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Binding of Human Plasminogen to *Borrelia burgdorferi*

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We studied the binding of plasminogen to *Borrelia burgdorferi*, a spirochete which causes Lyme disease and produces no endogenous proteases which digest extracellular matrix proteins. Using ^{125}I-labeled plasminogen, we demonstrated that *B. burgdorferi* bound human plasminogen and that this binding was inhibitable with unlabeled plasminogen. ^{125}I-labeled plasminogen binding by *B. burgdorferi* was also inhibited by the lysine analog ε-aminocaproic acid. There was no significant difference in the binding of Glu- or Lys-plasminogen to *B. burgdorferi*. Binding of plasminogen was similar in low-passage (infectious) and high-passage (noninfectious) isolates of *B. burgdorferi*. Plasminogen bound to the surface of *B. burgdorferi* could be converted into plasmin by a human urokinase-type plasminogen activator. ^{125}I-labeled plasminogen ligand blots of borrelial membrane proteins demonstrated two prominent binding proteins at ~70 and ~30 kDa. By Western blot (immunoblot), the 30-kDa protein was found to be outer surface protein A (Osp A) of *B. burgdorferi*. ^{125}I-labeled plasminogen binding to both the 70-kDa protein and Osp A was inhibited by ~90% with a 1,000-fold excess of unlabeled plasminogen. By scanning densitometry, the 70-kDa band bound >10 time more ^{125}I-labeled plasminogen than did Osp A. An Osp A-deficient mutant of *B. burgdorferi* and wild-type *B. burgdorferi* bound equal amounts of ^{125}I-labeled plasminogen. Ligand blots of membrane proteins from an Osp A-deficient mutant showed association of ^{125}I-labeled plasminogen at only the 70-kDa protein. Two-dimensional gel electrophoresis showed that the 70-kDa protein had a pI of ~5.3, clearly separable from Osp A. The association of host plasmin(ogen) with borrelial surface proteins provides a mechanism by which *B. burgdorferi* can digest extracellular matrix and disseminate.

Plasminogen is a 92-kDa glycoprotein which is the zymogen for the major mediator of fibrinolysis in humans. Plasminogen is converted to its active form, plasmin, by the splitting of a single peptide bond. Plasmin binds to its major substrate, fibrin, through discrete, triple-loop structures known as kringles. Both plasmin and plasminogen contain five kringles which can interact with ω-amino acids on fibrin and with certain plasminogen activation effectors (17). Recently, specific receptors for plasminogen have been described for macrophages, tumor cells, and some bacterial species (3, 15, 25, 26). Plasminogen binding to these bacteria also appears to be mediated by ω-amino acid binding sites located within the kringles (5, 25, 26).

Although its established role is in fibrin degradation, plasmin is more generally a serine protease with trypsin-like specificity. Since a number of cells can express surface plasmin activity, the ability of plasmin to act as a protease with broad substrate activity has led to the hypothesis that it may play a role in the spread of cells by facilitating the digestion of extracellular matrix proteins. Plasmin has been shown to degrade many of the components of the mammalian extracellular matrix, including fibronectin and laminin. Plasmin can also activate the conversion of procollagenase to collagenase, which would further enhance extracellular matrix protein digestion (1).

In serum, free plasmin is quickly inactivated by inhibitors such as α_1- and α_2-antiplasmin (8). However, plasmin bound to the surface of a cell or to fibrin is stabilized and protected against inactivation (15). The conversion of bound plasminogen to plasmin directly on the cell surface results in the generation of active plasmin protected against inhibition.

Borrelia burgdorferi is a spirochete which causes Lyme disease. It is transmitted to humans by the bite of an infected *Ixodes* tick. From the inoculation site, the bacteria spreads locally in the skin, causing the characteristic rash of erythema migrans. It can subsequently disseminate widely, causing varied manifestations including arthritis, carditis, and meningitis (23). Most other bacteria which have the abilities to spread in the skin and to disseminate produce enzymes which degrade extracellular matrix components (7, 18). In previous studies, we demonstrated the acquisition of human plasmin activity on the surface of *B. burgdorferi*. In this report, we characterize the binding of human plasminogen to *B. burgdorferi* and describe two major borrelial membrane proteins which mediate plasminogen binding.

MATERIALS AND METHODS

Borrelial isolates and cultivation. *B. burgdorferi* was grown at 32°C in modified Barbour-Stoenner-Kelly (BSK II) medium prepared as previously described (2). Cultures were monitored by dark-field microscopy for growth and contamination. The number of spirochetes were determined by dark-field microscopy and by measuring the optical density of suspensions at 595 nm and comparing them with standards. The strains used in these experiments included G39/40, a high-passage, noninfectious strain; 297, an infectious human meningeal isolate; NECK, an infectious human skin isolate; N40, an infectious strain isolated from ticks; and 7x297, a noninfectious mutant strain lacking outer surface proteins A and B (Osp A and B, respectively) (19). Both noninfectious, high-passage (more than 15 passages) and infectious, low-passage (fewer than 15 passages) isolates of 297 and N40 were used in these experiments. Borrelia were centrifuged for 2 min at 11,000 × *g*. The supernatant was removed, and the pellet of bacteria remaining was washed three times with phosphate-buffered saline (PBS) containing 0.2% bovine albumin (PBSA).

Reagents. Highly purified human Glu-plasminogen separated by lysine-Sepharose chromatography in the presence of aprotinin followed by treatment with aprotinin-Sepharose to remove plasmin was purchased from American Diagnostica, Inc. (Greenwich, Conn.). No impurity could be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Highly purified human Lys-plasminogen was a gift of Jack Henkin and Andrew Mazar (Abbott Laboratories, Abbott Park, Ill.). Human single-chain urokinase (uPA) was obtained from Calbiochem-Novabiochem Corp. (La Jolla, Calif.). Unless otherwise noted, all other reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.).

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Radiolabeling of plasminogen. Plasminogen was labeled with iodine-125 (New England Nuclear, Boston, Mass.) by the chloramine-T method (10). Briefly, 50 μg of plasminogen was suspended in 0.25 M phosphate buffer at pH 7.4. The plasminogen was then mixed with 4 μl of chloramine-T (2.5 mg/ml in 0.25 M phosphate buffer) and 30 ng of ^{125}I (17.4 Ci/mg). After 10 s at room temperature, 20 μl of sodium *meta*bisulfite (5 mg/ml in 0.25 M phosphate buffer) and 300 μl of PBS were added to stop the reaction. The labeled protein was separated from free ^{125}I by gel filtration on a Bio-Gel P-6DG desalting column (Bio-Rad, Melville, N.Y.). Labeled plasminogen was aliquotted and stored at −70°C until use. The specific activity of labeled plasminogen ranged from 4 to 6 mCi/mg.

Chromogenic P assay. Spirochetes (G39/40, 10^8) were suspended in 100 μl of PBSA to which Glu-plasminogen (10 mU) was added. The spirochetes were incubated for 1 h and then washed three times with PBSA to remove unbound plasminogen. The spirochetes were then resuspended in 100 μl of PBSA. The chromogenic plasmin substrate pyro-Glu-Gly-Arg-*p*-nitroanilide (0.25 M, 50 μl) was added to each tube as a chromatic substrate. Human uPA (10 U) was added to each tube to convert bound plasminogen to the active enzyme plasmin. After 1 h, the samples were centrifuged for 2 min at 11,000 × *g*, and the A_{405} of each sample was recorded. Plasmin activity was determined by comparison with standards. Each experiment was performed three times in duplicate.

SDS-PAGE zymography. *B. burgdorferi* (N40, low-passage, 10^8 spirochetes) were incubated with human Glu-plasminogen (100 mU) or PBSA alone at 0°C. A sham tube containing plasminogen but no borrelia was incubated under the same conditions. After 1 h, the samples were centrifuged, and the supernatant was removed. Pellets of spirochetes were washed three times with PBSA. The samples were then resuspended in PBSA and mixed with SDS-PAGE sample buffer (63 mM Tris base, 35 mM SDS, 0.14 M glycerol, 7.5 μM bromophenol blue) and loaded into lanes of a 10% polyacrylamide gel. A control sample of plasminogen (0.8 mU) was mixed with sample buffer and placed in another lane of the gel. Samples were then electrophoresed under nonreducing conditions (14). After SDS-PAGE, the gel was washed in 2.5% Triton X-100 and layered over a 1.0% agarose gel containing casein (2.0%) and uPA (50 U/ml) (22). The zymograms were allowed to develop at 37°C, and plasmin-dependent caseinolysis was photographed under dark-background illumination.

^{125}I-labeled plasminogen binding to *B. burgdorferi*. In duplicate, various numbers of *B. burgdorferi* (NECK, low passage) were incubated for 1 h with ^{125}I-labeled Glu-plasminogen (10 nM) at 4°C. Unbound ^{125}I-labeled plasminogen was separated from the bacteria either by centrifuging samples through oil at 11,000 × *g* or by washing the samples with PBSA, centrifuging the mixture at 11,000 × *g*, and removing the supernatant. The radioactivity associated with the bacterial pellet was measured in a gamma counter (Gamma 9000; Beckman Instruments, Inc., Irvine, Calif.). Experiments were performed three times in duplicate.

The binding of plasminogen with an N-terminal glutamic acid (Glu-plasminogen), which is the form found circulating in serum, was compared with the binding of plasminogen with an N-terminal lysine (Lys-plasminogen). The binding of ^{125}I-labeled Glu- and Lys-plasminogen to *B. burgdorferi* (NECK, low-passage, 10^8 spirochetes) was determined at multiple concentrations. All experiments were performed three times in duplicate.

To assess the ability of unlabeled Glu-plasminogen to inhibit binding of ^{125}I-labeled plasminogen, *B. burgdorferi* (NECK, low-passage, 5×10^8 spirochetes) were incubated at 4°C with ^{125}I-labeled Glu-plasminogen (10^{-9} M) either with or without unlabeled Glu-plasminogen at various concentrations. Experiments were performed three times in duplicate.

The ability of ε-aminocaproic acid (EACA) to inhibit the binding of ^{125}I-labeled plasminogen to *B. burgdorferi* was also determined. EACA (1.0 μM to 10 mM) was added to spirochetes (NECK, low passage, 5×10^7). The samples were incubated at 0°C with ^{125}I-labeled Glu-plasminogen (10 nM) for 1 h. The results were compared with binding in the absence of EACA. Each concentration of EACA was tested at least three times in duplicate.

Comparison of plasminogen binding to different strains of *B. burgdorferi*. In the mouse model of Lyme borreliosis, *B. burgdorferi* loses its ability to establish infection as it is passaged in culture. High- and low-passage 297, high- and low-passage N40, high-passage G39/40, and low-passage NECK were used for these experiments. Spirochetes of each strain were incubated for 1 h with or without Glu-plasminogen (100 mU). After 1 h, the samples were centrifuged for 2 min at 11,000 × *g*, and the supernatants were removed. Each sample was washed three times with PBSA. The pellets were then resuspended in PBSA. *B. burgdorferi* (10^7 organisms) from each sample was then placed into a well cut into a 4% casein–1% agarose gel. Human uPA (1 U) was added to each well. The casein zymogram was incubated at 37°C and caseinolysis was allowed to develop for 24 h. The gel was photographed under dark-background illumination.

Solubilization and fractionation of *B. burgdorferi*. *B. burgdorferi* (NECK, 7x297, and G39/40) were grown to log phase in 2 liters of BSK II medium. Cultures were centrifuged for 10 min at 10,000 × *g* to sediment the bacteria. The bacterial pellet was washed three times with PBS. The pellet was then suspended in solubilization buffer (1% Triton X-114, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] 5 mM $MgCl_2$, 100 μM phenylmethylsulfonyl fluoride [pH 7.4]) and mixed overnight at 4°C. The sample was then centrifuged at 10,000 × *g* at 4°C for 10 min. The supernatant containing the solubilized proteins was incubated at 37°C for 15 min to induce phase separation. After phase separation, the mixture was centrifuged at 1,500 × *g* at 25°C for 5 min. The aqueous phase was removed and discarded. PBS was added to the detergent fraction, and the mixture was incubated at 4°C for 15 min. Next, the mixture was centrifuged at 10,000 × *g* for 10 min to remove any remaining particulate matter. The process of phase separation was repeated. CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate} (0.5%) was added to the final detergent phase prior to aliquotting and storage at −20°C.

Binding of ^{125}I-labeled plasminogen to *B. burgdorferi* membrane proteins. Solubilized *B. burgdorferi* and detergent-phase proteins of solubilized *B. burgdorferi* were run on 10 to 15% polyacrylamide gradient SDS-PAGE and then electrophoretically transferred to nitrocellulose paper (14, 24). The nitrocellulose paper was blocked for 1 h with PBS containing 0.05% Tween 20 (PBST). The nitrocellulose paper was cut into strips and incubated with ^{125}I-labeled Glu-plasminogen at various concentrations for 1 h at 25°C. Some strips were incubated in the presence of unlabeled plasminogen. The strips were washed three times for 20 min each with PBST. The dried strips were mounted onto paper and autoradiographed (Kodak X-Omat cassette, Kodak X-Omat film). The autoradiograms were developed after 48 h scanned with a scanning densitometer (model 504-XL; Biomed Instruments, Inc., Fullerton, Calif.).

Two-dimensional gels were run on the detergent-phase proteins of solubilized *B. burgdorferi* as described by Celis and Bravo (6). The gels were subjected to isoelectric focusing in the first dimension followed by SDS-PAGE in the second dimension. Briefly, isoelectric focusing was performed by using polyacrylamide tube gels containing 9.2 M urea, 2% Nonidet P-40, ampholines (4% [pH 5 to 7] and 2% [pH 3 to 10]; LKB Pharmacia, Piscataway, N.J.), 3.5% acrylamide, TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine), and ammonium persulfate. Detergent-phase solutions were saturated with urea (9.2 M) prior to loading onto the gels. Loaded gels were overlaid with buffer (8.0 M urea, 1% ampholines [pH 3.0 to 10.0], 5% Nonidet P-40, 5% β-mercaptoethanol). NaOH (50 mM) was placed at the anode, and 25 mM H_3PO_4 was placed at the cathode. The proteins were focused at a constant voltage of 0.2 W per gel for 16 h. The completed gels were expelled from the capillary tubes and incubated in SDS-sample buffer for 5 min. The tube gels were then placed over 10 to 15% gradient acrylamide gels and electrophoresed. The completed gels were either serially stained with Coomassie blue and silver stained or transferred electrophoretically to nitrocellulose paper. The nitrocellulose paper was blocked with PBST, incubated with ^{125}I-labeled plasminogen for 1 h, and washed three times with PBST. It was then autoradiographed.

Immunoblot analysis of *B. burgdorferi* for Osp A. Strips of detergent-phase proteins bound to nitrocellulose paper were probed with 5332, a mouse monoclonal antibody against borrelial Osp A (a gift of Tom Schwann, Rocky Mountain Laboratories). After a 1-h incubation with the anti-Osp A antibody, the strips were washed three times with PBST. They were subsequently incubated for 1 h with a goat anti-mouse immunoglobulin G antibody conjugated with alkaline phosphatase (Promega). The strips were again washed three times with PBST and then developed with a 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium reagent (Vector Laboratories, Inc., Burlingame, Calif.).

RESULTS

Plasminogen binding to *B. burgdorferi*. The binding of plasminogen to *B. burgdorferi* was first studied with a functional assay employing chromogenic P as the substrate. The results are shown in Fig. 1A. The activity associated with 10^8 *B. burgdorferi* organisms incubated with 100 mU of plasminogen per ml was equivalent to the activity of ~305 μU of plasmin. No activity was seen for borrelia incubated without plasminogen or for borrelia exposed to uPA alone (data not shown). Figure 1B shows the results of an SDS-PAGE zymogram. The migration of the caseinolytic band on the gel was identical to that of free plasminogen. Again, no caseinolytic activity was present on bacteria which were not previously exposed to plasminogen. These results indicate that *B. burgdorferi* does not express endogenous plasminogen and that the enzymatic activity was due to the cleavage of human plasminogen associated with the spirochetes.

In related studies, we examined the association of radiolabeled plasminogen with *B. burgdorferi*. The amount of ^{125}I-labeled plasminogen that was bound increased as the number of borrelia increased, indicating that plasminogen binding was directly correlated with the presence of spirochetes, not with other nonspecific factors (data not shown).

Differences in the binding of Glu- and Lys-plasminogen have been noted for some bacteria but not others (12, 13, 27). Glu-plasminogen is the form of plasminogen found circulating in serum but is easily converted to Lys-plasminogen in a reac-

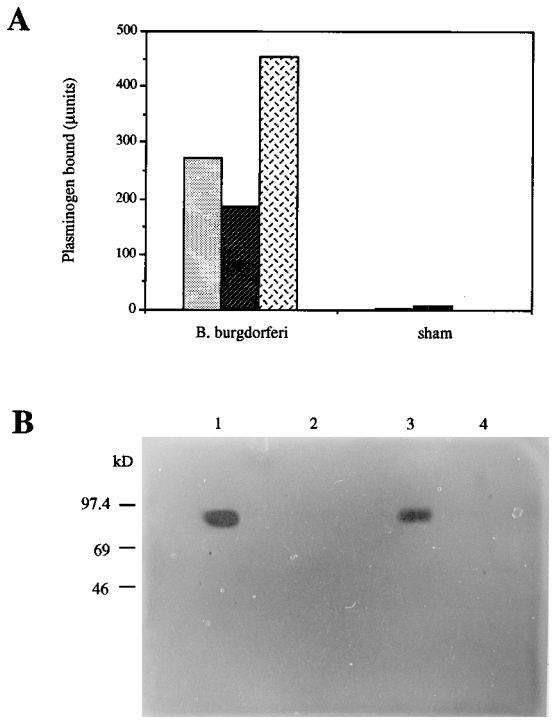


FIG. 1. Activation of *B. burgdorferi*-bound plasminogen to plasmin (A) *B. burgdorferi* was incubated with human Glu-plasminogen. The spirochetes were washed three times with PBSA prior to incubation with uPA. The activation of plasminogen to plasmin was monitored by measuring absorption at 405 nm with chromogenic P as the substrate. Each column represents one experiment done in duplicate. (B) *B. burgdorferi* incubated with human Glu-plasminogen as described for panel A was subjected to SDS–10% PAGE. The gel was then overlaid onto a 4% casein–1% agarose gel containing human uPA (50 U/ml). Clear zones indicate areas of caseinolysis by plasmin. Lanes: 1, *B. burgdorferi* incubated with human Glu-plasminogen; 2, solubilized material from a sham experiment (done without spirochetes); 3, plasminogen (0.4 mU) alone; 4, spirochetes not incubated with plasminogen.

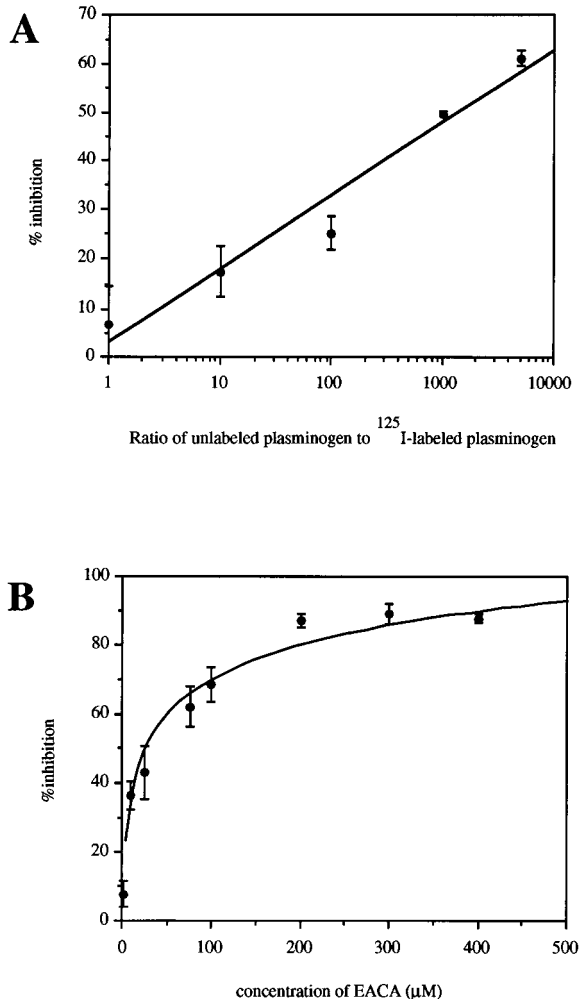


FIG. 2. Inhibition of ^{125}I-labeled plasminogen binding to *B. burgdorferi*. *B. burgdorferi* was incubated simultaneously with increasing concentrations (1 nM to 5.0 μM) of unlabeled plasminogen (A) or EACA (B) and a constant amount of ^{125}I-labeled plasminogen. After 1 h, the spirochetes were washed and the radioactivity in the remaining pellet was measured. Data are expressed as the percentage of inhibition compared with binding in the absence of inhibitor. Each point represents the mean of three experiments performed in duplicate. Error bars represent standard errors of the means.

tion that is catalyzed by plasmin. No difference was seen in the association of ^{125}I-labeled Glu- versus Lys-plasminogen by *B. burgdorferi* (data not shown).

The results of competition assays using unlabeled Glu-plasminogen to compete with ^{125}I-labeled Glu-plasminogen are shown in Fig. 2A. Inhibition of ^{125}I-labeled plasminogen association with *B. burgdorferi* increased with increasing amounts of unlabeled plasminogen. Inhibition reached 50% at a 1,000-fold excess of unlabeled plasminogen and >60% at a 2,500-fold excess. These results demonstrate that a major portion of the binding of Glu-plasminogen to *B. burgdorferi* is specific.

EACA, a lysine analog, was used to examine whether lysine binding sites on plasminogen play a role in its binding to *B. burgdorferi*. Binding of plasminogen to other bacteria is inhibited by EACA (25, 26). The results of experiments with *B. burgdorferi* are shown in Fig. 2B. EACA was a very efficient inhibitor of plasminogen binding to *B. burgdorferi*. Maximal inhibition was approximately 90% at a concentration of 0.2 mM EACA. Plasminogen bound to *B. burgdorferi* could also be eluted with EACA (data not shown).

Comparison of binding of Glu-plasminogen to different isolates of *B. burgdorferi*. Infectious and noninfectious strains of *B. burgdorferi* were tested for their binding of plasminogen (Fig. 3). Strains isolated from both ticks and humans were used. All strains and passages of *B. burgdorferi* which were incubated with Glu-plasminogen acquired caseinolytic activity in the presence of uPA. Lytic zones ranged in diameter from 4 to 6 mm at 24 h. In the absence of plasminogen, *B. burgdorferi* did not lyse casein.

Binding of ^{125}I-labeled plasminogen to solubilized proteins of *B. burgdorferi*. Autoradiograms of ligand blots containing proteins of *B. burgdorferi* incubated with ^{125}I-labeled plasminogen are shown in Fig. 4. Whole solubilized *B. burgdorferi* showed binding of ^{125}I-labeled plasminogen to 10 to 15 bands, with the major binding to an ~70-kDa protein. The membrane protein fraction of solubilized *B. burgdorferi* showed binding of ^{125}I-labeled plasminogen to only two major bands, 70 and 30 kDa. By scanning densitometry, the degree of association of ^{125}I with the 70-kDa band was more than three times higher than that of the 30-kDa band. In contrast, scanning of Coomassie blue-stained SDS-polyacrylamide gels showed that the ratio of protein at the 70-kDa band to that at the 30-kDa band was ~1:3.5, indicating that the increased binding to the 70-kDa band was due to a higher affinity or larger capacity for ^{125}I-

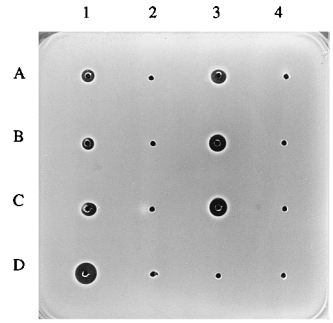


FIG. 3. Binding of plasminogen to various strains of *B. burgdorferi* assayed by casein plaque zymography. *B. burgdorferi* was incubated with plasminogen for 1 h and then placed in wells of a 4% casein–1% agarose gel. Wells contained uPA (1 U) to activate bound plasminogen to plasmin. Control wells contained *B. burgdorferi* strains not incubated with plasminogen. Rows: A, strain 297; B, N40; C, NECK. Columns: 1 and 3, *B. burgdorferi* incubated with plasminogen; 2 and 4, spirochetes not incubated with plasminogen; 1 and 2, low-passage strains; 3 and 4, high-passage strains. D1, plasminogen (1 mU) without spirochetes; D2, uPA alone; D3, suspension from a sham experiment done without spirochetes; D4, blank.

labeled plasminogen and was not a function of the amount of protein.

Inhibition of plasminogen association with borrelial membrane proteins by EACA or Glu-plasminogen was analyzed by scanning densitometry of the ligand blots. Inhibition at a 100-fold excess of unlabeled plasminogen was 55% for the 70-kDa protein and 65% for the 30-kDa protein. Inhibition at a 1,000-fold excess of unlabeled plasminogen was 89% for the 70-kDa protein and 90% for the 30-kDa protein. EACA (0.1 mM) inhibited essentially all binding of ^{125}I-labeled plasminogen to solubilized separated membrane proteins.

Two-dimensional gel electrophoresis confirmed binding of ^{125}I-labeled plasminogen to the 70-kDa band; binding to the 30-kDa band was variable and not seen in all experiments (Fig. 5). The pI of the 70-kDa band was ~5.3; the 30-kDa band was less focused and was estimated to have a pI of between 7.0 and 9.0.

Detergent-phase proteins separated by SDS–10 to 15% PAGE and transferred to nitrocellulose were immunoblotted with a monoclonal antibody against Osp A and demonstrated a band with a migration identical to that of the 30-kDa plasminogen-binding band (data not shown). To assess the relative binding of plasminogen to the 70-kDa protein and Osp A, mutants lacking Osp A (7x297) were obtained. Comparison of the association of ^{125}I-labeled plasminogen with mutant and

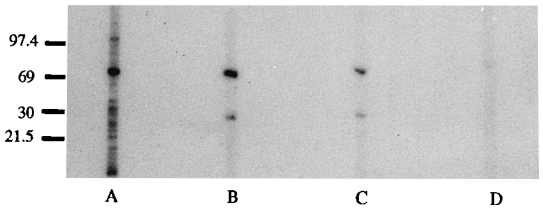
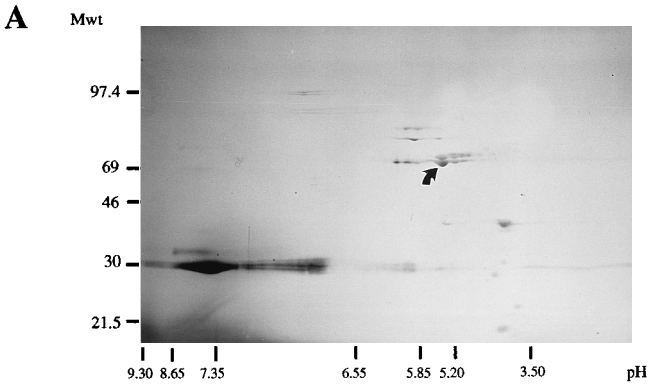


FIG. 4. Binding of ^{125}I-labeled plasminogen to solubilized proteins of *B. burgdorferi*. *B. burgdorferi* was solubilized with Triton X-114 and then phase separated into detergent and aqueous fractions. Samples of whole solubilized borrelia (lane A) and detergent-phase borrelial proteins (lanes B to D) were run on SDS–10 to 15% PAGE. Gels were transferred to nitrocellulose paper. Ligand blots were performed with ^{125}I-labeled plasminogen (1 nM) in the absence (lanes A and B) or presence of a 100-fold excess of unlabeled plasminogen (lane C) or 0.1 mM EACA (lane D). Strips were autoradiographed for 24 h. Size markers (in kilodaltons) are indicated on the left.



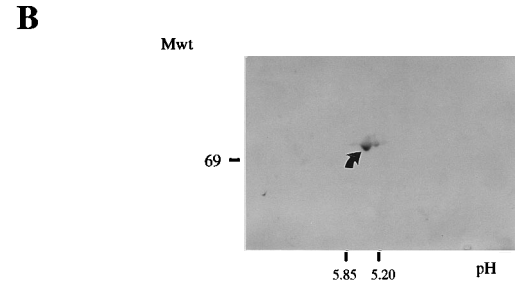


FIG. 5. Two-dimensional gel electrophoresis of detergent-phase proteins of *B. burgdorferi*. Detergent-phase proteins were subjected to two-dimensional gel electrophoresis as described in Materials and Methods. After electrophoresis, the gel was stained with Coomassie blue followed by silver stain (A) or transferred to nitrocellulose paper (B). The nitrocellulose paper was then incubated with ^{125}I-labeled plasminogen, washed, and autoradiographed. The arrow points to the 70-kDa plasminogen-binding protein. Size markers (in kilodaltons) are indicated on the left.

wild-type *B. burgdorferi* (NECK) showed no differences (Fig. 6A). Plasminogen binding by Osp A-deficient mutants was inhibited by ~50% by a 1,000-fold excess of unlabeled plasminogen. Plasminogen bound to Osp A-deficient mutants was converted into active plasmin by uPA, as assayed by the chromogenic P method (data not shown). Osp A-deficient mutants were also solubilized and separated into detergent and aqueous phases. The results of ^{125}I-labeled plasminogen ligand blots with the separated membrane proteins of this mutant are shown in Fig. 6B. Binding was seen at only the 70-kDa band.

DISCUSSION

The binding of human plasmin to bacteria was first reported for group A streptococcus by Lottenberg et al. in 1987 (15). It had been previously demonstrated that streptococci interacted with the human fibrinolytic system through the production of a plasminogen activator called streptokinase. Lottenberg and his collaborators showed that streptococci could not only produce the activator but could also bind the active enzyme, plasmin, to its surface. As group A streptococcal infections are often characterized by tissue invasion, the question was posed as to whether surface-bound plasmin could be used by streptococci in breaking down barriers to invasion. Since Lottenberg et al.'s original findings, plasmin(ogen) has been found to bind to the surface of other invasive bacteria, including *Staphylococcus aureus*, several enteric gram-negative species, *Neisseria meningitidis*, and *Neisseria gonorrhoeae* (5, 12, 13, 15, 20, 25, 26). At

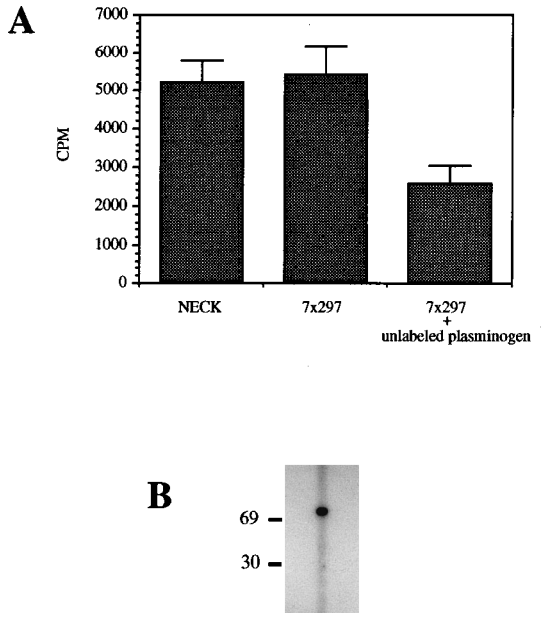


FIG. 6. Binding of plasminogen to Osp A-deficient mutant *B. burgdorferi* (7x297). *B. burgdorferi* (5×10^8 organisms) was incubated with ^{125}I-labeled plasminogen (1 nM). (A) After 1 h of incubation, the spirochetes were washed, and the radioactivity associated with the remaining pellet was measured in a gamma counter. All experiments were performed in triplicate, and error bars represent standard errors of the means. (B) Ligand blots of ^{125}I-labeled plasminogen to membrane proteins of 7x297. Strain 7x297 was solubilized in Triton X-114 and phase separated into detergent and aqueous phases. The detergent-phase proteins were then subjected to SDS-PAGE and transferred to nitrocellulose paper. Strips of the nitrocellulose paper were incubated with ^{125}I-labeled plasminogen (10^{-8} M), washed, and then autoradiographed. The positions of the molecular size proteins (in kilodaltons) are indicated on the left.

least two genes coding for plasmin and plasminogen receptors have now been isolated for group A streptococci. The plasmin receptor was found to be a 36-kDa protein with homology to glyceraldehyde 3-dehydrogenases (16). The isolated plasminogen-binding protein was a 43-kDa M protein (3).

The ability of *B. burgdorferi* to spread widely in the skin and tissue has been well documented. We have shown by functional and radioisotopic methods that *B. burgdorferi* binds a human zymogen, plasminogen. In addition, plasminogen bound to the surface of *B. burgdorferi* can be converted into active plasmin by human uPA. The characteristics of plasminogen binding to *B. burgdorferi* share many features with the binding of plasminogen to other organisms. The ability to inhibit plasminogen uptake with EACA suggests that the receptor-ligand interaction is likely mediated by ω-amino acids such as lysine. Lysine binding sites on plasminogen are thought to be responsible for the binding of plasminogen to fibrin, eukaryotic cells, and other bacteria (17). The concentration of EACA required to maximally inhibit plasminogen binding to *B. burgdorferi* is slightly less than the concentration required for *N. gonorrhoeae*, *N. meningitidis*, *Haemophilus influenzae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae* (0.2 versus ~1 mM) (25, 26).

Since our initial finding of plasmin binding and stabilization by *B. burgdorferi*, Osp A of *B. burgdorferi* has been reported to be a plasminogen-binding protein (9, 11). Our investigations confirm that an ~30-kDa protein with a migration pattern identical to that of Osp A binds plasminogen. However, the loss of Osp A does not affect plasminogen binding in the Osp A- or Osp B-deficient mutant 7x297. Plasminogen appears to bind to several borrelial membrane proteins, but binding is most prominent to a 70-kDa protein. Other bacteria which bind plasminogen have also been found to have multiple plasminogen-binding proteins, often with both high and low affinities (3, 5, 25).

The relationship of fibrinolytic proteins to bacterial virulence is not uniform. For *Yersinia pestis*, bacterial virulence appears to be related to the production of a plasminogen activator encoded by a plasmid-borne gene designated *pla*. The 50% lethal dose for mice injected subcutaneously with *pla*$^+$ *Y. pestis* (wild type) is around 10^6 times less than that with *pla* mutant *Y. pestis*; however, the 50% lethal doses for mice injected intravenously are similar for *pla*$^+$ and *pla* mutant *Y. pestis* (21). Clearly, this suggests a role for a plasminogen activator, and, by extension, plasmin, in the spread of *Y. pestis* through extracellular matrix proteins. Defining the role of plasmin(ogen) binding to streptococci, staphylococci, and other gram-negative rods has been more difficult, in part because these organisms also produce other proteases and protease activators which can lyse the extracellular matrix components and provide separate pathways for dissemination.

A role for plasmin(ogen) in the pathogenesis of Lyme disease is attractive for the following reasons. *B. burgdorferi* disseminates widely but, unlike the other bacteria which bind plasminogen, does not produce any collagenase, elastase, hyaluronidase, or plasminogen activators to degrade the extracellular matrix (11). In the absence of these activators, acquisition of the capacity to degrade extracellular matrix from the host would be an important alternative strategy for organism invasion and dissemination. The natural route of entry of *B. burgdorferi* through tick bites would allow the spirochetes to become exposed to high local levels of both host plasminogen and uPA. One characteristic feature of early Lyme disease is the skin lesion erythema migrans. Erythema migrans is most typically an expanding erythematous lesion with central clearing. Viable spirochetes are recoverable throughout the lesion (4). The use of a host enzyme with broad substrate activity, such as plasmin, could allow *B. burgdorferi* to disrupt basement membranes and to disseminate. The presence of host rather than bacterial enzymes on the surface of *B. burgdorferi* could also explain the relative absence of an acute inflammatory response seen in erythema migrans lesions. The absence of an inflammatory response in the presence of viable spirochetes is quite striking compared with the exuberant host response seen with other subcutaneously spreading organisms. The fact that there were no differences in plasminogen binding between infectious and noninfectious strains of *B. burgdorferi* indicates that surface acquisition of plasmin(ogen) is not the only determinant of virulence in the mouse model. This is not surprising since this model assesses the overall ability of spirochetes to establish an in vivo infection and not the many individual components of pathogenesis. The 70-kDa plasminogen-binding protein was conserved in all tested strains, suggesting an important functional role for this protein. Dissecting the contribution of plasminogen binding by *B. burgdorferi* to the pathogenesis of Lyme disease will require better understanding of the binding proteins and improved models to assess spirochete invasion.

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REFERENCES

1. **Alexander, C. M., and Z. Werb.** 1991. Extracellular matrix degradation, p. 255–302. *In* E. D. Hay (ed.), Cell biology of extracellular matrix. Plenum Press, New York.
2. **Barbour, A.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57:**521–525.
3. **Berge, A., and U. Sjobring.** 1993. PAM, a novel plasminogen-binding protein from *Streptococcus pyogenes*. J. Biol. Chem. **268:**25417–25424.
4. **Berger, B. W., R. C. Johnson, C. Kodner, and L. Coleman.** 1992. Cultivation of *Borrelia burgdorferi* from erythema migrans lesions and perilesional skin. J. Clin. Microbiol. **30:**359–361.
5. **Broder, C., R. Lottenberg, G. O. von Mering, K. H. Johnston, and M. D. B. Boyle.** 1991. Isolation of a prokaryotic plasmin receptor. J. Biol. Chem. **266:**4922–4928.
6. **Celis, J. E., and R. Bravo.** 1984. Two-dimensional gel electrophoresis of proteins: methods & applications, p. 3–36. Academic Press, Orlando, Fla.
7. **Duran-Reynolds, F.** 1942. Tissue permeability and spreading factors in infection. Bacteriol. Rev. **6:**197–252.
8. **Francis, C. W., and V. J. Marder.** 1990. Mechanisms of fibrinolysis, p. 1313–1323. *In* W. J. Williams, E. Beutler, A. J. Erslev, and M. A. Lichtman (ed.), Hematology. McGraw Hill Publishing Co., New York.
9. **Fuchs, H., R. Wallich, M. Simon, and M. Kramer.** 1994. The outer surface protein A of the spirochete *Borrelia burgdorferi* is a plasmin(ogen) receptor. Proc. Natl. Acad. Sci. USA **91:**12594–12598.
10. **Greenwood, F. C., W. M. Hunter, and J. S. Glover.** 1963. The preparation of [I-131] labeled human growth hormone of high specific activity. Biochem. J. **89:**114–123.
11. **Klempner, M. S., R. Noring, M. Epstein, et al.** 1995. Binding of human plasminogen and urokinase-type plasminogen activator to the Lyme disease spirochete, *Borrelia burgdorferi*. J. Infect. Dis. **171:**1258–1265.
12. **Kuusela, P., and O. Saksela.** 1990. Binding and activation of plasminogen at the surface of *Staphylococcus aureus*. Eur. J. Biochem. **193:**759–765.
13. **Kuusela, P., M. Ullberg, U. Saksela, and G. Kronvall.** 1992. Tissue-type plasminogen activator-mediated activation of plasminogen on the surface of group A, C, and G streptococci. Infect. Immun. **60:**196–201.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
15. **Lottenberg, R., C. Broder, and M. Boyle.** 1987. Identification of a specific receptor for plasmin on group A streptococcus. Infect. Immun. **55:**1914–1928.
16. **Lottenberg, R., C. Broder, M. Boyle, S. J. Kain, B. L. Schroeder, and R. Curtiss.** 1992. Cloning, sequence analysis, and expression in *Escherichia coli* of a streptococcal plasmin receptor. J. Bacteriol. **174:**5204–5210.
17. **Mayer, M.** 1990. Biochemical and biological aspects of the plasminogen activation system. Clin. Biochem. **23:**197–211.
18. **Morihara, K.** 1964. Production of elastase and proteinase by *Pseudomonas aeruginosa*. J. Bacteriol. **88:**745–757.
19. **Norton-Hughes, C. A., S. M. Engstrom, L. A. Coleman, C. B. Kodner, and R. C. Johnson.** 1993. Protective immunity is induced by a *Borrelia burgdorferi* mutant that lacks OspA and OspB. Infect. Immun. **61:**5115–5122.
20. **Rigner, M., K. H. Valkonen, and T. Wadstrom.** 1994. Binding of vitronectin and plasminogen to *Helicobacter pylori*. FEMS Immunol. Med. Microbiol. **9:**29–34.
21. **Sodeinde, O., Y. Subrahmanyam, K. Stark, T. Quan, Y. Bao, and J. Goguen.** 1992. A surface protease and the invasive character of plague. Science **258:**1004–1007.
22. **Sogowa, K., and K. Takahashi.** 1978. Use of fluorescamine-labeled casein as a substrate for assay of proteinases. J. Biochem. **83:**1783–1787.
23. **Steere, A.** 1989. Lyme disease. N. Engl. J. Med. **321:**586–596.
24. **Towbin, H., T. Stehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
25. **Ullberg, M., G. Kronvall, I. Karlsson, and B. Wiman.** 1990. Receptors for human plasminogen on gram-negative bacteria. Infect. Immun. **58:**21–25.
26. **Ullberg, M., P. Kuusela, B. Kristiansen, and G. Kronvall.** 1992. Binding of plasminogen to *Neisseria meningitidis* and *Neisseria gonnorrhoeae* and formation of surface associated plasmin. J. Infect. Dis. **166:**1329–1334.
27. **Wang, H., R. Lottenberg, and M. Boyle.** 1993. Analysis of plasmin(ogen) acquisition by clinical isolates of group A streptococci incubated in human plasma. J. Infect. Dis. **169:**143–149.