Haemophilus influenzae Protein E Binds to the Extracellular Matrix by Concurrently Interacting With Laminin and Vitronectin

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Nontypeable Haemophilus influenzae (NTHi) causes otitis media and is commonly found in patients with chronic obstructive pulmonary disease (COPD). Adhesins are important for bacterial attachment and colonization. Protein E (PE) is a recently characterized ubiquitous 16 kDa adhesin with vitronectin-binding capacity that results in increased survival in serum. In addition to PE, NTHi utilizes Haemophilus adhesion protein (Hap) that binds to the basement-membrane glycoprotein laminin. We show that most clinical isolates bind laminin and that both Hap and PE are crucial for the NTHi-dependent interaction with laminin as revealed with different mutants. The laminin-binding region is located at the N-terminus of PE, and PE binds to the heparin-binding C-terminal globular domain of laminin. PE simultaneously attracts vitronectin and laminin at separate binding sites, proving the multifunctional nature of the adhesin. This previously unknown PE-dependent interaction with laminin may contribute to NTHi colonization, particularly in smokers with COPD.

Haemophilus influenzae is an important human-specific pathogen that can be classified according to the presence of a polysaccharide capsule [1]. The encapsulated strains cause invasive disease, whereas the unencapsulated and hence nontypeable H. influenzae (NTHi) are mainly found in local upper and lower respiratory tract infections, albeit an increased incidence of invasive disease has been observed also by NTHi the last 5–10 years [2]. NTHi is after Streptococcus pneumoniae the most common bacterial pathogen in upper and lower respiratory tract infections and causes acute otitis media, sinusitis, and bronchitis [3–5]. In addition, NTHi is often found in patients with chronic obstructive pulmonary disease (COPD), both during stable disease as colonizers and during exacerbations [6].

An initial step in NTHi pathogenesis is adherence to the mucosa, basement membrane, and the extracellular matrix (ECM). The ECM of mammals comprises 2 main classes of macromolecules: the fibrous proteins that have both structural and adhesive functions (eg, laminin, collagens, and elastin) and the glycosaminoglycans that are linked to proteins in the form of proteoglycans [7]. The ECM stabilizes the physical structure of tissue and is involved in regulating eukaryotic cell adhesion, differentiation, migration, proliferation, shape, and structure. Bacterial interactions with the ECM play important roles in colonization of the host, and the ECM is not necessarily exposed to pathogens under normal circumstances. However, after tissue damage due to a mechanical or chemical injury or a bacterial–viral coinfection through the activity of toxins and lytic enzymes, the pathogen may gain access to the ECM.

Laminins are a family of heterotrimeric, cruciform-shaped glycoproteins of ∼400–900 kDa consisting of an
α, β, and γ chain [8]. There are different α, β, and γ chains, which combine into different laminin isoforms. The major role of laminin for the epithelium is to anchor cells to the basal membrane. Several pathogens bind laminin, including *H. influenzae*, *Yersinia enterocolitica*, *Mycobacterium tuberculosis*, and *Leptospira interrogans* [9–13]. *Moraxella catarrhalis* is another pathogen that via ubiquitous surface proteins (Usp) A1 and A2 interacts with laminin, and this interaction may play an important role in *M. catarrhalis* infection during exacerbations in patients with COPD [14].

NTHi expresses a number of surface structures that influence the process of adherence and colonization. Both pilus and nonpilus adhesins of *H. influenzae* have displayed adherence to ECM proteins. *Haemophilus* adhesion and penetration protein (Hap) is an adhesin that binds fibronectin, laminin, and collagen I [15]. Hap is ubiquitous among *H. influenzae* isolates and mediates adhesion to respiratory cells, invasion, and bacterial aggregation [16, 17]. In addition to Hap, Protein E (PE) is a low molecular weight (16 kDa) outer membrane lipoprotein with adhesive properties [18, 19]. PE induces a proinflammatory epithelial cell response resulting in an increased interleukin 8 (IL-8) secretion and intercellular adhesion molecule 1 (ICAM-1) upregulation that leads to an enhanced neutrophil adherence to epithelial cells [18]. The adhesive PE domain is located within the central part of the molecule (amino acids 84–108). In addition, PE binds vitronectin, and this interaction is important for attachment and for survival of NTHi in human serum [20]. We recently analyzed a large series of clinical NTHi isolates (*n* = 186), encapsulated *H. influenzae* strains, and culture collection strains [21]. PE was expressed in >98% of all NTHi independently of the growth phase, and was highly conserved in both NTHi and encapsulated *H. influenzae* (96.9%–100% identity without the signal peptide). The epithelial cell-binding region in the central part of the PE molecule (PE84–108), which also binds to human vitronectin [20], was completely conserved supporting a significant biological function [21].

NTHi is commonly found in patients suffering from COPD [22]. Smoking is associated with increased incidence and severity of COPD, and a previous study has demonstrated that the laminin layer in the basement membrane is significantly thicker in smokers than in nonsmokers [23]. This increased laminin expression thus may pave the way for laminin-binding respiratory pathogens and explain the increased incidence of NTHi in COPD patients. In this study, we show that NTHi binds soluble and immobilized laminin and that both PE and Hap are involved in this interaction. The specific laminin-binding region was defined within the N-terminal part of the PE molecule and the C-terminal globular domains of laminin are responsible for the binding to PE. Taken together, the PE-dependent adhesion to laminin may be important in NTHi pathogenesis, particularly in lower respiratory tract infections.

### MATERIALS AND METHODS

**Bacterial Strains and Culture Conditions**

NTHi3655 was a kind gift from R. Munson [24]. The NTHi wild type and mutants were cultured in brain–heart infusion (BHI) liquid broth supplemented with nicotinamide adenine dinucleotide (NAD) and hemin (both at 10 µg/mL), or on chocolate agar plates at 37°C in a humid atmosphere containing 5% CO2. NTHi3655Δpe was cultured in BHI supplemented with 17 µg/mL kanamycin (Merck), and NTHi3655Δhap was incubated with 3 µg/mL chloramphenicol (Sigma-Aldrich). Both kanamycin and chloramphenicol were used for growth of the NTHi3655Δpe/hap. NTHi were isolated from patients (*n* = 19; Southwest Skåne) with upper and lower airway infections, meningitis, and sepsis (Table 1) in 2007.

**Manufacture of Mutant NTHi Strains**

NTHi3655Δpe was as previously described [18]. To produce Hap-deficient mutants, the 5′-end of *hap* (accession number: U11024) was amplified as 2 cassettes introducing the restriction enzyme sites BamHI and SalI, or SalI and Xhol in addition to specific uptake sequences [25]. The polymerase chain reaction (PCR) products were cloned into pBluescript SK(+) and, a chloramphenicol resistance gene cassette was amplified introducing a SalI restriction enzyme site. The product was ligated into the truncated *hap* gene fragment. NTHi3655 and NTHi3655Δpe were transformed according to the M-IV method [26].

**Protein Labeling and Direct Binding Assay Using Iodine-125-Labeled ECM Proteins**

To analyze binding of NTHi to various ECM proteins, we labeled laminin-1 (from Engelbreth–Holm–Swarm mouse sarcoma; murine laminin shows 82% identity with human laminin [27]), human vitronectin, fibronectin, and fibrinogen (all from Sigma-Aldrich) with iodine using the chloramine T method [28]. The NTHi strains were grown overnight in BHI liquid broth and washed with phosphate-buffered saline containing 1% bovine serum albumin (PBS-1%BSA). We incubated bacteria (2 × 107) with iodine-125 (125I)–labeled ECM proteins or increasing concentrations of 125I-labeled laminin (0–800 kcpm) for 1 hour at 37°C. After incubation, bacteria were either washed with PBS-1%BSA followed by measurement of 125I-labeled laminin bound to the bacteria in a gamma counter or centrifuged (10,000 × g) through 20% sucrose. The sucrose tubes were frozen and cut, and we measured radioactivity in pellets and supernatants in a gamma counter. We calculated binding as amount of bound radioactivity (pellet) vs total radioactivity (pellet plus supernatant). In the competitive binding assay, we added laminin or fibrinogen at increasing concentrations (5–100 µg/mL) to the reactions.

**Transmission Electron Microscopy**

We used negative staining and transmission electron microscopy (TEM) to analyze binding of gold-labeled laminin to the surface...
of NTHi3655 wild type and mutants and binding of gold-labeled PE to laminin as described [29]. We labeled laminin and recombinant PE22160 with 5 nm colloidal thiocyanate gold [30].

**Binding of NTHi to Immobilized Laminin**

To analyze binding of NTHi to immobilized laminin, we coated glass slides with 20 μl g laminin or BSA, air-dried them at room temperature (RT), washed them twice with PBS, and incubated them with NTHi at late exponential phase (OD600 = 0.9) for 2 hours at RT. Thereafter, we washed the slides twice with PBS, and bound bacteria were Gram stained.

**Enzyme-Linked Immunosorbent Assay**

We coated microtiter plates (F96 Polysorb, Nunc-Immuno Module) with peptide fragments of PE (40 μM) (Innovagen), full-length PE (5–10 μg/mL), or BSA (10 μg/mL) in 0.1 M Tris-HCl (pH 9.0) overnight at 4°C. We washed plates with PBS-0.05% Tween and blocked them for 1 hour at RT with PBS-2%BSA. After washings, we added laminin (5–30 μg/mL) and vitronectin (5–30 μg/mL) in PBS-2%BSA and incubated plates for 1 hour at room temperature. We detected binding with rabbit antilaminin pAb or rabbit antivitronectin pAb and HRP-conjugated anti-rabbit pAb. We washed the wells, developed them with 20 mM tetramethylbenzidine or 0.1 M 1,2-phenylenediamine dihydrochloride (OPD, DakoCytomation), and finally measured the absorbance at 450 or 492 nm, respectively. In the competition assay, we incubated PE22–160 with laminin (5 μg/mL) that had been preincubated with increasing concentrations of PE41–68 (0–300 μg/mL), PE44–108 (0–250 μg/mL) or heparin (0–1000 μg/mL).

**Surface Plasmon Resonance**

The PE–laminin interaction was analyzed using surface plasmon resonance (Biacore 2000). We activated 2 flow cells of a CM5 sensor chip, 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 10 μl/min, after which we injected laminin (10 μl/mL in 10 mM sodium acetate buffer, pH 4.0) over flow cell 2 to reach 4000 resonance units (RU). We blocked unreacted groups with 20 μl of 1 M ethanolamine (pH 8.5). We prepared a negative control by activating and subsequently blocking the surface of flow cell 1. We studied binding for various concentrations of purified PE22–160 in the range of 40–1250 nM in flow buffer (50 mM HEPES, pH 7.5 containing 150 mM NaCl, 3 mM EDTA, and 0.005% Tween-20). We injected protein solutions for 8 minutes during the association phase at a constant flow rate of 15 μl/min and then allowed them to dissociate for 10 minutes. We injected the sample first over the negative control surface and then over immobilized laminin. We subtracted the signal from the control surface. Bound PE22–160 was removed during each regeneration step with consecutive injections of 10 μl each of 4 M MgCl2 and 2 mM NaOH. We used BiaEvaluation 3.0 software (Biacore) for data analysis.

**Statistics**

Results were assessed by the Student t test for paired data. P ≤ .05 was considered statistically significant (*, P ≤ .05; **, P ≤ .01; ***, P ≤ .001).

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**Table 1. Epidemiological Data and Clinical Diagnoses Related to the Strains of NTHi in the Study**

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Age, y</th>
<th>Gender</th>
<th>Culture site</th>
<th>Clinical diagnosis a</th>
</tr>
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<td>Male</td>
<td>Nasopharynx</td>
<td>Upper airway infection</td>
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<tr>
<td>KR 251</td>
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<td>Nasopharynx</td>
<td>Lower airway infection</td>
</tr>
<tr>
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<td>Nasopharynx</td>
<td>Upper airway infection</td>
</tr>
<tr>
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<td>Bronchoalveolar lavage</td>
<td>Pneumonia</td>
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<td>Male</td>
<td>Blood</td>
<td>Meningitis and severe sepsis</td>
</tr>
<tr>
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<td>Blood</td>
<td>Pneumonia with sepsis</td>
</tr>
<tr>
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<td>Blood</td>
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<tr>
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<td>Upper airway infection</td>
</tr>
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</table>

* Based on the clinical information provided by the referring physician in the cases of nasopharyngeal and bronchoalveolar lavage culture. In the cases of blood cultures, the information is based on a retrospective control of the medical journal (Regional ethical committee for medical research, Lund, Sweden [2009/536]).
RESULTS

Clinical Nontypeable \textit{H. influenzae} Isolates Bind Laminin

Several bacterial species have been shown to bind laminin and thereby interact with the ECM [9, 12, 14]. To analyze whether laminin binding is a common feature of NTHi, 19 clinical NTHi isolates and NTHi3655 were tested for binding of $^{125\text{I}}$-laminin. Of the NTHi strains, 16 significantly bound laminin, whereas 4 were low binders with a variable binding as compared with binding capacity of an \textit{Escherichia coli} laboratory strain (Figure 1A). Among all the clinical isolates tested, our virulent model strain NTHi3655 [24] showed the highest laminin binding capacity ($38.8\% \pm 7.1\%$ of added $^{125\text{I}}$-laminin). Thus, the capacity to bind laminin is shared by most of the clinical NTHi isolates tested.

PE-Deficient NTHi Shows a Significantly Decreased Binding to Laminin

Because PE is a recently discovered adhesin found in most \textit{H. influenzae} strains [18, 21], we investigated whether PE plays a role in adhesion to the ECM protein laminin. The high-capacity laminin-binding NTHi3655 wild type (Figure 1) was chosen for analysis of binding of the different radiolabeled ECM proteins: laminin, vitronectin, fibronectin, and fibrinogen (Figure 1B). In addition, a specific NTHi3655 mutant without PE [19] was included in the analysis. PE-expressing NTHi3655 bound significantly better both iodine-labeled laminin and vitronectin [20] than did the NTHi3655\textsuperscript{Dpe}, suggesting that PE was involved in the NTHi–laminin interaction. In contrast, both NTHi3655 and NTHi3655\textsuperscript{Dpe} bound fibronectin and fibrinogen to a similar extent, proving that PE is not the major bacterial receptor in these interactions but mainly is involved in the interaction with laminin and vitronectin.

PE and Hap Are the Major Laminin-Binding Proteins Expressed by NTHi

Increasing concentrations of iodine-labeled laminin were added to NTHi3655 and we found that bacteria bound laminin in a saturable manner (Figure 2A). In contrast, a significantly decreased laminin binding was observed with NTHi3655\textsuperscript{Dpe} as compared with the isogenic NTHi3655 wild-type strain. To test the specificity of the laminin binding to NTHi, bacteria were incubated with increasing concentrations of unlabeled laminin (5–100 $\mu$g/mL) with $^{125\text{I}}$-labeled laminin. Laminin inhibited the binding of $^{125\text{I}}$-laminin to NTHi3655 in a dose-dependent manner (Figure 2B) with $>70\%$ inhibited binding at 50 $\mu$g/mL of laminin. Because fibrinogen did not bind to PE (Figure 1B), this ECM protein was included as a negative control (Figure 2B). Thus, the binding between NTHi3655 and laminin was specific, and the PE-deficient NTHi showed a significantly decreased binding to laminin.

Because Hap is an NTHi adhesin that also binds laminin [15], we investigated the role of Hap in relation to PE. To accomplish this, Hap was mutated in NTHi3655 as well as in NTHi3655\textsuperscript{Dpe}. The wild-type NTHi3655 and specific mutants without PE or Hap were analyzed in the direct binding assay using $^{125\text{I}}$-laminin. We observed a significantly reduced laminin binding with all mutants compared with the wild-type counterpart (Figure 2C). Thus, both PE and Hap significantly contributed to the interaction with soluble laminin. \textit{E. coli} was a negative control in these experiments and showed background binding. These interactions were further confirmed by TEM using gold-labeled laminin (Figure 2D).

To investigate the attachment of bacteria to immobilized laminin, we applied the different NTHi3655 strains to glass slides coated with laminin. PE- and Hap-expressing NTHi3655

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Protein E (PE) plays a major role in nontypeable \textit{Haemophilus influenzae} 3655 (NTHi3655)--dependent binding of soluble laminin. A, Binding of laminin to a series of nasopharyngeal nontypeable \textit{H. influenzae} (NTHi) isolates. Bacteria ($2 \times 10^7$) were incubated with iodine-125 ($^{125\text{I}}$)--labeled laminin. The bound fraction of laminin was measured in a gamma counter. B, PE plays a major role in NTHi3655-dependent binding of soluble laminin and vitronectin, whereas other extracellular matrix (ECM) proteins are not bound by PE, as revealed with a wild-type strain (wt) and a PE-deficient mutant. Bacteria ($2 \times 10^7$) were incubated with various $^{125\text{I}}$-labeled ECM proteins. Binding was determined as percentage of bound radioactivity vs added radioactivity measured after separation of free and bound $^{125\text{I}}$-labeled proteins over a sucrose column. The mean values of 3 experiments with duplicates are shown with error bars indicating standard deviation (SD) (**, $P \leq .01$).}
\end{figure}
strains were found to adhere to the laminin-coated glass slides (Figure 3A), whereas the PE- or Hap-deficient mutants showed a reduced adherence compared with the wild-type strain (Figure 3B–D). E. coli bound only weakly to laminin (Figure 3E), and NTHi3655 wild type did not adhere to BSA that was included as an additional negative control (Figure 3F). Taken together, both Hap and PE were major laminin-binding NTHi proteins as shown in a series of different experimental setups.

The Laminin Binding Region Is Located Within the N-terminal Part of PE (Amino Acids 41–68)

To pinpoint the interaction of PE with laminin, we incubated recombinant PE22–160 with increasing concentrations of laminin. PE22–160 bound soluble laminin in a dose-dependent manner (Figure 4A). When the interaction between PE and laminin was studied using surface plasmon resonance with laminin immobilized on a CM5 chip, we revealed a dose-dependent binding with the affinity constant dissociation constant \(K_d = 1.54 \pm 1.01 \mu M\) (Figure 4B).

The major laminin-binding region was located within the N-terminal PE41–68 and the binding was dose-dependent and saturated (Figure 4C and D). In addition, PE84–108 also bound laminin but with a lower binding capacity (Figure 4C). To confirm these findings, we tested PE41–68 for its ability to inhibit PE22–160-binding to soluble laminin. PE41–68 was able to inhibit this interaction, and 150 \(\mu g/mL\) was required to reduce the binding by 50% (IC50) (Figure 4E). PE84–108 at low concentrations decreased the binding slightly but at higher concentrations no inhibition of the laminin-binding to PE22–160 was detected (Figure 4F).

To confirm that laminin and vitronectin bound simultaneously to different parts on the PE molecule, we analyzed concurrent binding. Immobilized PE22–160 was incubated with a mixture of laminin and vitronectin at different concentrations followed by separate detection of bound proteins with either antilaminin or antivitronectin pAbs in ELISA. Laminin did not interfere with the binding of vitronectin to PE22–160, as increasing concentrations of laminin did not affect the vitronectin binding (Figure 5A). Similarly, vitronectin did not affect the laminin–PE interaction as increasing concentrations of vitronectin did not quench laminin (Figure 5B). In conclusion, PE is a multifunctional adhesin containing 2 separate binding sites; the N-terminal region PE41–68 harbored the laminin-binding part of the molecule, whereas the vitronectin-binding region [20] in addition to the adhesive domain [18] were located within PE84–108 (Figure 5C).
Thus, the binding site of PE22 laminin to PE22 Increasing concentrations of heparin inhibited the binding of site [35], we performed inhibition experiments with heparin. The LG domains G4 and G5 contain an active heparin-binding C-terminal globular domain of laminin (Figure 6 TEM revealed that gold-labeled recombinant PE bound to the PE22–160 Interacts With the C-terminal Globular Domains of Laminin Laminin is a glycoprotein that is composed of an α-, β-, and γ-polypeptide chain joined together through a coiled coil with 1 long and 3 short arms (Figure 6A) [31]. Both human and murine laminin-1 contains the α1, β1, and γ1 chains, and the gene encoding for the human and mouse α1 and β1 chains shows an identity of 76 and 93%, respectively [33, 34]. The C-terminal end of the long arm of laminin is composed of 5 globular domains named laminin globular (LG) domains G1–G5 [32, 35]. TEM revealed that gold-labeled recombinant PE bound to the C-terminal globular domain of laminin (Figure 6B). Because the LG domains G4 and G5 contain an active heparin-binding site [35], we performed inhibition experiments with heparin. Increasing concentrations of heparin inhibited the binding of laminin to PE22–160 in a dose-dependent manner (Figure 6C). Thus, the binding site of PE22–160 on the laminin molecule was located within LG domains G4 or G5.

**DISCUSSION**

An initial step in the pathogenesis of *H. influenzae* is adherence to the mucosa followed by attachment to epithelial cells and the ECM in the respiratory tract. NTHi expresses a number of adhesins that are involved in the success of NTHi colonization in patients with, for example, COPD [36]. Here we demonstrate a specific binding of the ECM protein laminin to NTHi. Interestingly, the adhesin PE appeared to have a dominant role in *Haemophilus*-dependent laminin binding to the well-known laminin-binding protein Hap [15].

*H. influenzae* PE is a 16 kDa lipoprotein with adhesive properties [19]. NTHi without PE showed a significantly decreased laminin-binding compared with that of the isogenic wild-type strain. In addition, Hap from *H. influenzae* also binds laminin [15]. Hap is an adhesin that mediates adherence to epithelial cells, ECM, bacterial aggregates, and microcolony formation [37]. When PE or Hap was deleted in our model strain NTHi3655, we observed a significantly decreased binding to both soluble and immobilized laminin, suggesting that these 2 proteins are the major laminin-binding proteins. However, the double mutant that lacked PE and Hap weakly bound to both soluble and immobilized laminin, suggesting additional laminin-binding proteins expressed by NTHi. The expression of multiple laminin-binding proteins has also been shown for several other pathogens, eg, *L. interrogans*, *Streptococcus pneumoniae*, *Borrelia burgdorferi*, and *M. catarrhalis* [14, 38–44].

NTHi is among the most common pathogens found in exacerbations as well as in stable disease in patients suffering from COPD [22]. Among the major causal factors of COPD is smoking, and in smokers there are pathological changes such as loss of epithelial integrity, which results in exposure of the basement membrane where the laminin layer is thickened [23, 45]. The human lung contains several different forms of laminin, including the cruciform laminin-10 and laminin-11 [33]. Laminin-1 used in this study and laminin-10 both contain β1 and γ1 chains, suggesting an importance of the NTHi/laminin interaction during infections in the lung. Little is known about the distribution and alteration of laminin during pathological conditions such as COPD. In addition, whether bacterial infections can alter the laminin expression or composition remains to be studied. However, these patients also have an increased synthesis and deposition of ECM proteins, including laminin [46]. Smoking thus indirectly may promote a more efficient laminin-dependent NTHi colonization. Other pathogens that cause respiratory infections, such as *M. catarrhalis*, also possess laminin-binding proteins [14], suggesting adhesive mechanisms that are shared by several respiratory pathogens.

The major laminin-binding domain was found within PE41–68, and inhibition experiments with peptides confirmed that PE41–68 was responsible for the interaction. In a recent paper, we showed that PE84–108 binds to epithelial cells in addition to
The laminin binding region is located within protein E (PE) 1-68. A, Immobilized PE 22-160 (5 μg/mL) binds laminin in a dose-dependent manner. The background binding was subtracted from all samples. B, PE 22-160 bound laminin as shown by surface plasmon resonance. C, The active laminin-binding region is located within PE 41-68. D and E, The binding of PE 41-68 to laminin is dose-dependent and specific. F, PE 84-108 does not inhibit laminin binding. PE 22-160 (5 μg/mL) (A), peptides (40 μM) spanning the entire PE molecule (with an overlap of 4 amino acids) (C), or PE 41-68 (D) was incubated with laminin (5 μg/mL) (A, C) or increasing concentrations of laminin (0–5 μg/mL) (D), and binding was analyzed in enzyme-linked immunosorbent assay (ELISA). In E and F laminin (5 μg/mL) was added with increasing concentrations of PE 41-68 (2–300 μg/mL) or PE 84-108 (2–300 μg/mL) to microtiter plates coated with PE 22-160. In A and C–F, bound laminin was detected with a rabbit antilaminin pAb followed by horseradish peroxidase (HRP)-conjugated goat antirabbit pAb. The mean values of 3 independent experiments are shown with error bars indicating standard deviation (SD). Statistical significance of differences was estimated using Student’s t test. ***, P ≤ .001, *, P ≤ .05. B, Binding of PE to laminin was studied using surface plasmon resonance (Biacore 2000). Laminin was immobilized on a CM5 chip, and increasing concentrations of PE (40–1250 nM) were injected and sensorgrams recorded (inset). The sensorgram obtained for 1250 nM of Moraxella IgD binding protein (MID), a negative control, is shown as dotted line. Responses at equilibrium were plotted vs concentration of injected PE and the dissociation constant (K_D) = 1.54 ± 1.01 μM was calculated using a steady-state affinity equation in Biaevaluation 3.0.
human vitronectin [18, 20]. Competition assays with laminin and vitronectin confirmed that both proteins are able to bind PE simultaneously, showing different binding sites on the PE molecule. These results reveal that various regions of PE have different specific functions. Despite the small size (16 kDa), PE binds epithelial cells, vitronectin and laminin, suggesting that it is multifunctional (Figure 6D). Similar binding profiles have been shown for other bacterial proteins, eg, the M. catarrhalis UspA1/2 family that bind laminin, vitronectin, fibronectin, and C3 [14, 47–49]. Laminin is a glycoprotein that consists of an α-, β-, and γ-polypeptide chain joined together through a coiled coil with 1 long and 3 short arms [31]. The C-terminal end of the long arm is composed of 5 α-chain laminin globular (LG) domains G1–G5 and LG domains G4 and G5 contain a heparin-binding site.

Figure 5. Protein E (PE) is a multifunctional protein that binds both laminin and vitronectin via different regions. A, B, Laminin and vitronectin bind concurrently to PE. A, Increasing laminin concentrations did not influence vitronectin binding to PE. B, Increased concentrations of vitronectin did not influence laminin binding to PE. A, B, PE22−160 was immobilized and concurrent binding of laminin and vitronectin (micrograms added are shown) were detected by specific antibodies in enzyme-linked immunosorbent assay (ELISA). C, Illustration showing the multifunctional PE. The laminin-binding region is located within PE41−68, and the vitronectin and epithelial cell–binding regions are within PE84−108 [18, 20].

Figure 6. Protein E22−160 binds the C-terminal globular domains G4 and G5 of laminin. A, Schematic cartoon of laminin showing the composition of an α- (gray), β- (white), and γ-polypeptide chain (black) joined together through a coiled-coil with 1 long and 3 short arms [31]. The illustration is modified from McKee et al and Durbeej et al [31, 32]. The C-terminal end of the long arm is composed of 5 α-chain laminin globular (LG) domains G1–G5 and LG domains G4 and G5 contain a heparin-binding site. B, Protein E (PE) binds the C-terminal globular head of laminin. Gold-labeled PE22−160 was mixed with laminin and examined by electron microscopy. C, Increasing amounts of heparin inhibited the binding of laminin to PE22−160. In C, immobilized PE22−160 was incubated with laminin and increasing concentrations of heparin, and bound laminin was detected with a rabbit antilaminin pAb followed by horseradish peroxidase (HRP)-conjugated antirabbit pAb. The mean values of 3 independent experiments are shown with error bars indicating standard deviation (SD). Statistical significance of differences was estimated using the Student t test. ***, P < .001. D, Schematic picture of simultaneous binding of PE to laminin and vitronectin. PE-dependent binding of laminin may contribute to nontypeable Haemophilus influenzae (NTHi) adhesion and colonization of the host. The PE-dependent binding of the complement regulator vitronectin to the surface of NTHi protects against complement-mediated attacks and significantly contributes to the survival of NTHi in human serum.
PE bound the C-terminal globular domains of laminin as revealed by TEM. These domains also bind heparin [32, 35], and heparin inhibits the interaction between PE and laminin, confirming the involvement of the C-terminal globular domains G4 and G5. The ability to bind laminin, which is a major constituent of the ECM and basal membrane, suggests that bacteria may use this interaction for adherence to the lung parenchyma followed by an efficient colonization of the host. In fact, M. tuberculosis probably uses laminin as a target protein to facilitate adhesion to host epithelial cells [12]. Several pathogen-derived surface proteins that bind laminin are recently identified, eg, Sc1 from S. pyogenes, LipL53, Lsa21 and Lsa63 from L. interrogans, and CRASP-1 from B. burgdorferi [38, 40–42, 44].

In conclusion, we have shown that the adhesin and vitronectin-binding PE of NTHi has the basement-membrane glycoprotein laminin as a major target. The specific interaction with laminin may contribute to adhesion, bacterial colonization and spread. Laminin-binding proteins most likely play a larger role than previously anticipated both in the upper respiratory tract in children and in the airways of COPD patients. However, more investigations are required to fully delineate the importance of pathogen-dependent interactions with laminin.

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