in a total volume of 400 μl of VBS-gel. The mixture was incubated in a 37°C water bath for 45 min. A parallel series

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of samples containing bacteria with no added plasmin served as the negative control. Fifty microliters of a suspension of bacteria or bacteria plus plasmin was placed either directly onto a fibrin plate or onto a 0.22-μm-pore-size membrane filter (Millipore Corp., Bedford, Mass.) placed between the bacteria and the fibrin plate. The plates were incubated for 20 h at 37°C, and the degree of hydrolysis was scored by measuring the area of the zone of clearing from the underside of the plate. In each experiment, a control of free plasmin was included, and each estimate was done in duplicate.

Plasmin-inhibitor complex generation. Plasmin was generated from plasminogen as described above. Three 130-μl samples of the labeled enzyme were placed into separate microtubes (approximately 2×10^6 cpm per tube) and incubated with a 200-fold molar excess of PPACK, aprotinin, or pNpGB for 10 min at room temperature. The volume of each sample was increased by the addition of 200 μl of VBS-gel, and each was applied to a separate G-25 column (PD-10; Pharmacia Fine Chemicals) to remove free inhibitor. Fractions (500 μl) were collected and counted in an Autogamma counter to localize the modified [^{125}I]plasmin. Fractions containing the labeled protein-inhibitor complexes were pooled. Samples of the three labeled complexes were mixed with a 10-fold molar excess of α_2-PI in a final reaction volume of 400 μl for 10 min at room temperature. Plasmin and plasminogen were included as controls. The volumes of each solution were adjusted to 1.1 ml with VBS-gel and then used in a direct binding assay to group A streptococci. Each of the plasmin-inhibitor samples that was incubated with excess α_2-PI was monitored on nonreducing SDS-polyacrylamide gels for the formation of a high-molecular-weight complex (19).

Determination of plasmin activity while bound to bacteria. To five microtubes, each containing 100 μl of a 10% (wt/vol) solution of the group A streptococcus strain 64/14 in a total volume of 400 μl of VBS-gel, 10 nM plasmin was added and allowed to bind for 40 min at 37°C. Five other tubes containing plasmin but no bacteria and one tube containing bacteria alone were prepared as controls. The bacteria were pelleted and washed twice with 1 ml of VBS-gel and suspended in 400 μl of VBS-gel containing a 10-fold molar excess of pNpGB, PPACK, aprotinin, α_2-PI, or buffer alone. All samples were then incubated for 15 min at room temperature. The samples containing bacteria were pelleted by centrifugation, washed with 1 ml of VBS-gel, and suspended with vigorous vortexing in 400 μl of VBS-gel.

To each tube, 20 μl of an 8 mM solution of the chromogenic substrate H-D-Val-Leu-Lys-paranitroanilide was added to yield a final concentration of substrate in the reaction mixture of 400 μM. The tubes were mixed by vortexing and incubated at 37°C for 25 min. At that time, the enzyme reaction was quenched by the addition of 400 μl of 10% (vol/vol) acetic acid, the samples were centrifuged for 5 min at $10,000 \times g$, and the optical densities of the solutions at 405 nm were determined. The release of paranitroaniline from the synthetic substrate monitored at this wavelength was directly proportional to the enzymatic activity of plasmin. Control samples of substrate alone and substrate plus bacteria were included, and each estimate was done in duplicate.

RESULTS

Twenty beta-hemolytic streptococcal isolates were grown overnight at 37°C and tested for their ability to bind radiolabeled plasminogen, plasmin, urokinase, or trypsin as described in Materials and Methods. The results showed that all 20 group A isolates bound plasmin but failed to bind significant quantities of plasminogen or any of the other labeled proteins, i.e., less than 10% of the offered label. Plasmin binding was found to be dependent on the concentration of bacteria and was maximal within 2 min at 37°C. Preincubation with excess unlabeled plasmin prevented binding of the labeled plasmin. In the absence of unlabeled plasmin, strain 64/14 consistently bound approximately 60% of the radioactive plasmin offered and was used to analyze further the selective plasmin-binding activity.

In our initial attempts to characterize the differential binding of plasminogen and plasmin to a group A streptococcus, we compared the kinetics of generation of plasmin from plasminogen with the ability of labeled protein to bind to the bacteria. Conversion of plasminogen to plasmin occurs when a single arginine-valine bond is split in the zymogen by action of the enzyme urokinase (7). The zymogen activation reaction can be monitored on SDS-polyacrylamide gels after reduction of disulfide bonds. The zymogen molecule migrates as a single polypeptide chain with an M_r of approximately 90,000. The active enzyme plasmin migrates under these conditions as two distinct polypeptide chains (a heavy chain with an M_r of approximately 60,000 and a light chain with an M_r of approximately 25,000). The activation reaction can be stopped at any time by addition of a 10-fold molar excess of pNpGB. Consequently, it is possible to obtain plasminogen in various stages of activation and compare the ability of the labeled proteins to bind to a group A streptococcus. The results presented in Fig. 1A demonstrate that the activation of plasminogen to plasmin could be readily monitored. As the conversion of plasminogen to plasmin proceeded, an increase in the binding of labeled protein occurred which correlated with the concentration of plasmin in the reaction mixture (Fig. 1B).

The conversion of plasminogen to plasmin yields a serine active site that is not expressed in the zymogen. In the next series of experiments, the role of the active site in binding of the enzyme to the bacteria was assessed. Plasmin was treated with the active site titrant pNpGB, the small naturally occurring inhibitor aprotinin (5), the selective histidine-modifying agent PPACK (11), and the physiological regulator α_2-PI (14). The ability of the various inhibited forms of plasmin to bind to a group A streptococcus was measured. The results presented in Fig. 2 demonstrate that samples of plasmin treated with pNpGB, aprotinin, or PPACK were all capable of binding to the bacteria in the presence of α_2-PI. By contrast, unmodified plasmin incubated with the physiological inhibitor α_2-PI failed to bind. Each of the plasmin-inhibitor samples that had been incubated with excess α_2-PI was monitored on nonreducing SDS-polyacrylamide gels for the formation of a high-molecular-weight complex. The high-molecular-weight band observed in the third lane indicates the formation of a stable complex of plasmin with its physiological inhibitor α_2-PI (Fig. 2, upper panel). Pretreatment of plasmin with aprotinin, pNpGB, or PPACK inhibited the ability of the enzyme to react with α_2-PI, demonstrating that under the experimental conditions used the plasmin active site was modified.

The next series of experiments were designed to determine whether bacterium-bound plasmin was capable of retaining its enzymatic activity. Radiolabeled plasmin was generated and incubated with a suspension of group A streptococci for 40 min at 37°C. The bacteria with the associated plasmin were recovered by centrifugation, washed twice with buffer, and then tested for their ability to

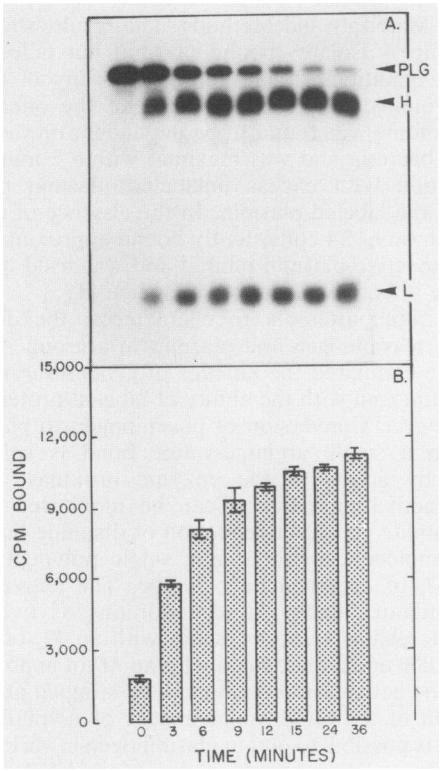

FIG. 1. Binding of plasmin to bacteria: comparison of the kinetics of generation of plasmin and its ability to bind to the group A streptococcus strain 64/14. Labeled plasminogen was converted to plasmin by treatment with urokinase. The kinetics of generation of plasmin was monitored on SDS-polyacrylamide gels under reducing conditions. The conversion of single-chain, high-molecular-weight plasminogen (PLG; M_r, approximately 90,000) into heavy (H; M_r, approximately 60,000) and light (L; M_r, approximately 25,000) chains of plasmin was monitored. At each time point, the ability of labeled proteins to bind to the group A streptococcus strain 64/14 was measured as described in Materials and Methods.

cleave the chromogenic synthetic substrate H-D-Val-Leu-Lys-paranitroanilide (as described in Materials and Methods). In these experiments, a control sample of bacteria alone failed to hydrolyze the chromogenic substrate, whereas bacteria preincubated with plasmin were found to cleave the substrate efficiently. The ability of bacterium-bound plasmin to be affected by a variety of different inhibitors was tested. The results shown in Fig. 3 demonstrate that addition of pNpGB, PPACK, or aprotinin to the bacterium-bound enzyme was capable of inhibiting its enzyme reactivity for the synthetic substrate. By contrast, addition of α_2-PI failed to reduce the enzyme activity (Fig. 3). All inhibitors were used in excess of that required to totally inhibit an equivalent concentration of plasmin in the fluid phase. Because α_2-PI failed to regulate the bacterium-bound enzyme, one might predict that the large molecule fibrin, the natural substrate of plasmin, would also be prevented from occupying the substrate pocket in the active site. To test this prediction, bacteria with plasmin bound to their surface were placed on a fibrin plate and their ability to mediate dissolution of the fibrin clot was measured. The results presented in Table 1 demonstrate that the bacterium-bound plasmin still retained its ability to cleave fibrin. These effects could not be accounted for by dissociation of plasmin from the bacteria, since clot lysis did not occur when the microbe-plasmin complex was separated from the clot by a

TABLE 1. Ability of bacterium-bound plasmin to solubilize a fibrin clot

Sample	Hydrolysis of fibrin[a]	
	Direct[b]	Indirect[c]
Bacteria alone	−	−
Bacterium-bound plasmin	++	−
Plasmin alone	+++	+++

[a] Under the experimental conditions described in Materials and Methods, reactions were graded as follows: +++, zone of clearing with a diameter of 1.0 to 1.5 cm; ++, zone of clearing of 0.5 to 1.0 cm; +, zone of clearing of 0 to 0.5 cm; and −, no clearing.
[b] Sample placed directly onto a fibrin plate.
[c] Sample placed onto a filter placed between the bacteria and the fibrin plate.

0.22-μm-pore-size Millipore filter (Table 1). Under these experimental conditions, unbound plasmin was capable of passing through the filter and causing fibrin degradation.

DISCUSSION

Plasminogen, an inactive zymogen, can be converted to the protease plasmin by a variety of plasminogen activators (3). This enzyme demonstrates broad substrate specificity, and in addition to fibrin it can activate the first component of

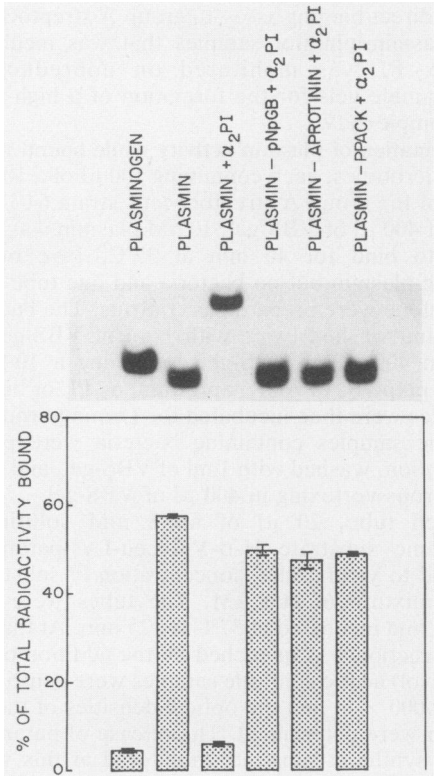

FIG. 2. Effect of inhibiting the active site of plasmin on its ability to bind to the group A streptococcus strain 64/14. The lower panel demonstrates the binding of the group A streptococcus to labeled plasminogen, plasmin, plasmin pretreated with excess α_2-PI, plasmin treated with excess pNpGB, plasmin treated with excess aprotinin, or plasmin treated with excess PPACK. The upper panel demonstrates the analysis of each of the plasmin-inhibitor samples that had been incubated with excess α_2-PI. Samples were monitored on nonreducing SDS-polyacrylamide gels for the formation of a high-molecular-weight complex.

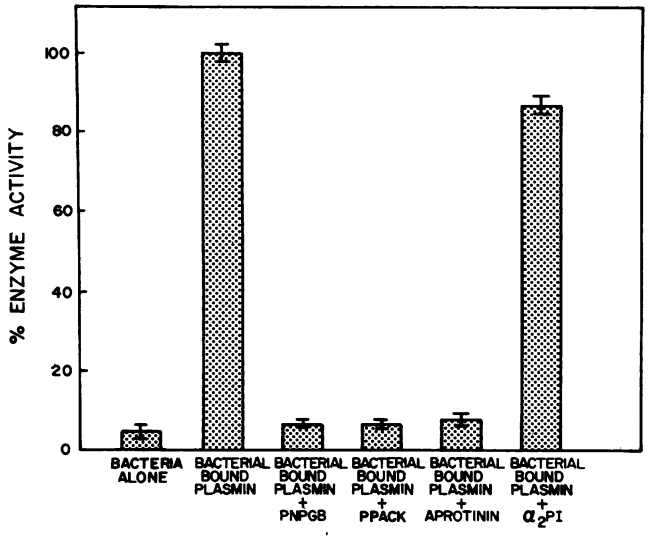

FIG. 3. Regulation of bacterium-bound enzyme activity by a variety of different serine protease inhibitors. Bacterial pellets were preincubated with plasmin, washed, and suspended in buffer containing excess pNpGB, PPACK, aprotinin, or α_2-PI or in buffer alone for 15 min at room temperature. After incubation with the inhibitor, the bacteria were pelleted and washed. Enzyme activity was then measured by the ability of the samples to hydrolyze the chromogenic substrate H-D-Val-Leu-Gly-paranitroanilide as described in Materials and Methods. The hydrolysis by bacterium-bound plasmin in the absence of any inhibitor represents 100% activity. Assays were done in duplicate.

the classical complement pathway and hydrolyze coagulation factors, components of basement membrane, and connective tissue (2, 9, 12). Furthermore, a variety of potent split products are generated as a consequence of plasmin activity, e.g., chemotactic fibrinopeptides (10). Effective regulation of plasmin activity is therefore important, to prevent tissue damage and inflammation. Normally the selective protease inhibitor α_2-PI regulates plasmin activity in humans (1).

Interaction of streptococci and streptococcal products with the fibrinolytic system has been recognized for many years (18). The observation that certain streptococci could lyse a fibrin clot led to the identification and isolation of streptokinase. This secreted protein is known to bind to human plasminogen and plasmin with a similar affinity (15). In this study, we have identified a surface receptor on certain group A streptococci that binds to plasmin while demonstrating no significant affinity for the zymogen form of the molecule, plasminogen. Thus the surface receptor we have identified is distinct from streptokinase.

Binding of plasmin to its bacterial receptor does not inhibit the ability of the enzyme to cleave either small synthetic substrates or its natural substrate, fibrin. Aprotinin, a naturally occurring tight-binding inhibitor of plasmin, and PPACK, which chemically modifies the histidine residue of the active site, can react with the bound plasmin and neutralize its enzymatic activity. These findings suggest that the catalytic portion of the plasmin molecule is not interfered with by the association with the bacteria. Of interest was the observation that the enzymatic activity of bacterium-bound plasmin could not be regulated by addition of its specific inhibitor, α_2-PI. This inhibitor is a potent inhibitor of plasmin in the fluid phase, forming a 1:1 stoichiometric complex between the enzyme and the inhibitor.

The failure of α_2-PI to regulate bacterium-bound plasmin provides the bacteria with a potential mechanism for tissue invasion by virtue of the ability of plasmin to hydrolyze components of connective tissue and basement membranes. Recent studies of the invasive characteristics and metastatic potential of tumor cells have suggested a key role for plasminogen activators in this process (4). The ability of certain group A streptococci to produce a plasminogen activator (e.g., streptokinase) and also to express a receptor for the activation product plasmin might account for certain of its invasive properties. Furthermore, since plasmin bound to a group A streptococcus is incapable of inhibition by α_2-PI, the bacterium has associated with it a nonregulatable proteolytic activity that may contribute to its tissue-invasive properties.

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