α-Enolase, a Novel Strong Plasminogen Binding Protein on the Surface of Pathogenic Streptococci*

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The plasminogen binding property of group A streptococci is incriminated in tissue invasion processes. We have characterized a novel 45-kDa protein displaying strong plasminogen binding activity from the streptococcal surface. Based on its biochemical properties, we confirmed the identity of this protein as α-enolase, a key glycolytic enzyme. Dose-dependent α-enolase activity, immune electron microscopy of whole streptococci using specific antibodies, and the opsonic nature of polyclonal and monoclonal antibodies concluded the presence of this protein on the streptococcal surface. We, henceforth, termed the 45-kDa protein, SEN (streptococcal surface enolase). SEN is found ubiquitously on the surface of most streptococcal groups and serotypes and showed significantly greater plasminogen binding affinity compared with previously reported streptococcal plasminogen binding proteins. Both the C-terminal lysine residue of SEN and a region N-terminal to it play a critical role in plasminogen binding. Results from competitive plasminogen binding inhibition assays and cross-linking studies with intact streptococci indicated that SEN contributes significantly to the overall streptococcal ability to bind plasminogen. Our findings, showing both the protected protease activity of SEN-bound plasmin and SEN-specific immune responses, provide evidence for an important role of SEN in the disease process and post-streptococcal autoimmune diseases.

Streptococcus pyogenes is responsible for a wide variety of human diseases that range from suppurative infections of the throat (pharyngitis), skin (impetigo), and underlying tissues (necrotizing fasciitis), to an often fatal toxic shock syndrome, and the post-streptococcal sequelae, rheumatic fever, and acute glomerulonephritis. Bacterial surface proteins play a major role in these disease processes by exhibiting a wide range of functions. As data have become available, it is clear that most surface proteins found on Gram-positive bacteria, particularly those on group A streptococci, have a great deal of structural similarities (1, 2). Proteins for which the function(s) has been defined have been found to be multifunctional, whereas in others a function has only been attributed to one of two or more domains (2, 3). Thus, the multifunctional characteristics of these surface proteins increase the complexity of the Gram-positive surface beyond what has been previously imagined.

We recently described one such multifunctional protein, streptococcal surface dehydrogenase (SDH), 1 as a major surface protein on group A streptococci and other streptococcal groups which is structurally and functionally related to glyceroldehyde-3-phosphate dehydrogenase (GAPDH) (4). SDH also has an ADP-ribosylating activity (5) and exhibits multiple binding activities to several mammalian proteins such as fibronectin and cytoskeletal proteins (4). A structurally and enzymatically similar streptococcal protein, Plr, was also identified on group A streptococci, based on its ability to bind plasmin (6). SDH, however, is a weak plasmin-binding protein (4). During our studies characterizing the SDH molecule, we reported that a 45-kDa protein was also found in high amounts on the surface of group A streptococci (4). While determining the relative plasmin binding activity of SDH with respect to other streptococcal surface proteins, we found that the 45-kDa protein had in fact strong plasmin binding activity.2

The plasminogen system displays a unique role in the host defense by dissolving fibrin clots and serving as an essential component to maintain homeostasis and vascular potency (7–9). Studies on the ability of Gram-positive bacteria to subvert the fibrinolytic activity of human plasminogen to their own advantage for tissue invasion have been largely focused on pathogenic streptococci and were described first as early as 1933 by Tillett and Garner (10). This property was subsequently attributed to the plasminogen activator, streptokinase (11), an extracellular 48-kDa protein secreted in culture supernatants (12). The role of pathogenic bacteria in tissue invasion utilizing this system has recently been reviewed (13).

In the present communication, we describe purification and characterization of the 45-kDa protein and show that it is the major plasminogen binding molecule on the surface of streptococci. We also show that this protein has significant sequence similarity with one of the important glycolytic enzymes, α-enolase, found generally in the cytoplasm. While bound on the surface of group A streptococci, this 45-kDa protein is found to retain its α-enolase activity, hence we named it SEN (streptococcal surface enolase). It is distinct from the 48-kDa streptokinase (12, 14), the 35.8-kDa SDH (4), the 41-kDa Plr (6), or the 45-kDa plasminogen-binding protein, PAM (15), all of which have been reported to bind plasmin to varying degrees. α-Enolase has not been previously identified on the surface of bacteria; however, it has been shown to be expressed on the surface

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1 The abbreviations used are: SDH, streptococcal surface dehydrogenase; EACA, e-aminocaproic acid; MES, 2-[N-morpholino]ethanesulfonic acid; Plr, plasmin receptor; plasminogen; Plt, plasminogen and plasmin; SEN, streptococcal enolase; PAG, polyacrylamide gel electrophoresis; PEP, phosphoenolpyruvate; PGE, 2-phosphoglycerate; PVDF, polyvinylidene difluoride; PMSF, phenylmethylsulfonyl fluoride; tPA, tissue type plasminogen activator; GAPDH, glyceroldehyde 3-phosphate dehydrogenase; BSA, bovine serum albumin; cfu, colony-forming units; ASD, azido-salicylamido(ethyl-1′-3′-dithiopropionate.

2 V. Pancholi and V. A. Fischetti, unpublished data.
of neuronal (16), cancer (17), and some hematopoietic cells (18, 19) as a novel plasmin(ogen) receptor. Here, in addition to the structural and functional characterization of SEN, we also describe the biological activity of SEN, the functional consequence of plasmin(ogen) binding to SEN, and the enzymatic activity of SEN-bound plasmin.

**Experimental Procedures**

**Bacteria**—Group A β-hemolytic streptococcal strains of various M types and standard strains used for streptococcal grouping were from The Rockefeller University Culture Collection (New York, NY) and listed as follows: M2 (D626), M4 (F694), M6 (D471), M9 (F690), M11 (F743), M14 (T14/46), M15 (D176A), M22 (D943), M25 (B554), M35 (C171), M40 (C270), M44 (C757), M49 (B910), M51 (A291), M58 (D632), M60 (D630), M61 (D336), M62 (D458), M63 (D459), M66 (D794), group B (0902), group C (C74), group D (D76), group E (K131), group F (F68C), group G (D166B), group H (F90A), group L (D167B), and group N (C559). These strains were grown overnight in Todd-Hewitt broth (Difco) and washed once with 50 mM ammonium bicarbonate and were used to prepare bacterial cell wall extracts.

**Human Plasminogen and Plasmin**—Purified human plasminogen and plasmin (lysine-plasmin) were purchased commercially (Sigma). Plasmin was also generated from plasminogen by incubation with urokinase (20 units/ml, Sigma) in HBS gel buffer (50 mM HEPES/NaOH, pH 7.4, containing 1 mM MgCl₂, 0.15 mM CaCl₂, and 0.1% gelatin) containing 40 mM lysine. Conversion of plasminogen to plasmin was maximal after 1 h at 37 °C. This method consistently converted more than 95% of the single chain zymogen molecule plasminogen to the heavy and the light chain of the plasmin molecule as reported earlier (20).

**Radiiodination of Plasminogen and Plasmin**—Purified plasminogen and plasmin were radiiodinated with Na[¹²⁵]I (17 Ci/mg, NEN Life Science Products) by the chloramine-T method, using IODO-Beads (Pierce) as described (4). The labeled proteins were separated from free iodine by passage over a G-25 column (PD-10, Amershams Pharmacia Biotech) and collection in HBS gel. The labeled proteins were stored at −70 °C. Plasmin was also generated from plasminogen by incubation with urokinase (20 units/ml, Sigma) in HBS gel that contained 40 mM lysine (20). More than 95% of the radioactivity was found to be retained with the plasmin. Upon precipitation, specific activity of the labeled plasmin and plasminogen was found to be essentially the same. Furthermore, the specific radioactivity of commercially available purified plasmin (Sigma) and urokinase-gener- 
ated plasmin was also the same. Typically, specific radioactivity of the [¹²⁵]I-labeled plasmin/plasminogen was achieved in a range of 1.2−2.0 × 10⁶ cpm µg⁻¹ protein.

**Blot Overlay System for Plasminogen Binding**—Proteins in the bacterial cell wall extracts were resolved by 12% SDS-PAGE gels and blotted electrophoretically onto a PVDF membrane as described (21, 22). Blots were incubated at room temperature for 3 h in a blocking HBST gel buffer (50 mM HEPES/NaOH, pH 7.4, containing 0.15 mM NaCl, 1% acidified BSA, 0.5% gelatin, 0.5% Tween 20, 0.04% NaN₃) and probed for 4 h at room temperature in the HBST gel buffer containing 2 mM PMPS and [¹²⁵]I-labeled human plasminogen or plasmin 3 × 10⁵ cpm ml⁻¹. The blotted proteins were washed several times with half-strength HBST gel buffer containing 0.35 mM NaCl, dried, and autoradiographed by exposure to Kodak X-OMAT AR film with an intensifying screen for 15 h at −70 °C.

**Extraction of Streptococcal Cell Wall-associated Proteins with Lysin or Mutanolysin**—M6 strain D471 was grown to stationary phase at 37 °C for 18 h in 4–6-liter batches of Todd-Hewitt broth. Bacteria were pelleted by centrifugation, washed, and resuspended in 50 mM phosphate buffer (1/50th of the original culture volume) containing 30% raffinose and 5 mM diithiothreitol and 5 mM EDTA. Streptococcal cell wall extracts using lysin (an amidase) enzyme (128 units/ml, Sigma) in 0.5% SDS-PAGE, electrophoresed onto a PVDF membrane, and probed with labeled plasmin(ogen). A strong plasmin(ogen) binding activity was found to be mainly associated with a 45-kDa protein of the sequentially fractionated cell wall extract with 40−60% saturation of ammonium sulfate (Fig. 1, A and B). For further purification, 40−60% ammonium sulfate precipitates were used as starting material. The dialyzed precipitate was concentrated (Centriprep-10, Amicon) and stored at −70 °C until further use. The concentrated sample was applied to a Mono Q FPLC column (HR10/10, Amersham Pharmacia Biotech) pre-equilibrated with 50 mM Tris/HCl buffer, pH 8.0. After washing with 5-column volumes of this buffer, bound proteins were eluted with a 70-ml linear NaCl gradient from 0 to 700 mM and then with a 20-ml linear NaCl gradient from 700 mM to 1 M. Protein elution profile in each fraction was determined by SDS-PAGE and by Coomassie blue stain. A duplicate gel was Western blotted and probed with [¹²⁵]I-labeled plasmin(ogen) as described above. The 45-kDa protein eluted at 630 mM NaCl. The pooled fractions containing the 45-kDa protein and exhibiting plasmin(ogen) binding activity were dialyzed against the starting buffer and re-chromatographed on the Mono Q column using the same conditions. The positive fractions were again pooled and concentrated to a volume of <1.0 ml, using Centriprep-30 and Centricon-30 concentra-
tors," the 45-kDa protein will be referred to as SEN (surface enolase).

The supernatants were analyzed by measuring absorbance at 240 nm as an investigation whether this protein is enzymatically active. The protein was visualized by staining with 0.1% Ponceau S (Sigma) in 1% acetic acid sequence homology of the 45-kDa protein with plasmin(ogen) binding activity were pooled. These fractions were then subjected to automated Edman degradation. Each sample contained approximately 5 µg of the protein as determined by the BCA protein estimation method (Pierce). A duplicate sample of PVDF membrane was added to each sample for peptide mapping (Lyo-tag system, Boehringer Mannheim), and the resulting peptide fragments were separated by capillary electrophoresis interphased with the matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Perspective Biosystems). N-terminal sequences of the two internal peptide fragments were then determined as described above. All microsequence analyses were performed at the Protein/Biotechnology Facility of the Rockefeller University.

α-Enolase Activity and Enzyme Kinetics—The strong N-terminal sequence homology of the 45-kDa protein with α-enolase prompted us to investigate whether this protein is enzymatically active. α-Enolase activity was measured essentially as described earlier by both the coupled assay (24) and the direct assay at A260 (25).

In the coupled assay, α-enolase activity was determined by measuring the transformation of NADH to NAD+. The enzymatic reactions were performed at 37 °C in 100 mM HEPES buffer, pH 7.0, containing 3.3 mM MgSO4, 0.2 mM NADH, 0.3 mM 2-phosphoglycerate (2-PGE), 1.2 mM ADP, 10.3 IU of lactate dehydrogenase, and 2.7 IU of pyruvate kinase in a final reaction volume of 1.0 ml. The reaction was started by adding 100 µl of the test solution containing α-enolase. The α-enolase activity was measured in a SDS-PAGE extra on a PVDF membrane. The presence of SEN in different cell fractions was monitored by affinity purified anti-SEN polyclonal antibodies against SEN were used as a positive control. Mice with high antibody titers were given a second dose of antigen intraperitoneally in distilled water. Mouse spleens were excised 3–5 days after the last booster. The spleen cell fusion to P3-NS1/LAG4–1(NS-1) myeloma cells was performed as described (27, 28). Hybridomas cloned by limiting dilution were grown in 2-liter rolling tissue culture flasks. From these cultures, secreted monocalonal antibodies were precipitated at 50% ammonium sulfate saturation. The precipitates were then dialyzed and purified using a protein A-Sepharose affinity column.

Production and Purification of Rabbit Polyclonal Antisera Against SEN—Polyclonal antibodies to SEN were prepared in New Zealand White rabbits immunized subcutaneously with 150 µg of purified SEN emulsified in complete Freund’s adjuvant (1:1) at multiple sites. Rabbits were boosted twice, each time with 150 µg of the purified protein in incomplete Freund’s adjuvant (1:1) at 3-week intervals. The rabbits were bled 10 days after the third immunization. All sera were filter-sterilized and stored at 4 °C.

To prepare SEN-specific IgG, the polyclonal serum was subjected to sequential purification on protein-A Sepharose CL-4B (Amersham Pharmacia Biotech) and SEN affinity columns. The affinity column was made by covalently linking approximately 2 mg of purified SEN to 0.5 g of CNBr-activated Sepharose 4B (Pharmacia). The affinity column was washed with 10 mM Tris/HCl buffer, pH 8.0, containing 10 mM MgSO4, and 10% glycerol, and eluted with 0.1 M NaCl. The elution was monitored by absorption at 280 nm. The eluted fraction was concentrated and affinity purified on the SEN affinity column using a strategy similar to that as described above. SEN-specific IgG was then used for immunohistochemical analyses.

Location and Nature of Plasmin(ogen) Binding Domain of SEN—To determine the location of SEN in streptococcal cells, an overnight culture of strain D471 was subjected to lysozyme digestion in 30% raffinose buffer to extract streptococcal cell wall-associated proteins as described above. From the resulting proteolysates, the membrane and cytoplasmic fractions were separated as described previously (5, 22). Proteins from each cellular fraction were resolved by SDS-PAGE extra on a PVDF membrane. The presence of SEN in different cell fractions was monitored by affinity purified anti-SEN polyclonal antibodies (75.0 nm g−1) and monoclonal antibodies (12 nm g−1) as described (4, 5).

Immune Electron Microscopy—Group A streptococci (D471) from the overnight TH broth cultures were harvested, washed, and adjusted to a concentration of 107 cfu/ml. An aliquot of 200 µl of the bacterial suspension was absorbed to the affinity purified anti-SEN(1A10) or with anti-SDH (4F12) monoclonal antibodies for 4 h followed by a 2-h incubation with colloidal gold (5- and 10-nm sized beads for anti-SEN and anti-SDH labeled bacteria, respectively) anti-mouse IgG (Amersham Pharmacia Biotech, 1:25) at room temperature. The labeled bacteria were then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 4 h at 4 °C. The fixed labeled bacteria were then processed for transmission electron microscopy as described (29).

Prevalence of SEN in Various Group A Streptococcal M Types and Streptococcal Groups—Proteins from the cell wall extract of several M serotypes after lysozyme digestion and those of various streptococcal grouping strains (group A–H, L, and N) after mutanolysin digestion were resolved by SDS-PAGE and transferred to a PVDF membrane. The blots were blocked, probed with affinity purified anti-SEN rabbit polyclonal antibodies (75.0 nm g−1) for 3–4 h, and the reactive protein bands were visualized as described (4, 5).

Location and Nature of Plasmin(ogen) Binding Domain of SEN—Polyclonal antibodies to SEN (10 µg) was treated with carboxypeptidase Y (Boehringer Mannheim) at a substrate to enzyme concentration ratio of 13.5:1 in 50 mM HEPES buffer, pH 7.0, at 70 °C for 16 h. Equilibrium dialysis of the digests and untreated SEN were resolved by SDS-PAGE, and their plasminogen binding activity was determined by the blot overlay method as described above. In another set of similar experiments, plasmin(ogen) binding activity was measured in the presence of 0.1 mM EACA or 0.1 mM lysine.

Ligand Binding Assays for Plasmin(ogen) Binding to Immobilized SEN—The ligand binding analysis was carried out using 96-well mi...
crotter (C8 Maxi Break-apart, Nagle Nunc International, Naperville, IL). The plates were coated with 100 μl of purified SEN in 0.05 M carbonate buffer, pH 9.6 (5 μg ml−1), for 3 h at 37 °C and were then kept at 4 °C overnight. The plates were washed and blocked with HBST gel blocking buffer for 4 h at room temperature. A serial 2-fold dilution of SEN labeled with plasminogen (ogen) was tested in triplicate. Non-specific binding was measured after the addition of a 200 nM excess of unlabelled plasminogen/plasmin or 0.1 M EACA. Non-specific binding was also evaluated in BSA-coated plates. Non-specific binding contributed between 4 and 10% of the total counts without these agents. Specific binding was calculated by subtracting non-specific binding (10%) from the total binding. The amount of free plasmin(ogen) was calculated by subtracting specifically bound plasmin(ogen) from the total amount of labeled plasmin(ogen) added. To determine the equilibrium dissociation constant (Kd), a nonlinear least square analysis of the total count offered versus the count bound was carried out using the curve fitting computer program from Sigma Plot. The values of bound plasmin(ogen) versus bound/free ratio were plotted, and the slope representing 1/Kd was determined. The percentage of plasmin(ogen) bound to immobilized SEN in wells, in the absence of any competitor, was treated as the maximum binding value. Percentage of the maximum binding at different concentrations of the competitors was plotted.

Cross-linking Studies—Sulfosuccinimidyl-2-[p-azido-salicylamido]ethyl-1-3-dithiopropionate (SAPSO) (Pierce, 300 mg) was iodinated (0.5 mg 125I-Na) with IODO-GEN (100 μg), and conjugation of [azido-salicylamido]ethyl-1,3-dithiopropionate to plasmin(ogen) (400 μg) was carried out in the dark essentially as described before (31) with minor modifications. 125I-labeled plasminogen-ASD was purified on a PD-10 column as described above. Cross-linking of 125I-labeled plasminogen-ASD with intact group A streptococci was performed as described (30). The specific binding of plasmin(ogen) to SEN was also determined in a competition assay in which a constant amount of 125I-plasminogen (0.77 pmol) and 125I-plasmin (1.37 pmol) was mixed with decreasing concentrations of free plasmin(ogen) (5 ng to 50 μg, i.e. up to 380 pm excess). The amount of plasmin(ogen) bound to immobilized SEN in wells, in the absence of any competitor, was treated as the maximum binding value. Percentage of the maximum binding at different concentrations of the competitors was plotted.

Identification of a Novel 45-kDa Plasminogen/Binding Protein in the Group A Streptococcal Cell Wall Extracts—By Western blot, we examined the plasmin(ogen) binding activity of proteins in a crude streptococcal cell wall extract using 125I-labeled plasmin and plasminogen (Fig. 1A). The results showed that, in addition to the weak plasminogen binding to the 39-kDa SDH molecule (4), a significantly stronger plasminogen binding occurred with a 45-kDa protein present in the streptococcal cell wall extract. We also found similar binding activities of SDH and the 45-kDa protein with 125I-labeled plasminogen (Fig. 1B). These findings identify a new protein with strong plasminogen (ogen) binding activity in the cell wall extract and confirm our previous report of the weak plasminogen/ogen) binding activity of SDH (4).

Purification of the 45-kDa Protein—The 45-kDa protein was partially purified from the cell wall extract by 40−60% ammonium sulfate precipitation. The protein was further purified by ion-exchange chromatography on a Mono Q column followed by a Superose-12 molecular sieve. With the latter, the peak elution volume having plasminogen/binding activity corresponded to that of a standard 150-kDa IgG molecule, suggesting that the native form of the 45-kDa protein is likely a multimer. Final purification was achieved on a Poros BUM hydrophobic column (Fig. 2A). The average yield of purified 45-kDa protein from a total of 10 liters (1.5 × 109 CFU/ml) of bacterial culture was 1.128 mg.

Terminal Amino Acid Sequence and Identification of the 45-kDa Protein as an α-Enolase Enzyme—N-terminal amino acid sequence of the 45-kDa protein revealed a single amino acid in the first 50 residues (Fig. 2B). N-terminal sequences of two internal peptides (Pep-1, molecular mass 1712.1 Da; and Pep-2, molecular mass 1683.5 Da) obtained after cleavage with a lysine-specific endopeptidase were also determined for 15 and 11 residues, respectively. The presence of a single amino acid at each sequence cycle for the intact 45-kDa protein and each
internal peptide verified the homogeneity of these molecules.

When the three sequences were compared with known sequences in the translated GenBankTM data base, 81% identity and ~90% homology was found with the first 50 N-terminal residues of α-enolases of Bacillus subtilis and those of prokaryotic (Fig. 2B) and eukaryotic origin (comparison not shown). Similarly, the two internal peptide sequences (Pep-1 and Pep-2) were compared with sequences in the GenBank™ data base. The results show a strong sequence identity (87.5–100%) with the N-terminal and peptide fragments of B. subtilis α-enolase (Bst-α-enolase). The location of the Pep-1 corresponds to the middle of the molecule whereas that of Pep-2 corresponds to the C-terminal region of the molecule.

α-Enolase Activity and Enzyme Kinetics—By establishing whether the sequence of the 45-kDa protein was that of α-enolase, we investigated whether it also possessed the activity of this glycolytic enzyme. In a coupled-enzyme assay, the 45-kDa protein converted terminal NADH to NAD in a dose-dependent manner (Fig. 2A). This indicated the conversion of pyruvate to lactate by lactate dehydrogenase and NADH, confirming the presence of the cytoplasmic enzymes lactate dehydrogenase and pyruvate kinase and found no activity (not shown). Based on these results, the 45-kDa protein is hereafter referred to as SEN.

Subcellular Location and Prevalence of SEN in Other M Types and Streptococcal Groups—By using affinity purified rabbit anti-SEN antibodies and monoclonal antibodies, we examined the distribution of SEN in various subcellular fractions of group A streptococci by Western blot analysis. The results showed that SEN is found in both the cell wall and cytoplasmic fractions, with negligible amounts in the membrane fraction (Fig. 4A). Furthermore, SEN was found to be present in comparable quantities in all M types examined and in all streptococcal groups except group N (Fig. 4B). The uniform antibody reaction that showed no obvious size heterogeneity among the SENs in different M types indicates that SEN is a conserved protein in all streptococci tested.

Plasminogen Binding Activity of SEN and Its Comparison with That of SDH—To compare the plasminogen binding activities of purified SEN and SDH, equal quantities of the purified proteins were separately resolved by SDS-PAGE and electro-blotted onto PVDF membranes. The blots were then probed with either 125I-plasminogen, 125I-plasmin, or 125I-plasminogen.
min derived from \(^{125}\)I-plasminogen by urokinase. The results showed that plasmin(ogen) bound weakly to SDH compared with SEN (Fig. 5A). The results are in agreement with our previous results (4) and with those shown in Fig. 1. Furthermore, SEN consistently showed significantly higher binding affinity for plasminogen than plasmin (Fig. 5A).

The plasmin(ogen) binding activity of SEN was found to be significantly decreased in the presence of 0.1 m lysine or 0.1 m EACA (not shown), suggesting that the exposed lysine residue(s) in SEN is likely responsible for this binding activity (Fig. 5B). Similarly, carboxypeptidase Y-treated SEN also showed significant decrease in binding activity to plasmin(ogen), indicating that the lysine-binding residues are likely located at the C-terminal end of the SEN molecule (Fig. 5C).

To determine the specific binding activity of plasmin(ogen) to SEN, a quantitative solid phase assay was carried out using 96-well microtiter plates and different concentrations of \(^{125}\)I-plasminogen and \(^{125}\)I-plasmin. Nonspecific binding of \(^{125}\)I-labeled plasminogen/plasmin to only BSA-coated wells or with SEN-coated wells in the presence of 0.1 m EACA or 200 molar excess of unlabeled plasminogen(ogen) did not exceed \(-10\%\) of the bound counts in the absence of these agents. Thus, to determine the specific binding of plasminogen(ogen) to SEN, only \(90\%\) of the total bound counts were considered for the calculation.

\(^{125}\)I-Labeled plasminogen and plasmin reacted specifically and strongly with SEN-coated wells. Scatchard analysis of the binding of plasminogen and plasmin to SEN was nonlinear, indicating the presence of more than one site for this interaction. Thus, for plasminogen, two equilibrium dissociation constants (\(K_{D,pol} = 1.3\) nm and \(K_{D,pl} = 7.4\) nm) were recorded. Although plasmin bound to SEN with relatively lower affinity, Scatchard analysis revealed a similar nonlinear curve with two distinct equilibrium dissociation constants (\(K_{D,pol} = 2.2\) nm, \(K_{D,pl} = 27\) nm) (Fig. 5D). The reaction between \(^{125}\)I-plasminogen/plasmin and immobilized SEN (solid phase on microtiter plates) was inhibited by unlabeled plasminogen/plasmin in a dose-dependent manner. At 156 molar excess of unlabeled plasminogen, approximately \(90\%\) inhibition in the binding of the labeled plasminogen to SEN was recorded. At a similar concentration of free plasmin, relatively less inhibition (75%) in the binding of the labeled plasmin was recorded. Together, these results revealed the specificity of the interactions and high affinity of plasminogen(ogen) to SEN, indicating that SEN may have more than one site for the binding of plasminogen(ogen) and may serve as the major plasminogen binding molecule on the surface of group A streptococci.

**Role of SEN in Plasminogen Binding Activity of Streptococci**—In addition to enzymatic and biochemical properties suggesting that SDH (4) and SEN are located on the streptococcal surface (Figs. 2–4) and to provide additional proof of their presence on the streptococcal surface, SEN- and SDH-specific mouse monoclonal antibodies (1A10 and 4F12, respectively) were used in indirect immune electron microscopy. As shown in Fig. 6A, both SEN and SDH molecules, reacting with their specific monoclonal antibodies (\(4\,\mu\)g/10^6 streptococci), were found on the surface of streptococci. Their binding patterns suggest that the distribution of these proteins is either in the form of a cluster or the epitopes recognized by specific monoclonal antibodies are not uniformly exposed on the cell surface. The latter argument is supported by the fact that even at higher concentrations of both monoclonal antibodies (up to 20 \(\mu\)g of IgG/10^6 streptococci), of the distribution of gold particles was the same as that seen with lower concentrations of monoclonal (data not shown).

By having confirmed the location of SEN and SDH on the streptococcal surface, the role of these proteins in plasminogen binding by streptococci was further investigated. Since plasminogen binding activity is dependent on the C-terminal lysine residue, we subjected intact streptococci to carboxypeptidase B digestion to remove the C-terminal lysine residues of the plas-
minogen-binding proteins. These results were compared with those obtained for the plasminogen binding activity of streptococcal cell wall proteins extracted from intact untreated and carboxypeptidase B-treated streptococci (Fig. 6B). In this analysis, carboxypeptidase B-treated streptococci showed a significant reduction in plasminogen binding activity. Similarly, by phosphor image analysis, the cell wall-associated SEN from the carboxypeptidase B-treated streptococci showed 20–30% less binding as compared with SEN associated with untreated streptococcal cell walls (Fig. 6B). In both treated and untreated streptococci, plasminogen binding activity of cytoplasmic SEN was found to be the same. The fact that carboxypeptidase B did not completely remove the plasminogen binding activity suggests that whereas the C-terminal lysine residue of a protein molecule may play an important role in its ability to bind plasminogen, the region N-terminal to the C-terminal lysine residue may also play a role in this binding. As described above, plasminogen binding activity of SDH was found to be significantly less than that of SEN, and carboxypeptidase B treatment did not seem to affect plasminogen binding activity.

In the blot overlay plasminogen binding assay, both SEN and SDH were found to bind plasminogen with a distinct high and low affinity; however, it is not clear whether SEN or SDH or both these molecules, while bound on the streptococcal surface, participate in plasminogen binding. To understand the individual roles of these molecules in streptococcal plasminogen binding activity, a cross-linking study was carried out using 125I-labeled plasminogen-ASD and intact streptococci. The presence of the labeled proteins was detected in the streptococcal cell wall extract and not in the corresponding cytoplasmic preparation, indicating that 125I-labeled plasminogen-ASD came in contact with only surface-exposed molecules. Furthermore, SEN, but not SDH, was found to be labeled, suggesting that SEN, because of its high affinity with plasminogen may be cross-linked with 125I-labeled plasminogen-ASD more efficiently as compared with SDH (Fig. 6C). These results were further confirmed by a competitive inhibition binding assay in which the plasminogen binding activity of intact streptococci was measured in the presence of purified SEN or SDH (Fig. 6C). The results showed that in the presence of low concentrations of purified SEN (≤1.6 μg), up to 50% of the plasminogen binding activity was inhibited and at higher concentrations (≥12 molar excess) up to 75% inhibition could be achieved (Fig. 6C). At similar low concentrations of SDH, plasminogen binding activity of streptococci was inhibited only to a minimal level (6%). Plasminogen binding activity of streptococci could be inhibited up to 50% by 12 molar excess of free SDH (Fig. 6C). Together these data indicate that SEN may play an important role in plasminogen binding.
FIG. 5. Plasminogen-binding properties of SEN. A, comparison between the plasminogen binding activity of purified SEN and that of SDH (4) (see also Fig. 1). One μg each of SEN, SDH, and cytoplasmic SDH (4, 5) were electroblotted in replicates onto a PVDF membrane. One gel was stained with Coomassie Blue. The PVDF membranes were each blocked and probed with either 125I-labeled plasminogen, 125I-plasmin, or 125I-plasmin (urokinase).

B

| Control | 0.1 M lysine |
|---------|-------------|
| 1 | 0.5 | 0.25 | 0.13 | 0.07 | 0.04 |

C

Carboxy-peptidase Y treatment (hour)

| 6 | 12 | 18 |

Enzyme(-) | Enzyme(+) | Enzyme (+) anti-SEN

D

Binding of Plasminogen to SEN

Binding of Plasmin to SEN

Competitive Inhibition

Fig. 5. Plasminogen-binding properties of SEN. A, comparison between the plasminogen binding activity of purified SEN and that of SDH (4) (see also Fig. 1). One μg each of SEN, SDH, and cytoplasmic SDH (4, 5) were electroblotted in replicates onto a PVDF membrane. One gel was stained with Coomassie Blue. The PVDF membranes were each blocked and probed with either 125I-labeled plasminogen, 125I-plasmin, or
role in streptococcal plasminogen binding and is the functional
plasminogen-binding receptor protein on the surface of group A
streptococci.

Protease Activity of Plasmin and Activated Plasminogen
Bound to Purified SEN—The proteolytic activity of free and
SEN-bound plasmin was determined by measuring their able
to cleave the chromogenic substrate Val-Leu-Lys-para-ni-
troanilide in the presence and absence of $\alpha_2$-antiplasmin, a fast
acting plasmin inhibitor. The results show that, either bound to SEN or free in solution, exhibits the same proteolytic activity (Fig. 7). However, SEN-bound plasmin was not inacti-
vated as rapidly in the presence of $\alpha_2$-antiplasmin as free plas-
min. After 4 h, SEN-bound plasmin still retained activity
whereas free plasmin was nearly completely inactivated (Fig. 7).

We next studied the proteolytic activity of SEN-bound plas-
imogen to plasmin after tPA activation in the presence and
absence of $\alpha_2$-antiplasmin. As expected, plasminogen (either
SEN-bound or free) exhibited no proteolytic activity (Fig. 7),
whereas tPA-activated plasminogen showed high activity, with
the SEN-bound form exhibiting somewhat higher activity (23% more) than an equal amount of free plasminogen. However,
after 4 h in the presence of $\alpha_2$-antiplasmin, SEN-bound tPA-
activated plasminogen exhibited a significantly elevated pro-
teolytic activity when compared with the activated form of free
plasminogen (Fig. 7). In contrast, $\alpha_2$-antiplasmin could not
inactivate streptokinase-activated free or bound plasminogen
as reported earlier (13, 34). Taken together, these results indi-
cate that plasmin bound to SEN is significantly resistant to
inactivation by $\alpha_2$-antiplasmin.

Opsonophaeytosis of Group A Streptococci in the Presence
of anti-SEN Antibodies—To understand the biological role of
SEN as an important streptococcal surface protein, we deter-
mined whether antibodies to SEN were able to opsonize these
organisms in vitro. The opsonic activity of anti-SEN antibodies
was measured in terms of the ability of streptococci to survive
in blood from a nonimmune individual who lacks type-specific
anti-M antibodies against the test strain. The results, as shown
in Fig. 8, A and B, revealed that affinity purified anti-SEN IgG antibodies effectively opsonized and enhanced the phagocytosis
of group A streptococci of two different serotypes (types 6 and
14). These results not only confirm the surface location of SEN
on streptococci but also suggest that anti-SEN IgG antibodies
may foster non-type-specific protection against streptococcal
infection.

DISCUSSION

The plasminogen binding property of pathogenic bacteria in
general, and of streptococci in particular, is suggested to be
one of the characteristics that may contribute to tissue invasion
and the overall pathogenicity of group A streptococci (13, 34).
Plasminogen binding is responsible for the degradation of intravascular clots and extracellular proteolysis in a wide va-
diety of physiological and pathological processes (7–9). In this
report, we identify and characterize a novel plasminogen-
binding protein, SEN, on the surface of group A streptococci.
Structurally and functionally this protein is an $\alpha$-enolase, one of
the key glycolytic enzymes, and is the second glycolytic
enzyme that we have identified on the surface of group A
streptococci. We reported earlier that streptococcal surface de-
hydrogenase (SDH), a major protein on the surface of these
organisms, is structurally and functionally related to the gly-
colytic enzyme glyceraldehyde-3-phosphate dehydrogenase and
is able to bind plasmin weakly (4). On the basis of latter
activity, another group has found a structurally and function-
ally similar protein, Plr (plasmamin receptor), from a clinical
group A streptococcal strain (6). The identification of this 45-
kDa plasminogen-binding protein as $\alpha$-enolase was based on its
strong sequence identity, both at its N-terminal end and within
two different internal peptides to other reported $\alpha$-enolases.
We have identified and isolated SEN from the M6 streptococcal
strain from which SDH was originally identified and purified (4).

Functional identity of SEN in its purified form, as well as on
the streptococcal surface, was confirmed by its ability to cata-
yze the conversion of 2-PGE to PEP in both direct and coupled
enzyme assays. The values for the enzyme kinetic constants,
$K_m$ and $V_{max}$, for purified SEN (Fig. 3) are comparable with
those of other reported $\alpha$-enolase enzymes (35, 36). Further-
more, both monospecific polyclonal and monoclonal antibodies
against SEN were found to be important tools for its cellular
location in group A streptococci and its presence on the surface
of other streptococci (Fig. 4).

From this and several other reports (6, 14, 15), it is apparent
that group A streptococci express more than one type of plas-
imogen binding receptor which acquires plasminogen through
various mechanisms (34). Clinical isolates and animal-
passaged strains have more plasminogen binding capacity
then the same strains passaged in the laboratory (37). Thus,
group A streptococci expressing several low affinity plasminogen
binding molecules may bind the equivalent amount of
plasminogen as strains that express fewer high affinity mole-
cule(s). In addition to strain specificity, M type specificity has
also been reported for the plasminogen-binding protein, PAM,
in group A streptococci (15). Although not characterized, at
least two types of plasminogen receptors (low and high affini-
ity) have been reported in group G streptococci (38), a chara-
teristic that may well be found on other streptococcal groups.
We report here that in group A streptococci, surface glyceral-
dehyde-3-phosphate dehydrogenase (SDH/Plr) is a weak plas-
imogen-binding protein and that SEN is a strong plasmino-
gen-binding protein (Fig. 5). By using anti-SEN antibodies, we
have identified $\alpha$-enolase-like molecules on the surface of both
capsulated and unencapsulated strains of Streptococcus pneu-
moniae, but not on staphylococci, suggesting that surface
$\alpha$-enolase (plasminogen binding) may be an important viru-
lence determinant for pathogens of the respiratory muco-
sa.

the labeled product (urokinase-plasmin) obtained from the urokinase-treated $^{125}$I-labeled plasminogen. The plasminogen/plasmin binding activity of SEN and SDH was then visualized by autoradiography. B, effect of the presence of lysine or plasminogen binding activity of SEN. Different
centrations (0.04–1.0 $\mu$g) of purified SEN was Western-blotted on PVDF membranes, and plasminogen binding activity as described above in
A was carried out in the presence and absence of 0.1 m lysine and visualized by autoradiography. C, effect of carboxypeptidase Y treatment on the
plasminogen binding activity of SEN. 50 $\mu$g of purified SEN was treated with carboxypeptidase Y (3.7 $\mu$g) for 6, 12, and 18 h. Enzyme treated (+) and untreated (−) preparations were electrobotted onto PVDF membranes, reacted with $^{125}$I-labeled plasminogen, washed, and autoradiographed. A duplicate PVDF membrane containing the enzyme-treated SEN was also stained with anti-SEN polyclonal antibodies (75 ng/ml) as described in
Fig. 4, A, D, to determine specific binding of plasminogen and plasmin to SEN. Microtiter plate (break-apart) wells coated with 0.5 $\mu$g/well SEN were incubated with increasing concentrations of aprotinin/PMSP-treated $^{125}$I-plasminogen and -plasmin. Non specific binding was assessed as
binding to BSA-coated wells and subtracted from the total binding. Non specific binding contributed ~10% of the total binding. Scatchard analysis of
the specific binding data both for plasminogen and plasmin is shown as an inset in the corresponding figure, respectively. Competitive inhibition
of the interaction between plasminogen/plasmin and SEN was assessed by determining the binding of the indicated amount of $^{125}$I-plasminogen/plasmin to immobilized SEN in the presence of increasing molar excess of unlabeled plasminogen or plasmin. Each data point represents the mean of
three different experiments. In each experiment, an average reading for each parameter was calculated from three individual wells.
-Enolase is one of the key glycolytic enzymes found generally in the cytoplasm; nevertheless, its presence on the surface of cells is not without precedent. Several eukaryotic studies have provided evidence that α-enolase-related molecules are expressed on the surface of several cell lines such as U937 human monocytoid (19), human breast tumor (17), peripheral blood monocytes, and neutrophils (18) and that these molecules contribute about 10% of the plasminogen-binding capacity of the cells. Recently, α-enolase has also been shown to be present as an abundant immunodominant antigen in the cell wall of Candida albicans (39, 40). In prokaryotes, however, the presence of cell-surface α-enolase has not been previously reported.

**Fig. 6. Role of SEN in overall plasminogen binding activity of streptococci.** A, immunogold electron microscopic detection of the location of SEN (magnification × 35,000) and SDH (× 25,700) on thin sections of group A streptococci pretreated with anti-SEN (1A10) and anti-SDH (4F12) monoclonal antibodies, followed by colloidal gold-labeled anti-mouse IgG (gold particle sizes for SEN and SDH are 5 and 10 nm, respectively). Control represents the sections obtained from streptococci pretreated with only anti-mouse conjugate. B, effect of carboxypeptidase B (CPB) on the plasminogen binding activity of streptococci. Plasminogen binding activity was determined on streptococci fixed to 96-well microtiter plates using increasing concentrations of 125I-plasminogen. Effect of the enzyme treatment on the plasminogen binding activity of streptococci was measured after treatment of some wells with fixed streptococci. Each point represents an average of values obtained from three individual wells. Effect of CPB treatment on the 125I-plasminogen (Pg-I125) binding activity of surface-exposed SEN and its cytoplasmic homolog was determined by the blot overlay method as described under “Experimental Procedures” and evaluated by a PhosphorImager (Molecular Dynamics). For the comparison of cell wall extracts from treated and untreated streptococci, each lane contains 50 μg of total protein. Similarly, for the cytoplasm, each lane contains 80 μg of total protein. Locations of SEN and SDH are shown by arrowheads. C, cross-linking studies using 125I-labeled plasminogen-ASD. After cross-linking of the labeled complex with streptococci, the reaction mixture was irradiated. Cell wall extract and cytoplasm were obtained from the irradiated streptococci as described under “Experimental Procedures.” Aliquots of cell wall proteins (75 μg/lane) and cytoplasmic proteins (50 μg/lane) were resolved by SDS-PAGE and transferred onto PVDF membranes. Proteins were visualized by Coomassie stain, and the presence of SEN and SDH in the cell wall extract and cytoplasm was detected by the immunostaining method. Autoradiograph showing the labeled streptococcal proteins on a duplicate blot was analyzed by a PhosphorImager. Competitive inhibition of the plasminogen binding of intact streptococci was carried out in the presence of increasing amounts of purified SEN and SDH. Binding of plasminogen to streptococci in the absence of any additive was taken as 100%. Plasminogen binding in the presence of different concentrations of SEN or SDH was determined as the percentage of total binding. Each point represents the average of 3–4 individual readings.
Our present findings on the plasmin(ogen) binding activity of SEN (Fig. 5) differ from those of the reported eukaryotic plasminogen binding α-enolases (18) in two major respects. (i) SEN exhibits significantly higher affinity for plasmin(ogen) \( (K_D = 1–4 \text{ nM}, \text{Fig. 5D}) \) as compared with that of eukaryotic enolase \( (K_D = 0.1–2 \text{ μM}) \). (ii) In contrast to eukaryotic enolase, SEN exhibited more than one interaction site for plasminogen and plasmin. Although plasminogen and plasmin showed comparable binding affinity to SEN in a solid phase assay, the latter consistently bound less efficiently on Western blots. We speculate that in addition to the C-terminal lysine binding site of the SEN molecule for plasmin(ogen), the region upstream of this site may also be responsible for the binding. This is supported by the fact that plasmin(ogen)-binding proteins that do not possess lysine residues at their C-terminal ends also show appreciable affinity for plasmin(ogen), possibly through other exposed lysine residues (38, 41).

The high affinity of intact group A streptococci for plasminogen \( (K_D = 0.2 \text{ nM}) \) has recently been attributed to the Plr protein, a member of GAPDH family (6); however, this activity may actually be due to the combined activity exhibited by several such binding proteins on the streptococcal surface. Recently, glyceraldehyde-3-phosphate dehydrogenase isolated and purified from Streptococcus equisimilis has been shown to have an equilibrium constant in the range of 220–260 nM for plasminogen and about 25 nM for plasmin (41). We reported earlier that SDH, also a member of GAPDH family, is a weak plasminogen-binding protein (4). In view of these reports and of other published reports showing low affinity plasminogen-binding proteins of group A streptococci (14, 15, 38), SEN may be the major plasminogen-binding protein on the surface of group A streptococci. Based on its ubiquitous presence on the surface of a variety of group A streptococcal serotypes and streptococcal groups (Fig. 4), we suggest that SEN, or an SEN-like molecule, may serve as a major plasmin(ogen)-binding molecule/receptor on the surface of nearly all pathogenic streptococci and would therefore play an important role in disease outcome.

Earlier reports that the Plr molecule (6, 42, 43) or other plasminogen-binding proteins (15, 41) are the streptococcal plasminogen-binding receptors, are based on the plasminogen binding activity of the purified natural or recombinant proteins using different methods. It is not clear, however, if Plr (6) or SDH (4) is in fact the streptococcal plasminogen-binding receptor and, if so, whether this binding activity represents the observed plasminogen binding activity exhibited by intact streptococci. Hence, in the present communication we investigated whether plasminogen binding activities of SEN and SDH are relevant to the observed streptococcal plasminogen binding activity. We first confirmed their surface location using immunoelectron microscopy with SEN- and SDH-specific monoclonal antibodies. Furthermore, we found a significant reduction in plasminogen binding activity of carboxypeptidase B-treated intact streptococci indicating that the streptococcal domain of SEN and/or SDH which contains the C-terminal lysine residue is exposed to the surface. In conjunction with these results, we found that the plasminogen binding activity of the surface-bound SEN, but not that of cytoplasmic SEN, was reduced after treatment with carboxypeptidase B, suggesting that SEN was accessible to this enzyme. The observation that
the enzyme-treated streptococci also showed significant plasminogen binding activity raises the possibility that the region which is N-terminal to the C-terminal lysine residues of SEN (and also that of SDH) may also play a role in plasminogen binding activity. From the cross-linking studies that were designed to determine whether SEN, SDH, or both the proteins play a role in streptococcal plasminogen binding, it was possible to determine that SEN is probably more exposed to the surface as compared with SDH, since the labeled ASD from the 125I-labeled plasminogen-ASD complex was found to cross-link to SEN rather than to SDH. A dose-dependent inhibition of the streptococcal plasminogen binding activity, even in the presence of low concentrations of free SEN, further confirmed that the high affinity of SEN for plasminogen inhibits the ability of the bacteria to bind to plasminogen. These results together indicate that SEN serves as a primary receptor for plasminogen binding. The question of how glyceroldehyde-3-phosphate dehydrogenase (SDH/Plr) (4, 6) and α-enolase (SEN) are transported through the cell membrane and sorted onto the cell surface without the presence of a signal sequence remains intriguing. Whether SEN, like eukaryotic surface α-enolases, is transported by internal signal sequences like that found with plasminogen activator inhibitor 2 (44) or by the post-translational acylation method (45) needs further investigation. It is possible that SEN and SDH are transported to the cell surface as a complex in conjunction with a secreted protein utilizing a specialized secretory system. What is clear, however, is that SEN and SDH, are members of the growing group of proteins which lack signal sequences but are transported to the surface and anchored to cells by an as yet undefined mechanism (44).

Like GADPH/SDH (4, 46), eukaryotic α-enolase has also been shown to be a multifunctional protein presenting a variety of activities besides its native glycolytic activity. Functions such as being a structural component of turtle lens, J-crystallin (47), a neurotropic factor (48), the ability to form a stable complex with Clostridium difficile toxin B (49), and the ability to bind to polynucleotides (50) have been recently reported for α-enolase. If SEN, like eukaryotic α-enolases (47–50) and SDH (4, 5), is also a multifunctional molecule, its physiological implications are at present equivocal.

Our finding that plasmin bound to SEN retains its proteolytic activity even in the presence of α,α-antiplasmin indicates that SEN may be an important streptococcal virulence determinant. Particularly in infected tissues, such a characteristic would enable the streptococcus to become more invasive by evading localization by the host's clotting pathway (34). The fact that α-enolase has recently been found to be secreted in the growth medium (39, 51) and that increased levels of fungal-specific α-enolase have been found in patients with invasive candidiasis (52) suggest that a similar phenomenon may exist in cases of invasive streptococcal infection. Understanding the properties of this molecule during growth and infection may prove useful in sorting out the complex pathogenic properties displayed by group A streptococci.

α-Enolase present in the cell wall of C. albibicans (40) has been designated as an abundant immunodominant antigen in cases of invasive candidiasis (39, 51). The fact that antibodies to SEN enhance the phagocytosis of group A streptococci of heterologous M types (Fig. 6) lends further support to the surface location of SEN and indicates that antibodies to surface molecules other than M protein are oposonic for group A streptococci, although M-specific antibodies are more effective (33). It is likely that the immune response to streptococcal α-enolase may also play an important role in the final outcome of a streptococcal infection in view of the fact that α-enolase is also present in several hematopoietic cells as a surface-expressed molecule (18). However, whether such an immune response does occur in humans during streptococcal infection is presently unknown.

The presence of α-enolase on the surface of streptococci and also on the surface of a variety of mammalian tissues including brain (neuron-specific enolase) (8, 18) adds new insight in the role of SEN-specific antibodies in post-streptococcal autoimmune diseases such as glomerulonephritis and neurological disorders such as Sydenham’s chorea, a major manifestation of rheumatic fever (53). Since anti-enolase-specific antibodies have been reported in systemic rheumatic diseases (54), and autoimmune polyglandular syndrome (55), the latter as a result of C. albibicans-specific enolase, as well as cell-mediated immunity to enolase in schizophrenia (56), a possible role of SEN in post-streptococcal autoimmune diseases cannot be ruled out.

On the basis of biochemical and biological properties, SEN together with SDH/Plr (4, 6) develop an emerging theme for a new class of bacterial surface proteins. In vitro cross-protective nature of the anti-SEN antibodies, coupled with the ubiquity of SEN on the streptococcal surface, may prove useful for an immunotherapeutic intervention against streptococcal diseases. In addition to this, its potential role in autoimmune disease suggests that SEN is an important biologically active molecule with a substantial role in streptococcal pathogenesis.

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