

Identification of an Extracellular Plasmin Binding Protein from Nephritogenic Streptococci

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Summary

Examination of the extracellular products of nephritis(+) and nephritis(-) group A streptococci revealed the presence of a 46-kD protein secreted by nephritogenic strains that binds to human plasmin. Immunological data revealed that this protein, called nephritis plasmin binding protein (NPBP), is not related to group A streptokinase nor to a recently described streptococcal dehydrogenase protein. The binding of human plasmin to this protein can be blocked by ϵ -amino caproic acid, indicating the importance of lysine groups in the binding process. Mutanolysin extracts of cell walls from these nephritogenic strains probed with anti-NPBP antibody were negative for cell wall-bound NPBP. Serological data with acute poststreptococcal glomerulonephritis (APSGN) and acute rheumatic fever sera indicated that the protein reacts preferentially with APSGN sera. Amino acid sequence analysis and immunological reactivity suggest NPBP is the streptococcal pyrogenic exotoxin B precursor, also previously described as zymogen (streptococcal proteinase precursor). The secretion of both group A streptokinase and a secreted plasmin binding protein in the same nephritogenic strain raises an intriguing hypothesis of the mechanism of action of this protein in APSGN.

Investigations spanning several decades have revealed that only certain strains of group A streptococci (1) are capable of causing acute poststreptococcal glomerulonephritis (APSGN). The acute onset of the disease coupled with the fact that "skin" or impetigo nephritis produces a disease identical in all respects to "throat" nephritis has suggested that nephritogenic streptococci might secrete a product unique to those streptococcal strains associated with nephritis.

Previous work in our laboratory has identified two extracellular products, called nephritis strain-associated protein (NSAP) and group A streptokinase, of similar molecular mass that are preferentially secreted by nephritogenic streptococci (2, 3). While NSAP was identified in 18 of 21 APSGN biopsies and reacted only with the sera of APSGN patients, streptokinase was not localized in 10 APSGN biopsies. Furthermore, streptokinase did not react uniquely with APSGN sera (4).

In view of the discrepancies noted above, we reexamined the question of what are the biological activities of the protein we have called NSAP. The reports by Lottenberg et al. and Broder et al. (5, 6), that a streptococcal cell wall plasmin receptor protein had plasmin binding properties and had approximately the same molecular mass as NSAP, prompted us to examine the extracellular products (ECP) of nephritogenic streptococci for a plasmin binding protein.

The present report demonstrates that the extracellular products of all nephritis-associated streptococci tested secrete a

plasmin binding protein not seen in nonnephritogenic streptococci. The size of this protein (46,000 daltons) is similar to that previously described for NSAP (2), and immunological and structural data confirm that it is not group A streptokinase.

Materials and Methods

Streptococcal Strains. All streptococcal strains were from The Rockefeller University collection and were part of the group of strains used in previous studies of APSGN (2, 3). Nephritis (+): F203d (type 1), A374 (type 12), B923 (type 12), A207 (type 2), B905 (type 4), and B512 (type 3). Nephritis (-): A456 (type 36), A830 (type 3), A990 (type 4), 1GL22 (type 30), 1GL22 (type 30), 1GL362 (type 19), and A834 (type 49). Nephritogenic strains were defined as those strains isolated directly from a patient with APSGN while the other strains were not associated with known nephritis.

Isolation of ECP. The method of isolation of ECP for each strain was carried out essentially as previously described (3) with the minor modification of adding 10% dialyzed Todd Hewitt medium (peptides <10,000 daltons) to the chemically defined medium for better growth. The ECP of all strains were adjusted to the same total protein concentration before use.

SDS-PAGE. All electrophoretic analyses were carried out according to the procedure of Laemmli (7) using 10% SDS gels.

Immunoblots. All ECP were immunoblotted using the procedure of Towbin et al. (8). The signal antibody, alkaline phosphatase-conjugated F(ab)₂ goat anti-rabbit serum, was diluted 1:1,000 (Tago, Inc., Burlingame, CA).

Plasmin Binding Protein (PBP) Determinations. Nitrocellulose paper, containing the transferred ECP of the various strains, was first blocked for 2 h in 10 mM Tris, 0.5% Tween, 1% crystalline BSA, pH 8.2, and then probed as follows: the paper was bathed in a solution containing human plasmin at 20 ng/ml (Sigma Chemical Co., St. Louis, MO), 1% BSA, 0.5% Tween, 0.04% sodium azide, pH 7.0, plus added PMSF (2 mM) for 18 h at room temperature. After several washes in blocking buffer to remove residual plasmin, the paper was probed with a rabbit anti-human plasmin IgG F(ab)₂ preparation at 1:100 dilution for 90 min at room temperature. After repeated washes in blocking buffer, the paper was exposed to the alkaline phosphatase-conjugated goat anti-rabbit F(ab)₂ antibody (1:1,000) for an additional 1 h and then developed with alkaline phosphatase substrate (Sigma Chemical Co.). Appropriate controls included preimmune rabbit serum, signal antibody alone, and first antibody without plasmin.

Human Sera. Human sera were obtained from The Rockefeller University collection. Sera from well-documented acute rheumatic fever (ARF) and APSGN patients were used. Controls were age matched and were obtained from the same geographical area as the patient sera.

Animal Sera. To obtain antiserum specific for human plasmin, rabbits were immunized with commercially available human plasmin (Sigma Chemical Co.) at a concentration of 1 mg/ml in incomplete adjuvant X2 ~1 mo apart. Serum was collected 10 d after the second injection and used at a 1:5,000 dilution. In some experiments, pepsin-digested antibody, F(Ab)₂, of these sera was used at a dilution of 1:100.

For the production of antibody to the NPBP, the pure protein band was identified on SDS-PAGE gels. The gel slices were cut out, lyophilized, pulverized, taken up in a small volume of PBS, and used for immunization of rabbits at 3-wk intervals. 10 d after the third immunization, animals were bled and tested for reactivity to the binding protein in immunoblots. The whole serum was diluted 1:4,000 for most experiments. A F(ab)₂ IgG fraction diluted 1:100 was used in some experiments.

Rabbit polyclonal antibodies to streptococcal proteinase zymogen as well as streptococcal pyrogenic toxin B (SPEB) were kindly provided by Drs. Liu (Center for Biologics Evaluation and Research, FDA, Bethesda, MD) and Ferretti (University of Oklahoma), respectively.

Cell Wall Extractions. Mutanolysin extraction of the nephritic(+) strains was carried out according to method of Lottenberg et al. (5). After extraction, the suspension was centrifuged for 10 min at 10,000 g and the supernatants of each strain were adjusted to same protein concentration.

Purification of NPBP. The dialyzed ECP (0.05 M Tris buffer, pH 8.4) of strain F203D was applied to a DEAE column and eluted stepwise with 0.1-, 0.3-, and 0.5-M NaCl gradients. The 0.1-M fraction containing the protein was dialyzed in 0.05 M Tris buffer (pH 9.5) and applied to a FPLC Mono Q column. The fractions containing the protein were combined, concentrated, and further purified to homogeneity by passage through a FPLC Superose 12 column. SDS-PAGE and immunoblots were used to monitor the purification and eventual purity of the preparation.

Purification of Group A Streptokinase. Group A streptokinase was purified as previously described (3). The purified material was used at 100 µg/ml and 10 µl of the material was added to each lane in the SDS-PAGE gels.

Protein Sequence Studies. The purified NPBP was run on a 10% SDS-PAGE gel transferred to PVDF Immobilon-P membrane (Millipore Corp., Bedford, MA), and the strip cut out after identification of the pure protein by Coomassie stain and immunoblot. The strip

containing the NPBP protein was sent to The Rockefeller University sequencing facilities for NH₂-terminal amino acid analysis.

ε-Amino-Caproic Acid (EACA) Studies. The purified NPBP material was run on a 10% SDS-PAGE, transferred to nitrocellulose paper, and cut into strips to which decreasing dilutions of the EACA solution (Sigma Chemical Co.) starting at 0.5-M concentration were added. The strips were then probed with the standard concentration of plasmin followed by the addition of the antiplasmin antibody as described above.

Results

Comparable aliquots (300 µg/ml) of the concentrated dialyzed ECP from nephritogenic and nonnephritogenic strains were run on SDS gels and stained with Coomassie blue (Fig. 1). As can be seen (arrows) a protein appears at 46 kD in all six nephritogenic ECP that is not seen in the six nonnephritogenic ECP. While the general ECP gel patterns differed extensively between nephritogenic and nonnephritogenic strains, the 46-kD protein was consistently present in all nephritogenic strains.

Immunoblots of the ECP from eight strains were used to determine the plasmin binding capacity of the proteins. As seen in Fig. 2 A, a distinct band appears at 46 kD only in the ECP of nephritogenic streptococci (lanes 1-4). Using similar immunoblots with antisera to streptokinase, the lower bands seen in lanes 2 and 3, which also bound plasmin, were clearly identified as streptokinase, a finding also noted by Broder et al. (6) in their studies.

To verify the presence of the NPBP in only the ECP of

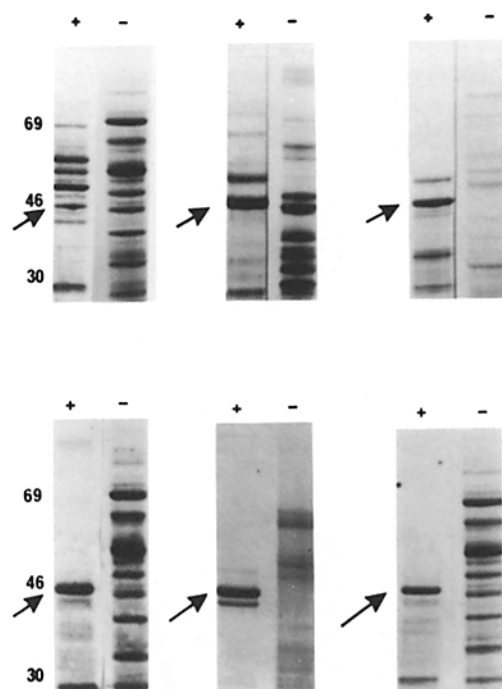


Figure 1. SDS-PAGE gels of the ECP of six nephritogenic(+) and six nonnephritogenic(-) strains. The arrows denote the presence of a protein band of 46 kD seen only in the nephritis(+) strains. All ECP were adjusted to a protein concentration of 300 µg/ml, and 6 µg of protein was loaded in each lane. Molecular mass markers (kD) are depicted on the left.

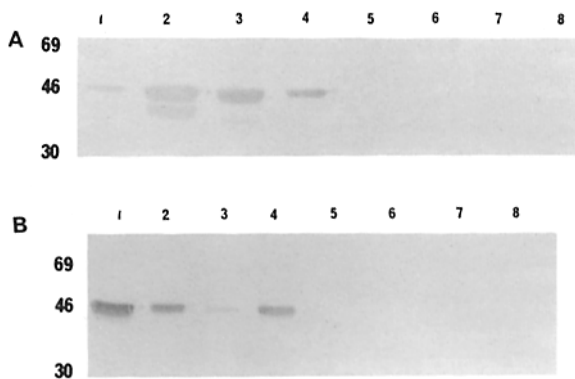


Figure 2. (A) The ECP of four nephritic(+) and four nonnephritic(-) strains were transferred to nitrocellulose paper and probed with 20 ng/ml of human plasmin for 24 h. After washing, the paper was probed with rabbit anti-human plasmin antibody followed by signal antibody and substrate. Note the presence of a positive band in the ECP of nephritis(+) strains (lanes 1-4) and the absence of these bands in nonnephritic ECP (lanes 5-8). The lower bands in lanes 2 and 3 were identified as streptokinase using monoclonal and polyclonal antibodies to group A streptokinase (data not shown). Lane 1, A207; lane 2, B512; lane 3, B923; lane 4, F203D; lane 5, A990; lane 6, A834; lane 7, A830; lane 8, A456. (B) The ECP of the same strains used as in A were transferred to nitrocellulose and probed with rabbit antibody to purified NPBP followed by the appropriate signal antibody. Lanes 1-4 represent ECP of nephritis(+) strains, while lanes 4-8 represent the ECP of the nephritis(-) strains. Note the presence of the band at 46 kD only in the ECP of nephritis(+) strains. Lane 1, A374; lane 2, F203D; lane 3, A207; lane 4, B923; lane 5, A990; lane 6, A834; lane 7, A830; lane 8, A456. In both experiments preimmune sera from rabbits as well as the signal antibody alone were negative.

nephritogenic strains, immunoblots were probed with antibody prepared against the purified NPBP. Fig. 2 B demonstrates that only the ECP of nephritic strains contain the NPBP and it is the same protein to which human plasmin bound (see Fig. 2 A). To determine whether lysine groups played an important role in the binding of plasmin to NPBP (a finding noted by Broeseker et al. [9] in their studies of the cell wall plasmin receptor protein), varying concentrations of EACA

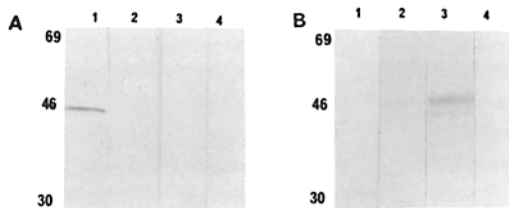


Figure 3. (A) Immunoblots of purified NPBP probed with various antibodies. Lane 1 probed with rabbit anti-NPBP antibody (kindly provided by Dr. Pancholi, The Rockefeller University); lanes 2 and 3, polyclonal and monoclonal antibodies to group A streptokinase; lane 4, probed with anti-SDH antibody (kindly provided by Dr. Pancholi). Note the positive reaction only in lane 1. (B) Immunoblots of group A streptokinase probed with various antibodies. Lane 1 is anti-SDH antibody; lanes 2 and 3 are monoclonal and polyclonal antibodies to group A streptokinase, respectively; lane 4 was probed with anti-NPBP antibody. Note positive reactions only in lanes 2 and 3 with antibodies specific for group A streptokinase.

were added to immunoblot strips of transferred NPBP before the addition of human plasmin. Concentrations of EACA as low as 1.0 mM effectively blocked the binding of human plasmin to NPBP (data not shown).

To be sure that NPBP was neither group A streptokinase (similar molecular mass) nor the streptococcal surface dehydrogenase (SDH) proteins recently described by Pancholi and Fischetti (10) and Lottenberg et al. (11), immunoblots using purified NPBP were probed with the appropriate antibodies. Fig. 3 A demonstrates that antibody to NPBP reacts specifically with purified antigen (lane 1). In contrast, polyclonal and monoclonal antibodies prepared against streptokinase (lanes 2 and 3) were negative. Similar negative results were obtained when antibody to SDH (lane 4) was used.

Using a Western blot with purified streptokinase (Fig. 3 B), the converse was also true. Antibody to SDH did not bind to group A streptokinase (lane 1), while both polyclonal and monoclonal antibodies bound to purified streptokinase (lanes 2 and 3). Finally, NPBP antibody did not bind to group A streptokinase (lane 4).

The NPBP protein eluted from SDS gels was transferred to nitrocellulose paper and probed with APSGN, ARF, and normal sera. Only APSGN sera recognized the plasmin binding protein when compared with ARF and normal sera (see Fig. 4).

The NH₂-terminal sequence of the NPBP was determined from a PVDF membrane (Millipore Co., Bedford, MA) containing the transferred protein. No sequence similarities were found with previously characterized streptococcal plasmin binding proteins, streptokinase (3), SDH (10), or the cell wall-bound plasmin receptor protein described by Lottenberg et al. (11). Comparisons of the NPBP peptide sequence with the Swissprotein data base showed complete identity with a region from the NH₂ terminus of streptococcal pyrogenic exotoxin B (SPEB) precursor (Fig. 5).

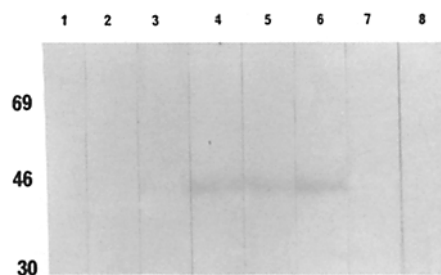


Figure 4. Immunoblots of nitrocellulose paper transfer of 10% SDS-PAGE gels of purified NPBP probed with various human sera. Lanes 1 and 2 are ARF sera; lanes 3-6 are APSGN sera; lanes 7-8 are normal sera. Note a strong band of reaction with NPBP in all four APSGN sera lanes. All human sera were used at a 1:1,000 dilution.

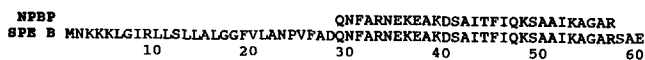


Figure 5. NH₂-terminal amino acid sequence of NPBP aligned with the NH₂ terminus of streptococcal pyrogenic exotoxin B (SPE B) precursor.

Immunoblots of purified NPBP and streptococcal zymogen probed with our anti-NPBP, as well as antibody to SPEB, and the antizymogen antibody revealed identical bands, thus confirming the structural data (Fig. 6 A).

Liu and Elliot (12) reported that streptococcal zymogen can be converted to active proteolytic form by treatment with the reducing agent dithiothreitol (DTT). To test for this activity, NPBP and purified zymogen (a gift from Dr. Liu) were treated for 2 h at 37°C in 0.5 mM DTT. After SDS-PAGE of the DTT-treated proteins, immunoblots stained with anti-SPEB identified showed that both zymogen and NPBP preparations were sensitive to the DTT treatment, as revealed by a change in their electrophoretic migration (Fig. 6 B). However, neither the reduced zymogen nor NPBP exhibited proteolytic activity (data not shown).

Finally, to determine whether nephritogenic streptococci contained NPBP on the cell surface of the nephritogenic strains similar to that described by Lottenberg et al. (5), Western blots of mutanolysin-extracted cell walls were examined. Using the NPBP antibody as an immunological probe, none of the strains known to secrete NPBP contained a similar molecule in the cell wall extracts (data not shown).

Discussion

When our results were compared with those reported by Lottenberg et al. (5) and Broder et al. (6), several important differences were noted. Their plasmin binding protein is a cell wall-associated protein of 41.8 kD and was found in all 20 random group A streptococcal strains tested (5). In contrast, our plasmin binding protein was secreted into the medium and was found only in the ECP of nephritogenic streptococci. Furthermore, our NPBP was 46 kD in contrast to the 41.8 kD described by them (5). Finally, the amino

acid analysis of their protein (11) and the one described by Pancholi and Fischetti (10) reveal extensive homology with glyceraldehyde-3-phosphate dehydrogenase while our protein does not. Further studies by Broder et al. (6), using one streptococcal strain, demonstrated that their protein was not secreted into the extracellular medium whereas the group A streptokinase secreted by that strain exhibited strong plasmin binding activity.

In confirmation of the structural data, immunoblots with appropriate antisera revealed that our 46-kD protein was antigenically distinct from both group A streptokinase and an SDH protein recently described by Pancholi and Fischetti (10), which also exhibits plasmin binding activity. SDH has the identical NH₂-terminal sequence of the plasmin binding protein reported by Lottenberg et al. (11). The NPBP reported here was also found to react with antibodies found only in APSGN sera (see Fig. 4), a finding not seen in immunoblots with group A streptokinase (4).

Whether this protein is in reality the NSAP originally described by Villarreal et al. (2) remains unclear. The molecular mass of our protein is similar to the 47-kD protein described previously by Villarreal et al. (2), and its preferential reactivity with APSGN sera is also similar. Unfortunately, antiserum prepared against NSAP is no longer available for direct comparisons. Future studies will determine whether this protein is also localized in the glomeruli of APSGN patients, similar to the studies described by Villarreal et al. (2).

An interesting and potentially important biological observation was the experiment by Lottenberg et al. (13) demonstrating that the active plasmin, once bound to the cell wall receptor protein, was not inactivated by α -2 antiplasmin, a physiological inhibitor of plasmin present in all human sera. Further studies by this group demonstrated that EACA at a concentration of 0.15 mM effectively blocked the binding of plasmin to its cell wall receptor, indicating the importance of lysine groups in the binding reaction (9). Similar experiments were carried out with our protein. Using varying concentrations of EACA, our results indicate that 1.0-mM concentrations of EACA totally blocked the binding of plasmin to NPBP in immunoblots.

The amino acid sequence of NPBP suggests this protein is the precursor of SPEB. Properties of this toxin include pyrogenicity and skin rash production, and it is a potent V β -specific T cell mitogen with properties of a superantigen (14). The amino acid sequence encoded by the SPEB structural gene has been shown to be very similar to the protein sequence described for the streptococcal proteinase precursor (15). It is now believed that the streptococcal proteinase, initially described by Liu and Elliot (12), and SPEB result from allelic variations of the same gene.

This study suggests a third activity for this protein through its ability to bind human plasmin. Through this function it may contribute in another way to the virulence of group A streptococci. The fact that the same strains secreting a plasmin binding protein concomitantly secrete group A streptokinase (3) raises an intriguing hypothesis concerning the pathogenic mechanisms whereby nephritogenic streptococci might initiate tissue destruction. Once in the tissues of the

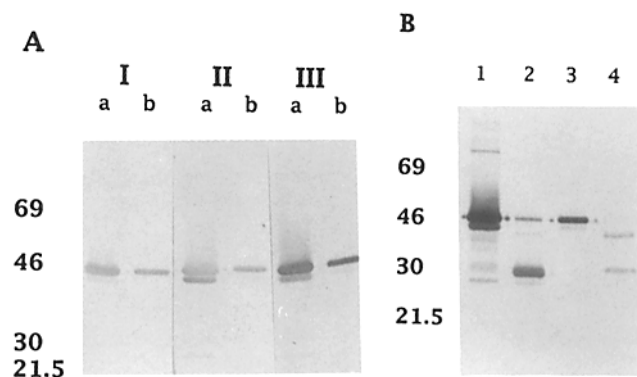


Figure 6. (A) Immunoblots of zymogen (lanes a) and purified NPBP (lanes b) probed with anti-NPBP (I), antizymogen (II), and anti-SPEB (III) antibodies. Note the presence of identical bands on immunoblots of SPEB, proteinase zymogen, and NPBP when probed with the three different antibodies. (B) Immunoblot of NPBP and zymogen showing the change in electrophoretic mobility after DTT treatment. Lane 1, zymogen incubated for 2 h at 37°C in reaction buffer (0.05 M Tris-HCl, pH 7.0); lane 2, zymogen incubated in reaction buffer with 0.5 mM DTT; lane 3, NPBP incubated in reaction buffer; lane 4, NPBP incubated in reaction buffer with 0.5 mM DTT.

susceptible host, group A streptokinase, known to be secreted by all nephritogenic strains, converts human plasminogen to the active plasmin moiety. The activated plasmin then binds to the plasmin binding molecule also secreted by the same nephritogenic strain. This activated NPBP-plasmin complex (also possibly protected from inactivation by α -2 antiplasmin) is thus capable of activation of the complement cascade, chemotaxis, and hydrolysis of connective tissues and basement membranes, all prime features of acute inflammation in APSBN. The fact that this plasmin binding protein is in reality an SPEB precursor raises the question as to whether the protease activity and/or its superantigen properties might

also contribute to the inflammatory damage seen in APSGN. Schlievert et al. (16) noted that nephritogenic group A isolates consistently produced SPEB either alone or in combination with the other two characterized pyrogenic exotoxins (SPEA and SPEC). These data emphasize the need to further investigate this toxin's contribution to nephritis.

Whether or not this secreted plasmin binding protein either alone or within circulating complexes of these patients is also localized in the glomeruli of APSGN patients and has particularly affinity for certain receptors in glomerular tissue is currently under investigation.

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