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Nontypeable *Haemophilus influenzae* Protein E Binds Vitronectin and Is Important for Serum Resistance¹

Teresia Hallström,* Anna M. Blom,† Peter F. Zipfel,‡ and Kristian Riesbeck²

Nontypeable *Haemophilus influenzae* (NTHi) commonly causes local disease in the upper and lower respiratory tract and has recently been shown to interfere with both the classical and alternative pathways of complement activation. The terminal pathway of the complement system is regulated by vitronectin that is a component of both plasma and the extracellular matrix. In this study, we identify protein E (PE; 16 kDa), which is a recently characterized ubiquitous outer membrane protein, as a vitronectin-binding protein of NTHi. A PE-deficient NTHi mutant had a markedly reduced survival in serum compared with the PE-expressing isogenic NTHi wild type. Moreover, the PE-deficient mutant showed a significantly decreased binding to both soluble and immobilized vitronectin. In parallel, PE-expressing *Escherichia coli* bound soluble vitronectin and adhered to immobilized vitronectin compared with controls. Surface plasmon resonance technology revealed a $K_D$ of 0.4 μM for the interaction between recombinant PE and immobilized vitronectin. Moreover, the PE-dependent vitronectin-binding site was located at the heparin-binding domains of vitronectin and the major vitronectin-binding domain was found in the central core of PE (aa 84–108). Importantly, vitronectin bound to the surface of NTHi 3655 reduced membrane attack complex-induced hemolysis. In contrast to incubation with normal human serum, NTHi 3655 showed a reduced survival in vitronectin-depleted human serum, thus demonstrating that vitronectin mediates a protective role at the bacterial surface. Our findings show that PE, by binding vitronectin, may play an important role in NTHi pathogenesis. *The Journal of Immunology*, 2009, 183: 2593–2601.

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³ Abbreviations used in this paper: MAC, membrane attack complex; C4BP, C4b-binding protein; ECM, extracellular matrix; NTHi, nontypeable *Haemophilus influenzae*; Hib, *H. influenzae* type b; Hsf, *H. influenzae* surface fibril; NHS, normal human serum; OMP, outer membrane protein; pAb, polyclonal antibody; PE, protein E; MID, Moresella IgD-binding protein.

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An initial step in the *H. influenzae* pathogenesis is adherence to the mucosa in the respiratory tract (15). Thereafter, adherence to the epithelial cells is a mechanism to circumvent the mucociliary clearance. Penetration of bacteria between host cells may also facilitate evasion from the immune system. Moreover, bacteria can reach the basement membrane and the ECM and may penetrate into deeper tissue layers and consequently into the circulation. In fact, *H. influenzae* expresses a number of surface structures that influence the process of adherence and colonization. Pili have been shown to exhibit adherence to human oropharyngeal epithelial cells, fibronectin, and other ECM proteins (16, 17). *Haemophilus* adhesion and penetration protein is another adhesin that has been reported to bind fibronectin, laminin, and collagen IV (18). Hsf is the major nonpilus adhesin found in encapsulated *H. influenzae* and this large outer membrane protein interacts with epithelial cells in addition to vitronectin (13, 19).

The recently discovered *H. influenzae* protein E (PE) is a low-molecular-mass (16 kDa) outer membrane lipoprotein with adhesive properties (20, 21). PE-expressing *H. influenzae* in addition to soluble recombinant PE(22–160) without a lipid moiety induces a proinflammatory epithelial cell response, resulting in an increased IL-8 secretion and ICAM-1 up-regulation that leads to an enhanced neutrophil adherence to epithelial cells. The adhesive PE domain is located within the central part of the molecule (aa 84–108) and preincubation of epithelial cells with this peptide blocks adhesion of several clinical _NTHi_ isolates. In the present study, we demonstrate that *H. influenzae* PE binds both immobilized and soluble vitronectin and that this interaction contributes to bacterial serum resistance. Importantly, vitronectin bound to the surface of _NTHi_ 3655 was functionally active and reduced MAC-induced hemolysis, and when _NTHi_ 3655 was exposed to vitronectin-depleted serum a significantly decreased bacterial survival was seen.

**Materials and Methods**

**Bacterial strains and culture conditions**

The _NTHi_ 3655 was a gift from R. Munson (The Ohio State University, Columbus, OH) (22) and the _NTHi_ 3655pe mutant was previously described (21). _NTHi_, wild-type, and mutant were routinely cultured in brain-heart infusion liquid broth supplemented with NAD and hemin (both at 10 μg/ml) or on chocolate agar plates. After 18 h of incubation at 37°C, CFU were determined.

**Antibodies**

Rabbits were immunized i.m. with 200 μg of recombinant PE(22–160) emulsified in CFA (Difco and BD Biosciences) and boosted on days 18 and 36 with the same dose of protein in IFA. Blood was drawn 2–3 wk later. To increase the specificity, the PE antiserum was affinity purified with Sepharose-conjugated recombinant PE(22–160) (23). To ensure that the polyclonal Ab (pAb) reacted with recombinant PE, the pAb was analyzed in ELISA. PE (100 ng/well) was immobilized overnight in microtiter plates (F96 Maxisorb; Nunc-Immuno Module) and incubated with increasing concentrations of the rabbit anti-PE pAb followed by HRP-conjugated goat anti-rabbit pAb (Dakopatts). The goat anti-human vitronectin and the FITC-conjugated donkey anti-goat pAb were purchased from Serotec. The HRP-conjugated rabbit anti-goat pAb was from Dakopatts.

**DNA cloning and protein expression**

The full-length PE(22–160) was expressed in _E. coli_ as described previously (21). Briefly, the PE DNA constructs were amplified by PCR introducing the restriction enzyme sites BamHI and HindIII. Genomic DNA from _NTHi_ 772 (H10175–H10178) was used as template (21). The homology between PE in _NTHi_ 772 and _NTHi_ 3655 is >98%. The sequence coding for the signal peptide was excluded. The PCR products were cloned into both pET26* and pET16. The resulting plasmids were transformed into _E. coli_ DH5α and thereafter into the expressing host BL21(DE3). The constructs were verified by sequencing using the BigDye Terminator Cycle Sequencing version 3.1 Ready Reaction Kit (Applied Biosystems). To produce recombinant PE(22–160) or expressing PE on the _E. coli_ surface, bacteria were grown to mid-log phase (OD₆₀₀ 0.6–0.8) followed by 1–3.5 h of induction with 1 mM isopropyl-1-thio-β-D-galactoside. Bacteria were sonicated and the recombinant proteins were purified using columns containing a nickel resin (Novagen and VWR International) according to the manufacturers’ instructions for native conditions.

**Serum bacterial assay**

NHS was prepared from pooled blood obtained from healthy volunteers with informed consent. Human serum was depleted from _C7_ or vitronectin by affinity chromatography using goat anti-human _C7_ pAb or goat anti-human vitronectin pAb coupled to a mix of protein A-Sepharose and protein G-Sepharose (GE Healthcare) overnight at 4°C. NHS was incubated with the Ab-coated Sepharose for 1 h at 4°C, followed by centrifugation to collect the depleted serum. The C7-depleted NHS was analyzed for activity in a hemolytic assay using rabbit erythrocytes. The vitronectin-depleted serum was analyzed for the presence of vitronectin and for complement activity by Western blot and hemolytic assay, respectively. The _NTHi_ 3655 wild-type (10⁷) and the corresponding PE-deficient mutant (10⁷) were diluted in DGGV (2.5 mM veronal buffer (pH 7.3) containing 0.1% (w/v) gelatin, 1 mM MgCl₂, and 0.15 mM CaCl₂) and incubated in 10% NHS, 10% heat-inactivated NHS (50 min at 56°C), and NHS (5–20% in a final volume of 100 μl at 37°C). To analyze the effect of MAC-deficient NHS or the effect of vitronectin, the _NTHi_ 3655 was incubated with 10% C7-depleted NHS or 10% vitronectin-depleted NHS. At different time points, 10-μl aliquots were removed and spread onto chocolate agar plates. After 18 h of incubation at 37°C, CFU were determined.

**Protein labeling and direct binding assay using ¹²⁵I-labeled vitronectin**

Vitronectin (Sigma-Aldrich) from human plasma was labeled with 0.05 mol of iodine (GE Healthcare) per mol protein using the chloramine-T method (24). The _NTHi_ 3655 wild-type and PE-deficient mutant strains were grown in brain-heart infusion liquid broth until mid-log phase and washed in PBS-1% BSA (Sigma-Aldrich). Bacteria (2 × 10⁷) were incubated with increasing concentrations (0.6–60 ng/ml) of ¹²⁵I-labeled vitronectin at 37°C for 1 h. After incubation, the bacteria were centrifuged (10,000 × g) through a 20% sucrose column. The tubes were frozen and cut, and radioactivity in the pellet and supernatant was measured in a gamma counter. Binding was calculated as amount of bound radioactivity (pellet) vs total radioactivity (pellet plus supernatant). In the competition assay, the _NTHi_ 3655 wild-type was preincubated with the goat anti-vitronectin pAb (5 μg/ml) or increasing concentrations of the rabbit anti-PE pAb (0–30 μl) followed by addition of ¹²⁵I-labeled vitronectin.

**Flow cytometry analysis**

The capacity for _NTHi_ to bind vitronectin and the PE protein expression were analyzed by flow cytometry. The _NTHi_ 3655 strain and the PE-deficient mutant from overnight cultures were grown in broth until mid-log phase and washed once in PBS containing 2% BSA (PBS-2% BSA). Bacteria (10⁷) were incubated with increasing concentrations of vitronectin (0.1–10 μg/ml) or NHS (0.1–10%) for 1 h at 37°C. After washings, bacteria were incubated with goat anti-vitronectin pAb followed by incubation with the FITC-conjugated donkey anti-goat pAb. After three additional washes, bacteria were analyzed in a flow cytometer (EPICS XL-MCL; Beckman Coulter). To analyze the expression of PE, bacteria were incubated with rabbit anti-PE pAb followed by washing with PBS-2% BSA and incubation for 30 min on ice with FITC-conjugated goat anti-rabbit pAb (Dakopatts). In the competition assays, the _NTHi_ 3655 wild type was incubated with increasing concentrations of heparin (Lee, Lövens Kemiske Fabrik), NaCl, PE(84–108) (PKRKYRSVRQXYKILNCANYHLTQVR) (Innovagen), PE(140–160) (LYNAAQICANYGKAFSVDKK) (Innovagen), or BSA (100 μg/ml) followed by addition of 2 μg/ml vitronectin.
Binding of H. influenzae to immobilized vitronectin

Glass slides were coated with 2 μg of human plasma vitronectin or 2 μg of BSA, air dried at room temperature, and then washed twice with PBS (13). The slides were incubated with bacteria harvested at the late exponential phase (OD600 = 0.9) for 2 h at room temperature, washed twice with PBS followed by standard Gram staining.

ELISA

Microtiter plates (F96 Polysorb; Nunc-Immuno Module) were coated with peptide fragments of PE (Innovagen), full-length PE (10 μg/ml), or BSA (10 μg/ml) in 0.1 M Tris-HCl (pH 9.0) overnight at 4°C. Plates were washed with PBS-0.05% Tween 20 and blocked for 1 h at room temperature with PBS-2% BSA. After washings, vitronectin (5 μg/ml) in PBS-2% BSA was added and incubated for 1 h at room temperature. Thereafter, the plates were washed and incubated with goat anti-human vitronectin pAb for 1 h. After additional washings, HRP-conjugated rabbit anti-goat pAb was added and incubated at room temperature for 40 min. The wells were washed, developed with 20 mM tetramethylbenzidine and 0.1 M potassium citrate, and finally the absorbance was measured at 450 nm with a MRX Microplate reader (Dynatech Laboratories).

Surface plasmon resonance

The interaction between PE(22–160) or PE(84–108) and vitronectin was further analyzed using surface plasmon resonance (Biacore 2000; Biacore and GE Healthcare). The individual flow cells of a CM5 sensor chip were activated, each with 20 μl of a mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 0.05 M N-hydroxy-sulfosuccinimide at a flow rate of 5 μl/min, after which vitronectin diluted to 20 μg/ml in 10 mM sodium acetate buffer (pH 4.0) was injected over individual flow cells to reach 2000 resonance units. Not reacted groups were blocked with 20 μl of 1 M ethanolamine (pH 8.5). A negative control was prepared for each chip by activating and subsequently blocking the surface of flow cell one. The association kinetics was studied for various concentrations of purified PE(84–108) and the synthetic peptide PE(84–108) (30–1700 nM), or the negative controls Moraxella IgD-binding protein (MID) (1000–1200) (25) and MID(1841–1868) (26) in 10 mM HEPES (pH 7.5) supplemented with 150 mM NaCl, 3 mM EDTA, and 0.005% Tween 20. Protein solutions were injected for 120 s during the association phase at a constant flow rate of 30 μl/min. The sample was injected first over the negative control surface and then over immobilized vitronectin or recombinant MID(1000–1200). Signal from the control surface was subtracted. The dissociation was followed for 120 s at the same flow rate. In all experiments, two 10-μl injections of 2 M NaCl were used to remove bound ligands during a regeneration step. The BiaEvaluation 3.0 software (Biacore) was used to analyze sensorgrams obtained. Response units obtained at the plateau of sensorgrams were plotted against concentrations of injected protein and used for calculation of equilibrium affinity constants.

Hemolytic assay

Hemolytic assays with rabbit erythrocytes were used to determine the complement activity of the C7-depleted NHS and the vitronectin-depleted NHS. Rabbit erythrocytes (2 × 10⁶ cells/ml; Rockland and Bio-Trend Chemikalien) were incubated with 10% C7- or vitronectin-depleted NHS in Mg-EGTA buffer (20 mM HEPES, 144 mM NaCl, 7 mM MgCl₂, and 10 mM EGTA; pH 7.4). After an incubation for 15 min at 37°C, the erythrocytes were centrifuged, and the amount of lysed erythrocytes was measured at 414 nm. To analyze the MAC regulatory effect of PE-bound or PE-free C7-depleted NHS, a titration of the serum concentration was performed, the NTHi 3655 survival in C7-depleted NHS was measured at 414 nm.

FIGURE 1. NTHi equipped with PE has a significantly better survival in human serum. NTHi 3655 and the NTHi 3655Δpe mutant were incubated with 10% NHS (A), 10% heat-inactivated serum (B), or increasing concentrations of NHS (5–20%) (C). D, NTHi 3655 survived in C7-depleted human serum. The NTHi 3655 was incubated in the presence of 10% NHS or 10% C7-depleted serum both diluted in DGVB ++ . At the indicated time points, bacteria were collected and spread on chocolate agar plates to allow determination of surviving bacteria. The Number of CFU (CPM) at the initiation of the experiment was defined as 100%. The mean values from three independent experiments are shown with error bars indicating SD. *, p ≤ 0.05 and **, p ≤ 0.01.

Statistics

Results were assessed by Student’s t test for paired data. A value of p ≤ 0.05 was considered to be statistically significant (*, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001).

Results

PE is important for NTHi survival in human serum

To analyze whether the outer membrane protein PE may have a role in NTHi survival upon exposure to NHS, the wild-type strain NTHi 3655 was mutated by introduction of a kanamycin resistance gene cassette in the gene encoding for PE (21). The wild-type NTHi 3655 and the PE-deficient mutant (NTHi 3655Δpe) were thereafter tested in a serum bactericidal assay. Interestingly, the mutant devoid of PE was significantly more sensitive to NHS compared with the isogenic wild-type strain (Fig. 1A). After 10 min of incubation with 10% of NHS, ~60% of the NTHi 3655Δpe was killed compared with none of the wild type. Both the wild-type strain and the mutant were resistant to heat-inactivated serum (Fig. 1B). Moreover, when a titration of the serum concentration was performed, the NTHi 3655Δpe was more sensitive to all concentrations of serum compared with the wild-type strain (Fig. 1C). Thus, these experiments suggested that PE plays a significant role in NTHi serum resistance. To investigate that the terminal pathway and MAC were responsible for the killing of NTHi 3655, the survival of NTHi 3655 in C7-depleted NHS, i.e., terminal pathway-deficient serum, was analyzed. NTHi 3655 was resistant to C7-depleted NHS, suggesting that the MAC is responsible for the killing of NTHi 3655 in NHS (Fig. 1D).
PE from H. influenzae binds human vitronectin

To determine whether H. influenzae PE is important for vitronectin-binding, NTHi 3655 and the PE mutant were incubated with soluble 125I-labeled vitronectin at increasing concentrations using a direct binding assay (Fig. 2A). The NTHi 3655 bound vitronectin in a dose-dependent and saturable manner. In contrast, a significantly decreased vitronectin binding was observed with NTHi 3655Δpe compared with the isogenic NTHi3655 wild-type strain. However, when NTHi 3655 and the PE-deficient mutant were incubated with higher concentrations of vitronectin (1–10 μg/ml) and the binding was analyzed by flow cytometry, we found that the PE-deficient NTHi 3655Δpe mutant also bound vitronectin (Fig. 2B), although to a significantly lower level than the wild type. These results suggested existence of an additional NTHi protein, which mediates bacterial vitronectin binding. Similar results were obtained by flow cytometry when NHS was used as a source of vitronectin (Fig. 2C). Flow cytometry analysis with an affinity-purified anti-PE pAb confirmed that the H. influenzae PE mutant (NTHi 3655Δpe) was PE deficient (Fig. 2D) (20). Moreover, a pAb against human vitronectin inhibited the binding of vitronectin to NTHi 3655 as examined in a direct binding assay (Fig. 2E). To further show that PE was involved in the interaction between vitronectin and NTHi 3655, the pAb against PE was used for blocking. This Ab inhibited ~50% of the binding (Fig. 2F). Thus, PE is important for NTHi-dependent vitronectin binding, but most likely also another vitronectin-binding outer membrane protein exists.

To further demonstrate that PE interacted with soluble vitronectin, E. coli-expressing PE at the surface was included in our study and analyzed by the direct binding assay (Fig. 3A) and flow cytometry (Fig. 3B). E. coli-expressing PE bound vitronectin ~4-fold better than the E. coli control that was transformed with an empty vector (Fig. 3A). When analyzed by flow cytometry, E. coli-PE bound vitronectin (10–100 μg/ml) in a dose-dependent manner as analyzed by flow cytometry, whereas NTHi 3655 wild type bound purified vitronectin and vitronectin from NHS in a dose-dependent manner. In contrast, the NTHi 3655 wild type (wt) bound vitronectin in a dose-dependent manner. In contrast, the NTHi 3655 wild type was incubated with 125I-labeled vitronectin and anti-vitronectin pAb against human vitronectin inhibited the binding of vitronectin to NTHi 3655. An anti-vitronectin pAb inhibited the binding of 125I-labeled vitronectin to NTHi 3655. F. Anti-PE pAb inhibited ~50% of the binding of 125I-labeled vitronectin to NTHi 3655. In A, the wild type and isogenic mutant were incubated with 125I-labeled vitronectin at increasing concentrations (0.6–60 ng/ml) and in D and E, the wild type was incubated with 125I-labeled vitronectin and anti-vitronectin pAb or anti-PE pAb. Binding was determined as percentage of bound radioactivity vs added radioactivity measured after separation of free and bound 125I-labeled vitronectin over a sucrose column. In B, Bacteria were incubated with increasing concentrations of vitronectin in a direct binding assay. Bacteria were incubated with higher concentrations of vitronectin (1–10 μg/ml) and the binding was analyzed by flow cytometry, we found that the PE-deficient NTHi 3655Δpe mutant also bound vitronectin (Fig. 2B), although to a significantly lower level than the wild type. These results suggested existence of an additional NTHi protein, which mediates bacterial vitronectin binding. Similar results were obtained by flow cytometry when NHS was used as a source of vitronectin (Fig. 2C). Flow cytometry analysis with an affinity-purified anti-PE pAb confirmed that the H. influenzae PE mutant (NTHi 3655Δpe) was PE deficient (Fig. 2D) (20). Moreover, a pAb against human vitronectin inhibited the binding of vitronectin to NTHi 3655 as examined in a direct binding assay (Fig. 2E). To further show that PE was involved in the interaction between vitronectin and NTHi 3655, the pAb against PE was used for blocking. This Ab inhibited ~50% of the binding (Fig. 2F). Thus, PE is important for NTHi-dependent vitronectin binding, but most likely also another vitronectin-binding outer membrane protein exists.

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manner, whereas a significantly decreased binding was detected in the control (Fig. 3B). When flow cytometry mean fluorescence intensity values were compared, E. coli-PE bound vitronectin 15-fold better than the E. coli control when incubated with the highest concentration of vitronectin (100 μg/ml; Fig. 3C).

Since vitronectin has been suggested to function as a receptor for bacteria (27), attachment of bacteria to immobilized vitronectin was investigated. NTHi 3655 and the isogenic PE mutant were applied to vitronectin-coated glass slides followed by standard Gram staining. NTHi 3655 wild type adhered to the immobilized vitronectin (Fig. 4A), whereas the NTHi 3655Δpe mutant barely bound (Fig. 4B). To further prove that PE interacted with immobilized vitronectin, the vitronectin-binding capacity of PE-expressing E. coli was analyzed. In similarity to PE expressing H. influenzae, E. coli-PE adhered to the vitronectin-coated glass slides (Fig. 4C), whereas only a few bacteria were detected when the E. coli control was analyzed (Fig. 4D). Neither NTHi nor E. coli adhered to the BSA-coated glass slides that were included as a negative control. The mean values of bacteria from 10 low-power fields are shown with error bars indicating SD. Glass slides were coated with vitronectin or BSA and incubated with the bacteria. After several washes, bacteria were Gram stained. **, p ≤ 0.01 and ***, p ≤ 0.001.

The interaction between PE and vitronectin is based upon nonionic interactions and is inhibited by heparin

The vitronectin molecule harbors several different functional regions that are involved in cell attachment, collagen, and glycosaminoglycan binding. Vitronectin contains three heparin-binding domains (1, 28). To further investigate the nature of the interaction of vitronectin to NTHi, competition experiments were performed. The NTHi 3655 wild type was preincubated with vitronectin (2 μg/ml) and increasing concentrations of heparin, NaCl, or BSA followed by an anti-vitronectin pAb. A, The interaction between NTHi and vitronectin is inhibited by increasing concentrations of heparin. The vitronectin binding of NTHi in the absence of heparin, NaCl, or BSA was defined as 100%. B, The interaction between NTHi and vitronectin is not based on ionic interactions. C, BSA did not affect the binding of vitronectin to NTHi 3655. A FITC-conjugated anti-goat pAb was subsequently added as a secondary layer followed by flow cytometry analysis. The mean values of three experiments are shown with error bars indicating SD. **, p ≤ 0.01; and ***, p ≤ 0.001.

**FIGURE 4.** PE is important for adhesion to immobilized vitronectin. A, The H. influenzae wild type adhered at a high density to vitronectin-coated glass slides. B, The NTHi 3655Δpe mutant adhered poorly to vitronectin. C, E. coli expressing PE at the bacterial cell surface adhered significantly to vitronectin. D, Wild-type E. coli adhered poorly to immobilized vitronectin. E, NTHi 3655 did not bind to BSA that was included as a negative control. F, The mean values of bacteria from 10 low-power fields are shown with error bars indicating SD. Glass slides were coated with vitronectin or BSA and incubated with the bacteria. After several washes, bacteria were Gram stained. ***, p ≤ 0.001.

**FIGURE 5.** The effect of heparin and ionic strength on the binding of vitronectin to NTHi 3655. Bacteria were incubated with vitronectin (2 μg/ml) and increasing concentrations of heparin, NaCl, or BSA followed by an anti-vitronectin pAb. A, The interaction between NTHi and vitronectin is inhibited by increasing concentrations of heparin. The vitronectin binding of NTHi in the absence of heparin, NaCl, or BSA was defined as 100%. B, The interaction between NTHi and vitronectin is not based on ionic interactions. C, BSA did not affect the binding of vitronectin to NTHi 3655. A FITC-conjugated anti-goat pAb was subsequently added as a secondary layer followed by flow cytometry analysis. The mean values of three experiments are shown with error bars indicating SD. **, p ≤ 0.01; and ***, p ≤ 0.001.
NaCl (1 M) concentration (Fig. 5B). BSA, that was included as a negative control, did not interfere with the binding (Fig. 5C). The affinity of the interaction between PE(22–160) and vitronectin was estimated using surface plasmon resonance technology. The equilibrium affinity constant ($K_D$) was found to be 0.4 µM (Fig. 6). No interaction with vitronectin was seen upon injection of the negative controls recombinant MID(1000–1200) or MID(1841–1868) at any concentration tested (data not shown).

The major vitronectin binding region is located in the central part of PE (aa 84–108)

To define the vitronectin-binding domain of PE, a series of peptides (with an overlap of four amino acids) spanning the entire PE(22–160) molecule was synthesized. The PE fragments were immobilized onto microtiter plates and vitronectin-binding was quantified by ELISA. One major vitronectin-binding domain was found within amino acids PE(84–108), which is located in the middle part of the molecule (Fig. 7). However, two additional vitronectin binding sites, i.e., PE(41–68) and PE(64–88), were most likely involved in the PE-dependent vitronectin binding, albeit these sequences had a lower binding capacity compared with PE(84–108). We could also detect a clear binding of PE(84–108) to vitronectin immobilized on the surface of a Biacore chip, but the nature of the obtained sensorgrams did not allow calculation of an accurate $K_D$ value (data not shown).

The interaction between vitronectin and the most efficient binding domain (PE(84–108)) was further confirmed using ELISA and a competition assay. Saturated conditions of the interaction between vitronectin and PE(84–108) were first defined in the ELISA (Fig. 8A). Thereafter, NTHi 3655 was incubated with vitronectin in the presence of increasing concentrations of PE(84–108) or PE(140–160) followed by flow cytometry analyses. PE(84–108) specifically inhibited the binding between NTHi 3655 and vitronectin (Fig. 8B). A total of 50 µg/ml PE(84–108) was required to block the NTHi 3655-vitronectin interaction by 75%. PE(140–160) was used as a negative control and this peptide did not block the binding between NTHi 3655 and vitronectin (Fig. 8B). Taken together, the interaction between PE and vitronectin was depending on the middle part of the PE molecule and was specific as revealed by the competition experiment.

Vitronectin bound to the surface of NTHi 3655 is still active and able to inhibit the MAC formation

Vitronectin regulates the terminal pathway by inhibiting membrane binding of C5b-7 and therefore inhibits the MAC assembly on the cell surface and protects it from lysis (1, 2). To analyze whether vitronectin in the presence of PE still inhibits the cytolytic activity of MAC, a hemolytic assay with the purified terminal complement proteins C5b-6, C7, C8, and C9 was performed. In the fluid phase, PE-bound vitronectin inhibited the cytolytic activity of C5b-9/MAC in a concentration-dependent manner (Fig. 9A). In contrast, PE(22–160) alone did not inhibit the cytolytic activity of MAC. Vitronectin, used as a positive control, blocked lysis, whereas factor H, used as a negative control, did not inhibit the lysis. Furthermore, to determine whether surface-bound vitronectin was still active and able to inhibit MAC, NTHi 3655 was preincubated with vitronectin and the capacity to inhibit MAC was

![FIGURE 6. PE(22–160) bound vitronectin as shown by surface plasmon resonance. PE was injected at least twice at increasing concentrations (20–1200 nM) over a CM5 chip with bound vitronectin and in parallel over a control flow cell that was used to subtract nonspecific signal. The amount of bound PE was measured in arbitrary response units (RU, insert). The response obtained for each concentration of PE at equilibrium (Res) was plotted against the concentration of PE, which allowed for estimation of the $K_D$.](http://www.jimmunol.org/)

![FIGURE 7. The active vitronectin-binding domain of PE is located within PE(84–108). Peptides spanning the entire PE molecule are shown. All fragments were tested for binding to vitronectin by ELISA. The peptides were coated on microtiter plates and incubated with 5 µg/ml vitronectin. Bound vitronectin was detected with a goat anti-human vitronectin pAb followed by HRP-conjugated anti-goat pAbs. Results are the mean values of three experiments and error bars indicate SD. The significance values were calculated using PE(140–160) as a reference for a nonbinding fragment. *, $p \leq 0.05$ and ***, $p \leq 0.001$.](http://www.jimmunol.org/)

![FIGURE 8. The binding between PE(84–108) and vitronectin is specific. A. To define saturating conditions, PE(84–108) (5 µg/ml) coated in microtiter plates was incubated with increasing concentrations of vitronectin as indicated. B. Bacteria were incubated with vitronectin (2 µg/ml) and increasing concentrations of PE(84–108) or PE(140–160) as indicated followed by a goat anti-human vitronectin pAb. FITC-conjugated anti-goat pAb was subsequently added followed by flow cytometry analysis. The vitronectin binding of NTHi in the absence of competitor was defined as 100%. The mean values of three experiments are shown with error bars indicating SD. *, $p \leq 0.05$; **, $p \leq 0.01$; and ***, $p \leq 0.001$.](http://www.jimmunol.org/)
that suffer from chronic obstructive pulmonary disease (30–34). In addition, NTHi is a frequent cause of acute otitis media in children and frequently colonizes patients with bronchiectasis (35). In this study, we describe for the first time a direct interaction between vitronectin, which is a regulator of the terminal complement pathway, and the outer membrane protein PE from NTHi. This previously unknown interaction is of importance for NTHi survival in contact with the complement system. A generally important feature for pathogens that allows them to cause disease is their ability to avoid and resist the bactericidal activity of the complement system. A pathogen having the capability to survive in human blood has the potential to spread to other sites of the body. NTHi is a pathogen that only rarely is associated with bacteremia. Hence, the binding of vitronectin most likely occurs at the mucosal surface. Reports of the presence of complement components in the respiratory tract of healthy individuals are scarce. However, several studies indicate the importance of complement in the respiratory tract during infections. During inflammation, the permeability of the mucosa increases and plasma, including complement proteins and immunoglobulins, enter the airway lumen (36–38). This process designated plasma exudation is likely the first line of the mucosal defense. In patients with chronic otitis medium with effusion, local complement activation in the middle ear mucosa has been observed, including an intense deposition of C3 (39). In addition, factor H, factor H-like protein 1, and factor H-related protein 1/2/3/4/5 are complement components found in middle ear effusions of patients with otitis media (40). Moreover, complement activity can also be detected in the ECM during inflammation (37).

Numerous microbes interact with the complement system in several ways to protect themselves from complement attacks. One efficient strategy used by several pathogens is binding of complement inhibitors, including vitronectin, factor H, factor H-like protein 1, and C4BP (41, 42). We have previously demonstrated that Hib binds vitronectin (13). This interaction contributed to serum resistance of Hib strains and was linked to Hsf protein expression. Moraxella catarrhalis ubiquitous surface protein A2 is another surface molecule that has been found to bind vitronectin and significantly contributes to serum resistance (43).

In this study, we demonstrate that NTHi devoid of PE had a markedly reduced survival in NHS compared with the wild type. The initial increase in the number of wild-type bacteria after 5 min suggests that NTHi uses NHS as a substrate for growth. Interestingly, this phenomenon has been seen before with other NTHi strains. One example is the C4BP binding strain NTHi 506 which also initially grew better after incubation with human serum (11). In parallel, in a study performed by Hooi et al. (44), a similar increase in the number of bacteria was observed when low concentrations of NHS (1.25–5%) were added to a NTHi strain. In our experimental set up, upon prolonged incubation (~40 min), the NTHi were killed by the complement system in NHS. However, even if the majority of the bacteria is sensitive to human serum, if just a few virulent bacteria survive, these might be able to multiply and initiate an infection. When bacteria were incubated in C7-depleted NHS, which is deficient in the terminal pathway, the NTHi 3655 strain survived, confirming that activation of the terminal pathway and consequently MAC formation is responsible for the killing of NTHi.

Furthermore, a direct binding assay showed that PE was important for binding of soluble vitronectin. This finding is in contrast to a previous study, in which no binding of soluble vitronectin to H. influenzae was found (12). The reason for this discrepancy is at present unclear. However, in parallel to NTHi, Hib also binds soluble vitronectin (13). In addition to the direct binding assay, flow cytometry experiments showed a significantly decreased vitronectin binding to the NTHi 3566Δpe compared with the isogenic wild
type when low vitronectin concentrations were used. However, the PE-deficient NTHi bound vitronectin at higher concentrations, suggesting the presence of a second vitronectin binding protein independently of PE. Moreover, when human serum was used as a source of vitronectin, similar results were obtained.

In addition to rendering the bacteria more resistant to complement-induced lysis, the bacteria-vitronectin interaction also contributes to the attachment and invasion into host cells. The vitronectin binding of Pseudomonas aeruginosa and Neisseria gonorrhoeae is involved in attachment and internalization of the bacteria into human epithelial cells (27, 45). Since vitronectin is also a component of the ECM, binding of H. influenzae to exposed ECM components may contribute to bacterial adherence, which is an essential step in the bacterial pathogenesis. These interactions appear to contribute to the spread of bacteria through tissue barriers into secondary sites of infection. Previous studies have shown that H. influenzae can interact with ECM and reconstituted basement membranes from cultured human epithelial cells (17). Binding to ECM proteins makes the bacteria able to reach deeper tissue layers of the mucosa. We demonstrate that the H. influenzae wild-type and PE-expressing E. coli transformants both bound to immobilized vitronectin. In contrast, when PE was deleted in H. influenzae, a significantly decreased binding was observed. Thus, PE-mediated bacterial attachment to vitronectin may be an important factor in initial colonization and bacterial spread to new sites of infection.

Three heparin-binding domains of the vitronectin molecule (residues 82–137, 175–219, and 348–376) have been identified (1, 28). We found that heparin inhibited the binding between PE-expressing H. influenzae and vitronectin. However, the interaction between PE and vitronectin is not dependent on ionic interactions between amino acids. In addition to NTHi PE, Hsf from encapsulated H. influenzae has been shown to interact with vitronectin (13). Hsf also interacts with both soluble and immobilized vitronectin and this interaction is inhibited by heparin and contributes to bacterial serum resistance. Despite the fact that PE is a low molecular mass protein (16 kDa) compared with Hsf (245 kDa), it is a potent binder of vitronectin. We compared the vitronectin-binding capacity of PE and Hsf and found that these two outer membrane proteins were approximately equally efficient in vitronectin-binding capacity (T. Hallström and K. Riebeck, unpublished observation).

To define the vitronectin-binding domains in PE, peptides spanning the entire PE molecule were analyzed for vitronectin binding in ELISA. PE(84–108) displayed the highest affinity for vitronectin and showed a dose-dependent binding to vitronectin. A relatively large area of PE (aa 41–108) is involved in binding to vitronectin. However, the highest signal in the ELISA analysis was obtained with the peptide comprising PE(84–108), which includes the adhesive domain mediating attachment to epithelial cells (20, 21). PE(84–108) has been shown to have affinity for the lung carcinoma cell line A549, the conjunctival epithelial cell line Chang, and the pharynx-derived cell line Detroit. Another proof of its importance for the interaction with epithelial cells is that PE(84–108) at ≥10 μg/ml significantly inhibited the binding between vitronectin and NTHi. The precise binding domain of PE on the vitronectin molecule is at present unknown and is an impetus for future studies.

The formation of the lytic pore, i.e., MAC, occurs by transition of hydrophilic complement components C5b, C6, C7, and C8, resulting in polymerization of C9. Vitronectin inhibits both the assembly and formation of MAC by inhibiting the C5b-7 complex at its membrane binding site (2). The insertion of the complex into the cell membrane is thereby inhibited, preventing lysis of the microbe. Vitronectin bound to recombiant PE(22–160) and to the surface of the bacteria (NTHi 3655) maintained its inhibitory activity and inhibited lysis in a hemolytic assay. Consequently, this inactivation protects the pathogen. In addition, when bacteria were incubated in serum lacking vitronectin but with the complement system preserved, a statistically significant decrease in survival was seen with NTHi 3655. Thus, this demonstrated that binding to vitronectin protects NTHi against the attack of the complement system.

In recent years, it has been demonstrated experimentally that H. influenzae interferes with the complement system in several ways. NTHi has been shown to interact with C4BP, factor H, and factor H-like protein 1, the soluble inhibitors of the classical/lectin and alternative pathways, and thereby uses these interactions as a protection against complement-mediated attacks (11, 14). In the present study, we have identified a novel interaction between PE of NTHi and vitronectin, the regulator of the terminal pathway. PE significantly contributes to NTHi serum resistance by interacting with vitronectin, and consequently contributes to NTHi virulence. This interaction may also contribute to bacterial colonization and spread of NTHi. These observations provide an excellent background for in vivo studies using animal models to fully establish the in vivo significance of the vitronectin binding in NTHi pathogenesis.

Disclosures
The authors have no financial conflict of interest.

References


