Haemophilus influenzae Uses the Surface Protein E To Acquire Human Plasminogen and To Evade Innate Immunity

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**Haemophilus influenzae** Uses the Surface Protein E To Acquire Human Plasminogen and To Evade Innate Immunity

Diana Barthel,* Birendra Singh, † Kristian Riesbeck, † and Peter F. Zipfel*,*†

Pathogenic microbes acquire the human plasma protein plasminogen to their surface. In this article, we characterize binding of this important coagulation regulator to the respiratory pathogen nontypeable **Haemophilus influenzae** and identify the **Haemophilus** surface protein E (PE) as a new plasminogen-binding protein. Plasminogen binds dose dependently to intact bacteria and to purified PE. The plasminogen–PE interaction is mediated by lysine residues and is also affected by ionic strength. The **H. influenzae** PE knockout strain (nontypeable **H. influenzae** 3655Δpe) bound plasminogen with ~65% lower intensity as compared with the wild-type, PE-expressing strain. In addition, PE expressed ectopically on the surface of *Escherichia coli* also bound plasminogen. Plasminogen, either attached to intact *H. influenzae* or bound to PE, was accessible for urokinase plasminogen activator. The converted active plasmin cleaved the synthetic substrate S-2251, and the natural substrates fibrinogen and C3b.

Plasminogen, which has a plasma concentration of ~200 μg/mL, is synthesized and secreted by liver cells and is also present in extracellular body fluids. (4) Proteolytic processing of plasminogen by human urokinase-type (uPA), by tissue-type plasminogen activators, and also by the bacterial streptokinase and staphylokinase generates the active protease plasmin (5–7). Plasmin as a key enzyme in fibrinolysis and homeostasis degrades components of the extracellular matrix (ECM) and the basement membrane, including fibrinogen, fibrin, and laminin (8). In addition, plasmin activates matrix metalloproteases and elastase, which regulate wound healing, tissue remodeling, tumor metastasis, and angiogenesis (9, 10).

**Haemophilus influenzae** is a Gram-negative pathogen that frequently colonizes the human respiratory tract. These opportunistic bacteria are divided into encapsulated and unencapsulated strains; the latter are also termed nontypeable *H. influenzae* (NTHi) (11–13). Encapsulated *Haemophilus* strains are subdivided into serotypes a–f, with *H. influenzae* strain b being the most virulent (14). *H. influenzae* strain b strains often cause bacteremia, pneumonia, and acute bacterial meningitis, and occasionally osteomyelitis, epiglottitis, and joint infections (11, 15, 16). Strain NTHi 3655 is often associated with local upper and lower respiratory tract infections, including acute otitis media, sinusitis, and chronic obstructive pulmonary disease (11, 17, 18). *H. influenzae* is after *S. pneumoniae* the second most common cause of chronic obstructive pulmonary disease (19).

Protein E (PE) of *H. influenzae* is a 16-kDa outer membrane lipoprotein with adhesive properties (20). PE mediates adhesion to and activates human epithelial cells. The pe gene is constitutively expressed in NTHi and is highly conserved among clinical NTHi isolates (21). PE uses the central 24-aa-long core domain (i.e., aa 84–108) to contact human epithelial cells and to induce proinflammatory responses. PE also binds human vitronectin, an ECM component, protein E and terminal complement regulator (22).

Infections are generally associated with local inflammation and epithelial cell damage that results in the exposure of the underlying ECM (23–25). Bacterial pathogens acquire plasminogen when in contact with human plasma or body fluids. Surface-bound plasminogen enhances interaction with host ECM components, and mediates adhesion and colonization (7, 8). Plasminogen attached to most microbial surfaces is accessible for human and also for bacterial activators that form the active serine protease plasmin. *H. influenzae*, similar to other pathogenic bacteria, including hemolytic group A, C, and G streptococci and pneumococci, use plasminogen to adhere and attach to the ECM (26, 27). Currently, spartase is the only identified plasminogen-binding protein of *H. influenzae* (28, 29). However, most pathogenic microbes including *Borrelia burgdorferi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and the yeast *Candida albicans* express several plasminogen-binding proteins (7, 30–39).

Given the central role of human plasminogen for bacterial immune evasion and ECM interaction, we wanted to define the role...
of plasminogen for immune escape of *H. influenzae*. First, we characterized plasminogen binding of NTHi and identified PE as a novel and major plasminogen-binding protein. Plasminogen bound to intact bacteria or to recombinant PE was accessible for uPA. uPA-activated plasmin cleaved the synthetic chromogenic substrate S-2251 and the natural substrates fibrinogen and also C3b. A linear stretch of 28 N-terminal aa was identified as the major linear plasminogen binding region. Thus, the Gram-negative, nontypeable bacterium *H. influenzae* uses the PE surface protein to acquire human plasminogen and uses surface-bound plasmin for complement evasion, innate immune escape, and ECM degradation.

**Materials and Methods**

**Bacterial strains, reagents, and cell culture**

NTHi strain 3655 and the isogenic PE knockout strain (NTHi 3655pe) were grown in brain heart infusion (BHI) liquid broth, supplemented with NAD and hemin (each 10 μg/ml). In addition, bacteria were plated on chocolate agar plates and incubated in a humid atmosphere at 37°C and 5% CO₂. The NTHi 3655pe mutant was grown in the presence of 20 μg/ml kanamycin (20). *Escherichia coli* BL21 (DE3) were grown in Luria–Bertani liquid broth, and PE transfectants were cultivated with 50 μg/ml ampicillin (20).

**Proteins and Abs**

Human plasminogen, uPA (Haemochorm Diagnostica), and vitronectin (CompTech) were used for binding studies, and complement protein C3b (CompTech) and fibrinogen (Calbiochem) were used for functional assays. Human proteins were identified with goat antiserum specific for plasminogen (Acris Abs), vitronectin (CompTech), or C3b (CompTech), or rabbit antisera specific for fibrinogen (Calbiochem). Corresponding secondary antisera (rabbit antisera specific for goat or goat antiserum specific for rabbit) were purchased from Dako and the lysine analog ε-amino caproic acid (eACA) from Sigma.

For expression of recombinant PE25-160, *E. coli* were transfected with the corresponding vector and protein expression was induced with iso-propyl-b-D-thiogalactopyranosil for 3.5 h. Bacteria were lysed, and after centrifugation, the pellet was resuspended in cold PBS supplemented with lysoyzme (0.1 mg/ml), sonicated, and then centrifuged again. Soluble recombinant PE was purified under native conditions by nickel chromatography (Novagen) (20).

*[125I] labeling and protein binding to bacteria (direct binding assay)*

Plasminogen was labeled with [125I] by the Chloramide-T method (20). For labeling, 0.05 M [125I] (Amersham Biosciences, Buckinghamshire, U.K.) was used per mole of protein. The labeled proteins were separated from free [125I] by PD10 columns (GE Healthcare Biosciences, Uppsala, Sweden).

Wild-type NTHi 3655 and the knockout strain were grown in BHI media until OD₆₅₀ = 1.0; then bacteria were washed in PBS supplemented with 2.5% BSA. After counting, bacteria (10⁶ cells/ml) were seeded in 96-well microtiter plates. [125I]plasminogen was added at the indicated concentrations, and the mixture was incubated for 30 min at 37°C. Thereafter, bacteria were pelleted at 4200 x g for 10 min and washed twice in PBS + 2.5% BSA to remove unbound plasminogen. Cells were harvested in a 96-well plate harvester (Tomtec, Hamden, CT), and the radioactivity was counted using a liquid scintillation counter (TriPak, Microbeta 1450; Perkin Elmer). All assays were performed in triplicate, and each assay was repeated at least three times.

**Flow cytometry**

Plasminogen binding to bacteria was analyzed by flow cytometry. Wild-type NTHi 3656 bacteria were grown in BHI, washed with PBS supplemented with BSA (PBS-1% BSA), and incubated with plasminogen, used at concentrations from 0.75 to 3.0 μg. After two washing steps, goat antiserum specific for human plasminogen was added and bound to the coated bacteria for a further 30 min at room temperature. After washing, Alexa 647-conjugated goat antiserum was added. Following two additional washing steps, bacteria were analyzed by flow cytometry (Accuri, St. Ives, Cambridge, U.K.). All incubation steps were performed in a volume of 100 μl PBS-1% BSA, and the same buffer was used for all washing steps.

**Protein-binding assays**

Purified PE or synthetic peptides (each at 0.5 μg in 50 μl PBS) that represent the entire PE sequence and have an overlap of 4 amino acids were immobilized onto a 96-well microtiter plate (Nunc) overnight at 4°C (20, 22). After washing three times with PBS-T (PBS + 0.05% Tween 20), blocking buffer 1 (Applied Biosystems) was added and the mixture was incubated for 2 h at 37°C. Then plasminogen (0.5 μg in 50 μl PBS) was added either alone or together with the lysine analog eACA or NaCl. After incubation for 1.5 h at room temperature, unbound protein was removed by washing (three times with PBS-T), and bound plasminogen was identified after incubation with appropriate antiserum (goat plasminogen antiserum, 1:1000). After washing with PBS-T, HRP-conjugated goat antiserum (1:2000 dilution in PBS-T) was added and the mixture was incubated for 1 h at room temperature. After three additional washing steps and addition of tetramethylbenzidine, the reaction was stopped by addition of 2 M H₂SO₄, and the absorption was determined at 450 nm using a microtiter plate reader. All further binding assays were performed at physiological NaCl levels of 150 mM.

The effect of vitronectin on plasminogen binding to immobilized PE was analyzed by ELISA. Plasminogen (used at 1 μg) and vitronectin were combined at different molar ratios and then bound to immobilized PE (0.5 μg/well). Similarly, plasminogen, used at different molar levels, was combined with a constant amount of vitronectin (1 μg/well) and added to immobilized PE. In addition, normal human serum (NHS; 20%, diluted in Dulbecco’s phosphate buffered saline) was used for PE binding under physiological conditions. After incubation and extensive washing, each bound human serum protein was detected with specific antiserum.

**Plasminogen binding and protein absorption assay**

Wild-type NTHi 3655, the knockout strain NTHi 3655pe, wild-type *E. coli*, or *E. coli* expressing PE was immobilized in buffer supplemented with plasminogen (20 μg/ml) or 10% heat-inactivated NHS for 1.5 h at 37°C. After extensive washing, bacteria were treated with elution buffer (60 mM Tris, 2% SDS, 25% glycerol) for 20 min at 37°C. The supernatant was collected, separated by SDS-PAGE, and transferred to a membrane by semidyblotting. Plasminogen was identified with a goat antisera (antiplasminogen 1:2000 in PBS-T) together with an appropriate HRP-goat antiserum (1:2000 in PBS-T).

**Plasminogen activation and plasmin activity**

The proteolytic activity of activated plasmin was determined as described previously (37). In brief, purified PE (0.25–2.0 μg dissolved in 50 μl carbonate-bicarbonate buffer) was immobilized onto a microtiter plate. After blocking with PBS supplemented with 2% BSA, plasminogen (0.6 μg/well) was bound. After incubation at 1.5 h at room temperature, unbound plasminogen was removed by washing with PBS-T. Then uPA (4 ng/well) together with the chromogenic substrate S-2251 (N-vallyl-leucyl-lysine-p-nitroanilide dihydrochloride, 100 μg/well; Sigma Aldrich) dissolved in reaction buffer (64 mM Tris, 350 mM NaCl, 0.01% Triton X; pH 7.5) were added. The plate was incubated at 37°C for 24 h, and cleavage of the chromogenic substrate was followed over a period of 24 h by measuring the absorbance at 405 nm.

Similarly, the activity of plasmin attached to the bacterial surface was analyzed. To this end, plasminogen (2 μg) was bound to intact bacteria (1 x 10⁹ cells) and the effect of the lysine analog eACA (5 mM) on binding and activation was analyzed for 1.5 h at 37°C. After washing with PBS, uPA (1 μg) and the chromogenic substrate S-2251 dissolved in reaction buffer was added and substrate cleavage was recorded.

**Fibrinogen and C3b degradation**

Degradation of the natural substrate fibrinogen or C3b by PE-bound and activated plasminogen was assayed. Plasminogen (5 μg/well) was bound to immobilized PE (5 μg/well) and after blocking, fibrinogen (0.5 μg, plasminogen depleted; Calbiochem) or C3b (0.5 μg) together with plasminogen activator uPA (4 ng/well) were added. The mixture was incubated at room temperature. At the indicated time points, aliquots were removed and the reaction was stopped by adding reducing buffer. Then the samples were heated for 5 min at 95°C, separated by SDS-PAGE, and transferred to a membrane (40). The degradation products were monitored by Western blotting using rabbit fibrinogen or goat C3 antisera and the corresponding secondary HRP-conjugated antiserum.

**Statistics**

Results were assessed by Student *t* test for paired data. A *p* value ≤0.05 was considered to be statistically significant (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001*).
Results

Plasminogen binds to intact H. influenzae

Binding of plasminogen to intact H. influenzae strain NTHi 3655 was assayed. Bacteria were incubated with [125I]plasminogen, and after extensive washing, bound plasminogen was determined by counting the radioactivity. Plasminogen bound to intact NTHi 3655 and binding was dose dependent (Fig. 1A). Plasminogen binding was also observed using flow cytometry (Fig. 1B).

To analyze whether lysine residues are relevant for plasminogen binding to intact bacteria, we assayed the effect of εACA. This lysine analog inhibited plasminogen binding to NTHi 3655, and the effect became dose dependent. εACA, at a concentration of 1 mM, inhibited plasminogen binding by 75% (Fig. 1C). In addition, the effect of ionic strength was assayed. NaCl inhibited plasminogen binding. At the physiological level (i.e., 150 mM), NaCl decreased plasminogen binding by 25%, and at a concentration of 1 M, NaCl inhibited the interaction by almost 60% (Fig. 1D). Thus, plasminogen binding to NTHi 3655 bacteria depends on lysine residues and is affected by ionic strength.

Plasminogen also bound to bacteria, which were collected at different growth stages, and binding was of similar intensity. Thus, plasminogen binding to H. influenzae is independent of the growth phase, and the bacterial plasminogen-binding protein(s) seems to be expressed constitutively (data not shown).

PE is a plasminogen-binding protein of NTHi

Because NTHi 3655 isolates express PE on their surface (21), this bacterial surface and adhesion protein was considered as a candidate ligand for plasminogen. Therefore, binding of plasminogen to recombinant PE was assayed. Plasminogen bound to immobilized, full-length, recombinant PE22–160 and binding were dose dependent. Plasminogen binding was saturated at a concentration of 50 μg/ml, which is ~4-fold lower than the serum level (Fig. 2A).

Plasminogen binding to Haemophilus bacteria is mediated by lysine residues and is ionic strength dependent. We therefore asked whether the plasminogen–PE interaction is similarly affected by these agents. First, the effect of εACA was tested. This lysine analog inhibited plasminogen binding to immobilized PE and at 1 mM εACA inhibited binding by 60% (Fig. 2B). Similarly, NaCl affected the interaction. At the physiological level of 150 mM, NaCl reduced plasminogen binding to PE by 25%, and at 1.0 M, by 75% (Fig. 2C). Thus, plasminogen binds to PE of H. influenzae; binding is mediated by lysine residues and is of ionic nature.

Plasminogen binds to a H. influenzae PE knockout strain with lower intensity and binds to ectopically expressed PE

To further prove that PE of NTHi 3655 is a plasminogen-binding protein, we studied plasminogen binding to a PE knockout strain (NTHi 3655 Δpe) (22). [125I]plasminogen bound to NTHi 3655 Δpe with low intensity. When compared with the wild-type, PE-expressing strain, binding was reduced by 65% (Fig. 3A). To provide further proof that PE binds plasminogen, we assayed binding to PE expressed on the surface of E. coli (20). [125I]plasminogen binding to ectopically expressed PE was 4-fold higher as compared with non–PE-expressing bacteria (Fig. 3B).

To further characterize the relevance of PE for plasminogen attachment, we assayed binding of both purified and serum-derived plasminogen to knockout, to wild-type NTHi 3655, and similarly to PE-expressing and nonexpressing E. coli. Bacteria were incubated either in purified plasminogen or in human serum. After extensive washing, bound proteins were eluted, separated by SDS-PAGE, transferred to a membrane, and plasminogen was detected by Western blotting. Purified, as well as serum-derived, plasminogen bound to wild-type NTHi 3655 (Fig 3C, lane 1) and to PE-expressing E. coli (Fig. 3D, lane 2). Both purified and serum-derived plasminogen bound with much lower intensity to the PE knockout strain (Fig. 3C, lane 2) or to the non–PE-expressing E. coli (Fig. 3D, lane 1). The lower binding to the PE knockout strain and also the strong binding to E. coli that ectopically express PE was assayed by ELISA. Plasminogen, used at increasing concentrations (10–200 μg/ml), was bound to immobilized PE (0.5 μg/well), and after extensive washing, bound plasminogen was detected with goat plasminogen and HRP-goat antisera by measuring the absorbance at 450 nm. B. Plasminogen was bound to PE and the effect of εACA was assayed. Bound plasminogen was detected with goat plasminogen antisemur and HRP-goat antisemur. Plasminogen binding to PE in the absence of εACA was set at 100%. C. The inhibitory effect of the lysine analog εACA for binding of [125I]plasminogen to NTHi 3655 was evaluated. Bound plasminogen was determined by counting the radioactivity. The signal obtained in the absence of εACA was set at 100%. D. The effect of NaCl on [125I]plasminogen binding to NTHi 3655 was evaluated. Plasminogen binding was determined by counting the radioactivity, and the amount of plasminogen bound in the absence of NaCl was set at 100%. At the physiological level, plasminogen binding was reduced by 25% (shown by ↓). Mean values of three independent experiments are shown; error bars indicate SD. For flow cytometry, the result of a typical experiment out of three is shown. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
show that PE is one major plasminogen-binding protein of *H. influenzae*. The reduced but existing binding to the PE knockout strain is in agreement with the existence of at least one additional plasminogen protein of *H. influenzae*, that is, aspartase (29).

**Localization of the plasminogen binding region in PE**

To localize the plasminogen binding region in the PE protein, we synthesized short linear peptides, with a length of 17–25 aa and an overlap of 4 residues. These synthetic peptides, which span the entire PE protein, were immobilized onto a microtiter plate and plasminogen binding was assayed. Plasminogen bound to full-length PE, bound with highest intensity to PE41–68, and also bound to peptides PE21–45, PE54–88, and PE84–108. Plasminogen did not bind to peptides PE104–128, PE124–148, and PE148–160 (Fig. 4). Thus, the 28 N-terminal residues (i.e., aa 41–68) represent the major linear plasminogen binding region of PE.

**Plasminogen and vitronectin compete for PE binding**

PE binds plasminogen and also vitronectin. The major plasminogen binding region was localized to the N terminus of PE, and similarly the major vitronectin binding region of PE was localized within the N terminus, to a linear stretch of 24 residues that represent 84–108 aa (20).

Because the N terminus of PE includes the major binding regions for these two human plasma proteins, we next asked whether plasminogen or vitronectin bind simultaneously to the bacterial PE protein and whether the two human proteins compete for PE binding. Both plasminogen and vitronectin bound to immobilized PE. However, plasminogen used at increasing levels affected binding of vitronectin to PE. At a 10-M excess, plasminogen bound to PE and vitronectin binding was reduced by ~20% (Fig. 5A, 5B, and 5C).

**FIGURE 3.** Binding of plasminogen to the *H. influenzae* PE knockout strain and to ectopically expressed PE. *A*, Plasminogen binding to the PE knockout strain (NTHi 3655Δpe) was assayed in a direct binding assay. [125I]Plasminogen was bound to NTHi 3655Δpe or to wild-type NTHi 3655. After extensive washing, bound plasminogen was quantitated by counting the radioactivity. Binding of [125I]plasminogen to the wild-type strain was set at 100%. Plasminogen binding to the PE knockout strain was reduced by 65%. *B*, Plasminogen bound to PE ectopically expressed by *E. coli*. Plasminogen bound to PE-expressing *E. coli* with about four times higher intensity. The mean values of three experiments and SDs are shown for *A* and *B*. *C*, Binding of purified and serum-derived plasminogen to the wild-type strain (*lane 1*) and to the PE knockout strain (*lane 2*) was assayed using an absorption assay. After extensive washing, bound proteins were eluted, separated by SDS-PAGE, transferred to a membrane, and assayed using an absorption assay. After extensive washing, bound proteins were eluted, separated by SDS-PAGE, transferred to a membrane, and assayed using an absorption assay. After extensive washing, bound proteins were eluted, separated by SDS-PAGE, transferred to a membrane, and assayed using an absorption assay. After extensive washing, bound proteins were eluted, separated by SDS-PAGE, transferred to a membrane, and assayed using an absorption assay.

**FIGURE 4.** Localization of the plasminogen-binding region in PE. Plasminogen binding was analyzed to full-length PE and to linear synthetic peptides that have a length of 17–25 aa and an overlap of four residues, and that span the entire PE sequence. After extensive washing, bound plasminogen was detected with a goat plasminogen antiserum together with HRP-goat antiserum, and the absorbance at 450 nm was determined. Gelatin was used as control. Results represent mean values of three independent experiments, and error bars show SD. The significance values were calculated using PE140–160 as a reference for a nonbinding protein.

**FIGURE 5.** Binding of plasminogen and vitronectin to PE. *A*, Plasminogen and vitronectin compete for PE binding. Vitronectin was combined with different amounts of plasminogen, the mixture was bound to immobilized PE, and after washing, both bound vitronectin and plasminogen were detected with specific antisera (i.e., goat plasminogen or goat vitronectin). Binding to a gelatin-coated surface in the absence of PE was used as negative control. *B*, Binding was also analyzed in a reverse setting. Plasminogen together with increasing amounts of vitronectin were bound to immobilized PE, and binding of both proteins was assayed. Again, binding to a gelatin-coated surface in the absence of a specific ligand was used to determine background binding. Both plasminogen and vitronectin, when used at the physiological ratio of 1:2.5 bound to PE (*A, B, third column pair; ↓*). *C*, Binding of both plasminogen and vitronectin derived from NHS to immobilized PE was assayed. NHS was added to immobilized PE, and after washing, bound proteins were detected by specific antisera (i.e., goat plasminogen or goat vitronectin). The mean value of four independent experiments and the SD are indicated. *p ≤ 0.05, ***p ≤ 0.001.
A representative experiment out of three independent assays is shown. Thus, PE has still free binding sites for the competing protein plasminogen. A similar result was observed when a reverse setting was used (Fig. 5B). Again, both human plasma proteins bound to immobilized PE. In this setup, increasing levels of vitronectin inhibited plasminogen binding to PE in a stronger extent. Thus, the binding of PE to vitronectin is benefited compared with plasminogen.

When the two human proteins were applied at the molar ratio that corresponds to their plasma levels (i.e., 1:2.5), both plasminogen and vitronectin bound to PE (Fig. 5B). In addition, binding of both human plasma proteins to immobilized PE was confirmed when NHS was used as a protein source (Fig. 5C). Thus, at the physiological level, PE binding sites are accessible for both plasminogen and vitronectin; however, the two human plasma proteins also compete for binding.

Plasminogen bound to PE or to intact bacteria is converted to functionally active plasmin

The protease plasmin cleaves fibrin clots, ECM components, and the complement protein C3b (8). We therefore asked whether plasminogen bound to PE is accessible for the activator uPA and can be converted to the active protease plasmin. To this end, plasminogen was bound to immobilized PE; after extensive washing, the activator uPA was added and the activity of newly generated plasmin was assayed. Plasminogen bound to PE activated by uPA and cleaved the chromogenic substrate S-2251 in a dose-dependent manner (Fig. 6A).

In addition, we assayed whether plasminogen bound to the bacterial surface is also activated by uPA. To this end, plasminogen was bound to wild-type NTHi 3655 or to the PE knockout strain, and after activation by uPA, cleavage of the synthetic chromogenic substrate S-2251 was assayed. In this case, newly generated plasmin cleaved the chromogenic substrate S-2251. This effect was dose dependent (Fig. 6B, squares). Plasminogen binds with lower intensity to the PE knockout strain and in this setup, when treated with uPA proteolytic activity was reduced by ~50% (Fig. 6B, diamonds). Similarly, in the presence of εACA, which blocks plasminogen binding to PE, proteolytic activity was reduced by ~60% (Fig. 6B, triangles). Thus, plasminogen bound to the surface of intact H. influenzae is activated to the active protease plasmin.

In addition, the activity of plasminogen bound to PE was assayed. Plasminogen was bound to immobilized PE and after washing, uPA and the substrate fibrinogen were added. After incubation, the reaction mixture was separated by SDS-PAGE, transferred to a membrane, and fibrinogen was detected by Western blotting. Bound plasmin when activated to plasmin cleaved the natural substrate fibrinogen and cleavage was time dependent. Cleavage of fibrinogen started after 15 min, and the substrate fibrinogen was totally cleaved after 2 h (Fig. 6C).

To prove the biological relevance of plasmin(ogen) at the bacterial surface, we assayed cleavage of the natural substrate fibrinogen. Again, plasminogen was bound to intact bacteria. After washing, the coated bacteria were transferred to a microtiter plate, and uPA and fibrinogen were added. After incubation, the reaction mixtures were separated by SDS-PAGE, transferred to a membrane, and fibrinogen, as well as fibrinogen cleavage fragments, were identified by Western blotting. Fibrinogen cleavage started after 1 h, and after 24 h, the fibrinogen bands were absent (Fig. 6D).

Thus, plasminogen bound to PE or to intact bacteria is converted to active plasmin.

**FIGURE 6.** Plasminogen bound to PE and to intact bacteria is functionally active. A. Plasminogen was attached to PE that was immobilized at different concentrations. After extensive washing, the activator uPA (4 ng/well) together with the chromogenic substrate S-2251 were added, and conversion of the chromogenic substrate was assayed by measuring the absorbance at 405 nm. Assays performed in the absence of uPA were used as negative control. The mean values of three independent experiments and the SDs are indicated. B. Similarly, plasminogen was bound to intact bacteria, that is, wild-type NTHi 3655 or to the PE knockout strain. After washing, the activator uPA and the chromogenic substrate S-2251 were added together with the inhibitor εACA. Plasminogen bound to bacteria where no uPA was added was used as control. A representative experiment out of three independent assays is shown. *p ≤ 0.05, **p ≤ 0.01. C. Degradation of the natural substrate fibrinogen by plasmin(ogen) bound to immobilized PE. Plasminogen was bound to immobilized PE and after washing, uPA together with fibrinogen were added. At the indicated time points, samples were removed, separated by SDS-PAGE, transferred to a membrane, and degradation of fibrinogen was assayed by Western blotting using a rabbit fibrinogen antisera and a HRP-rabbit antisera (lanes 1–7). D. Plasminogen was bound to intact bacteria (i.e., NTHi 3655), and after extensive washing, uPA and fibrinogen were added. At the indicated time points, samples were removed, separated by SDS-PAGE, and fibrinogen degradation was followed by Western blotting (lanes 1–7). A representative experiment out of three independent assays is shown.
**Plasminogen bound to PE cleaves the complement protein C3b**

To assay whether plasminogen affects complement activation, we assayed the role of PE-bound plasminogen for C3b cleavage. First, plasminogen was bound to immobilized PE; after extensive washing, uPA and C3b were added. After incubation, the reaction mixtures were separated by SDS-PAGE and C3b cleavage was analyzed by Western blotting. Plasminogen activated by uPA cleaved C3b in a time-dependent manner, and three major cleavage fragments of 68, 40, and 30 kDa were identified (Fig. 7A, lane 1–3). These cleavage fragments were detectable after 1 h, and after 24 h, C3b was completely cleaved (Fig. 7A, lane 4). Similarly, C3b cleavage by plasmin bound to intact bacteria was assayed, and after 1 h of incubation, the same cleavage products were identified (Fig. 7B, lanes 1, 2). Thus, plasminogen bound either to PE or to intact bacteria and when activated by uPA cleaved the human complement protein C3b.

**Discussion**

In this article, we identify the adhesion protein PE as a novel and major plasminogen-binding protein of NTHi. Plasminogen bound to purified PE and to intact bacteria. In both situations, bound plasminogen was accessible for the activator uPA, and when converted to plasmin cleaved the synthetic chromogenic substrate S-2251 and the natural substrates fibrinogen and C3b. Thus, plasminogen attached to the surface of *H. influenzae* and activated to plasmin allows the pathogen to control complement by degrading C3b, to interact with ECM and to degrade the ECM components like fibrinogen. Plasminogens assist in innate immune control of *Haemophilus*, facilitates bacterial survival in an immunocompetent host, and likely also spreads into deeper tissue layers.

The mechanisms by which *H. influenzae* evades host innate immune control and invades and colonizes host tissue are not completely understood. Here, we show that *Haemophilus* uses the human plasma protein plasminogen for immune evasion. Plasminogen bound to NTHi 3655 remains inactive, thus demonstrating that *H. influenzae* lacks bacterially encoded plasminogen activators. However, surface-bound plasminogen is accessible for the human activator uPA, which generates active plasmin, and converted plasmin cleaves the synthetic chromogenic substrate and also the natural substrates fibrinogen and C3b. The activation of plasminogen and the activity of plasmin bound to intact bacteria or PE is different due to the binding capacity, sterical situation, and access of the substrates in two different systems.

In the respiratory tract, *H. influenzae* adheres to the mucosal matrix and this step is crucial for pathogenesis (41) *H. influenzae*, similar to other pathogens, expresses surface proteins that are relevant for tissue adherence, complement activation, ECM interaction, and ECM degradation. Both plasminogen and plasmin activators are present in human lung tissue, thus indicating that acquisition of plasminogen and processing to active plasmin can occur in the respiratory tract and seem relevant for bacterial infection in vivo (42).

Plasminogen bound to NTHi 3655. Because clinical NTHi isolates express the adhesion protein PE, we hypothesized that PE is a plasminogen-binding protein (21). Consequently, binding of plasminogen to recombinant PE, to the PE knockout strain, and to PE expressed on the surface of *E. coli* was tested. Plasminogen bound to recombinant PE and to PE ectopically expressed on the surface of *E. coli*. Similarly, serum-derived plasminogen also bound to NTHi 3655 and to PE-expressing *E. coli*. In contrast, plasminogen binding to the PE knockout strain was reduced by 65%. Thus, PE is a novel and major plasminogen-binding protein of *H. influenzae*. The interaction both to intact bacteria and to purified PE depends on lysine residues, is ionic strength dependent, and thus resembles the binding features of other pathogen proteins, including *P. aeruginosa* Tuf, *B. burgdorferi* CRASP, and *C. albicans* Gmp1 (33, 37).

PE binds plasminogen and also the terminal complement inhibitor and adhesion protein vitronectin (20). At a physiological level, both human plasma proteins bind to PE. However, vitronectin competes with plasminogen for the free PE binding sites. The N-terminal plasminogen and vitronectin binding regions of the PE protein (i.e., residues 41–68 and 84–108, respectively) are positioned in close vicinity. This close proximity of the major binding sites explains why the two human plasma proteins bind alternatively and why they compete for PE binding.

This competitive effect suggests a role for the two human plasma proteins for complement control (43). The two PE-bound human plasma proteins allow complement control at the C3 level by bound and activated plasmin and inhibition of the terminal pathway by bound vitronectin.

Several pathogenic microbes use human plasminogen for immune evasion (44). In addition to *H. influenzae*, the Gram-negative pathogenic bacteria *B. burgdorferi* (31, 45), *P. aeruginosa* (33), and *H. pylori* (35), the Gram-positive bacteria *S. pneumoniae* (30) and *S. aureus* (46), and also human pathogenic fungi, including *C. albicans* (36, 47) and *A. fumigatus* (48), bind plasminogen. *H. influenzae*, similar to many microbial pathogens, expresses at least two plasminogen binding proteins, PE and aspartase. Thus, *H. influenzae* uses PE protein to acquire human plasminogen and uses surface-attached plasmin for immune and complement escape.

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**Disclosures**

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**References**


Wilson, R. 1998. The role of infection in COPD. Chest 113(Suppl. 4): 242S–248S.


