The nine residue plasminogen-binding motif of the pneumococcal enolase is the major cofactor of plasin-mediated degradation of extracellular matrix, dissolution of fibrin and transmigration

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Summary
The glycolytic enzyme α-enolase represents one of the non-classical cell surface plasminogen-binding proteins of Streptococcus pneumoniae. In this study we investigated the impact of an internal plasminogen-binding motif of enolase on degradation of extracellular matrix and pneumococcal transmigration. In the presence of host-derived plasminogen activators (PA) tissue-type PA or urokinase PA and plasminogen S. pneumoniae expressing wild-type enolase efficiently degraded Matrigel or extracellular matrix (ECM). In contrast, amino acid substitutions in the nine residue plasminogen-binding motif of enolase significantly reduced degradation of ECM or Matrigel by mutated pneumococci. Similarly, recombinant wild-type enolase but not a mutated enolase derivative that lacks plasminogen-binding activity efficiently degraded ECM and Matrigel, respectively. In particular, bacterial cell enolase-bound plasmin potentiated dissolution of fibrin or laminin and transmigration of pneumococci through a fibrin matrix. In conclusion, these results provide evidence that the enolase is the major plasminogen-binding protein of pneumococci and that the nine residue plasminogen-binding motif of enolase is the key cofactor for plasin-mediated pneumococcal degradation and transmigration through host ECM.

Keywords
Pneumococci, plasmin activity, enolase, fibrinolysis, degradation

Introduction
Plasminogen (PLG; ~ 90 kDa) is the single-chained pro-enzyme of the broad spectrum serine protease plasmin. PLG, which is abundant in human plasma and extracellular fluids in a concentration of 2 µM (1) as well as being associated with virtually every cell surface in the body, comprises a pre-activation peptide of ~ 8 kDa and five homologous disulfide-bonded triple-loop kringel structures (K1–5; 65 kDa) and a serine-protease domain (2). Conversion of the single-chained pro-enzyme to active plasmin is mediated by proteolytic activation through the mamalian plasminogen activators (PA), tissue-type plasminogen activator (tPA) and urokinase (uPA) (3). Plasmin is involved in intravascular fibrinolysis (4, 5) degradation of extracellular matrix (ECM) material, and is relevant for cell invasion (6). Moreover, plasmin plays a key role in the activation of metalloproteinases, and is thus implicated in wound healing, tissue remodelling (7, 8), tumor metastasis (9), and angiogenesis (10). The kringle modules contain lysine-binding sites that mediate the binding of PLG to fibrin and cellular plasminogen receptors including α-enolase (11).

Likewise, various bacterial pathogens bind PLG through its lysine-binding kringle domains to cell surface receptors. In particular, Streptococcus pneumoniae is a human pathogenic microorganism causing life-threatening diseases such as pneumonia, bacteremia and meningitis and plasmin(ogen) binding to outer-cell surfaces was found to be mediated by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and α-enolase (12–15). The enzymatic activity of bound plasmin may facilitate bacterial invasion and dissemination in the infected host (16–18). α-enolase can reassociate with the bacterial cell surface resulting in enhanced PLG-binding capacity that was initially traced to the C-terminal lysine residues of α-enolase and inhibited by ε-amino-caproic acid (12). Deletion of the C-terminal lysine residues (binding site 1 or BS1; 433KK434) in recombinant α-enolase abolished PLG binding under denaturing conditions, but PLG bind-
ing was not impaired under non-denaturing conditions. A nine residue internal PLG-binding site (binding site 2 or BS2) represented by FYDKERKVY was subsequently identified and comprises residues 248 to 256 of α-enolase (13). Structural analysis of pneumococcal α-enolase indicated an octameric oligomer, whereby the C-terminal lysine residues were located in an interdimer groove and are involved in the oligomerization of the enolase (19). The BS2 is exposed to the octamer surface and proposed as the primary site for plasminogen binding (19).

The impact of pneumococcal cell outer surface recruitable plasmin activity and the impact of PLG-binding by α-enolase related to bacterial transmigration, however, remain to be uncovered. In this study, we demonstrate that in particular the α-enolase located on the surface is responsible for the recruitment of proteolytic activity. In addition, we identified the nine residue PLG-BS2 of pneumococcal enolase as the key cofactor for plasmin mediated degradation of Matrigel, ECM and dissolution of fibrin. This activity facilitates pneumococcal transmigration through host ECM, since a mutant α-enolase lacking this binding site hardly promotes bacterial transmigration.

Materials and methods

Bacterial strains and proteins

*S. pneumoniae* serotype 2 strain D39 and its isogenic mutants D39eno<sup>del</sup>, D39eno<sup>int</sup>, and D39eno<sup>int/del</sup> were cultured at 37°C in Todd-Hewitt broth (Oxoid) supplemented with 0.5% yeast extract (THY) to OD<sub>600</sub> 0.4 or grown on blood agar plates (Becton Dickinson). Construction of the mutants and the deletion of PLG-BS1 (KK<sub>434</sub>) at the C-terminus of amino acid substitutions in PLG-BS2 (<sup>256</sup>FYDKERKVY<sup>256</sup>) of enolase was described previously (13). Expression and purification of His<sub>6</sub>-tagged fusion proteins was performed as described previously (12, 13). The respective plasminogen-binding activities of the isogenic mutants as well as the recombinant proteins Eno<sup>int/del</sup> are summarized in Table 1 (13). Mutants were grown in media containing 10 µg/ml erythromycin. *Escherichia coli* M15[<sup>pREP4</sup>] (Qiagen) was used as the host strain for recombinant pQE expression plasmids and cultured at 37°C on Luria-Bertani (LB) agar or grown on LB-agar containing 100 µg/ml ampicillin and 50 µg/ml kanamycin.

Human proteins

Human plasminogen (PLG) was purified as described (20) with an additional ion-exchange step (21). The purity was controlled by SDS-PAGE and immunoblot analysis using goat anti-plasminogen antibody (1:200; Affinity Biologicals, Ontario, Canada) and peroxidase labeled anti-goat antibodies (1:1000; Sigma, Germany). The following reagents were obtained from the respective suppliers: Human tissue plasminogen activator (tPA; Calbiochem, La Jolla), two chain form urokinase plasminogen activator (uPA; Chemicon, Temecula, USA), α<sub>2</sub>-antiplasmin (Calbiochem), aprotinin (Fluka), and phenyl-methyl-sulfonylfluoride (PMS chemicals, Roth). Enolase-coated microspheres

Recombinant wild-type enolase or mutated enolase<sup>int/del</sup> was coated on fluorosbrite<sup>™</sup> carboxy microspheres (3 µm diameter, Polysciences Inc.) according to the manufacturer’s instructions. Briefly, a solution of 20 µl of fluorosbrite<sup>™</sup> carboxy microspheres containing 2x10<sup>5</sup> beads was incubated overnight at 4°C with 100 µg recombinant enolase protein. Prior to use beads were blocked for 2 h with 1% bovine serum albumin in PBS. Immobilization of enolase proteins was verified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gels followed by staining with Coomassie brilliant blue or immunoblot analysis with anti-enolase antibodies as described (12).

Plasminogen-binding and plasmin activity assay

*S. pneumoniae* (10<sup>5</sup> CFU/ml) or enolase-coated microspheres were preincubated with 40 µg/ml plasminogen in PBS for 15 min at 37°C. In degradation assays and transmigration assays PLG (bound to bacterial cell outer surface, microspheres or in its soluble form) was activated with 0.24 KIU tPA or 0.06 KIU uPA (KIU: Kallikrein Inhibitor Unit). The plasmin activity assay was performed in 96 microtiter plates. Bacteria were washed to remove unbound PLG and added per well in a volume of 100 µl 50 mM Tris-HCl, pH 7.5 (TBS). Plasminogen activator and 30 µl of chromogenic substrate solution containing 0.54 mM D-valyl-leucyl-lysine-p-nitroanilididihydrochloride (S-2251, Fluka) were added to bacteria in order to start conversion of PLG to plasmin and to measure plasmin activity. Endpoint measurements were conducted after 1h. Absorbance was measured at 405 nm at indicated time intervals at 37°C using a Multiskan Ascent (ThermoLabsystems). Alternatively, to distinguish between bacterial surface-bound plasmin activity and activity of released plasmin into the supernatant, PLG-preincubated pneumococci were incubated for 15 min or 1h with tPA or uPA. Afterwards plasmin activity of both the sedimentated bacteria and the supernatant was measured. To measure activation of PLG bound to recombinant enolase, 50 µg of protein were coated per well overnight at 4°C. Binding of 3 µg PLG in TBS was performed at room temperature for 1 h, followed by incubation with the chromogenic plasmin substrate S-2251 and PA as described above. Activation of PLG by tPA or uPA was carried out with 0.04 KIU each. In kinetic experiments activation of PLG was measured in intervals of 1 min. In control experiments, no cofactor was present or ε-aminocaproic acid (EACA), or 500 KIU serine protease

Table 1: Plasminogen-binding activity of *S. pneumoniae* wild-type D39, isogenic mutants D39eno<sup>del</sup>, D39eno<sup>int</sup> and D39eno<sup>int/del</sup> and recombinant enolase proteins. Amino acids substituted in binding site 2 (BS2) of mutated enolase proteins are underlined. ΔKK: C-terminal lysine residues are deleted.
inhibitor aprotinin was added. To investigate the impact of the PLG-BS2 of enolase on plasmin formation, the synthetic peptide FYDKERKVYD (10 µg) or peptide FYDLGLRLVYD (10 µg) with multiple amino acid substitutions at position 4, 5, and 7, respectively, was used. Values were corrected by subtraction of background values and represent mean ± SD (n=3).

**Preparation of [35S]-radiolabeled NCI-H292 ECM**

The epithelial cell line NCI-H292 (CRL-1848, ATCC), derived from a human lung mucopidermoid carcinoma, was cultured to confluence in wells of 24 well tissue culture plates (Greiner, Germany) for 3 days in RPMI 1640 medium (PAA Laboratories, Germany) supplemented with 2 mM L-glutamine and 10% fetal calf serum (FCS) at 37°C under 5% CO2. The cell layer was washed with sterile PBS and cultured in methionine- and cysteine-free cell culture medium containing 10% FCS, 2 mM glutamine and 10% RPMI 1640 medium (c.c. pro, Neustadt, Germany) followed by addition of 21 µCi of L-[35S]-methionine and 9 µCi of L-[35S]-cysteine (Amersham) and incubation for 18 h at 37°C and 5% CO2-atmosphere. Preparation of the radiolabeled ECM of NCI-H292 was performed after detergent treatment of cell cultures as described (22). Briefly, cells were washed several times with 10 mM Tris-HCl, pH 8.0 followed by 30 min incubation with 10 mM Tris-HCl, pH 8.0 containing 0.5% sodium deoxycholate at room temperature. The cell debris was removed and the remaining ECM was incubated for 5 min with 10 mM Tris-HCl pH 8.0 supplemented with 10 U/ml DNase I. Finally, the ECM was washed three times with PBS, pH 7.4. The absence of cells and cell debris was confirmed by microscopy.

**Iodination of Matrigel**

Reconstituted basement membrane, prepared from mouse Engelbreth Holm Swarva sarcoma, (Matrigel™ Becton Dickenson), was diluted to a concentration of 1 µg/µl and 100 material were radiolabeled with 125Iodine by a standard chloramin T method (23). Wells of a 24 cell culture plate (Greiner) were coated with 2 x 10⁶ CPM/ml of [125I]-Matrigel by over night incubation at room temperature.

**Degradation of [125I]-Matrigel and [35S]-labeled ECM of NCI-H292 cells**

Pneumococci grown to mid-log phase were incubated in 0.5 ml HEPES-DMEM (low glucose; PAA Laboratories) containing 1% FCS and 20 µg PLG for 15 min at 37°C. Degradation of radiolabeled ECM and Matrigel was performed as described (24). Briefly, bacteria (untreated or pretreated with PLG) were washed two times with HEPES-DMEM and 6 x 10⁶ pneumococci in a volume of 600 µl were added to the wells containing the prepared radiolabeled ECM or Matrigel. Viability of pneumococci was confirmed during the experiment. Alternatively, 2 x 10⁷ of enolase-coated microspheres were preincubated with 20 µg PLG, washed twice with PBS and 1 x 10⁸ beads each were employed for the degradation assay. Degradation was performed in the absence or presence of 0.24 KIU tPA or 0.06 KIU uPA at 37°C. In control experiments 500 KIU aprotinin or 2 IU proteinase K were added. Further controls included wells without PA or 2 µg PLG together with 0.24 KIU tPA but without bacteria. Degradation was quantified measuring the released radioactivity, whereby subsamples of 25 µl were taken from the supernatant at different time intervals for up to 8 h and transferred into 2 ml of scintillation liquid (Ultima Gold). Radioactivity was measured in a liquid scintillation counter (Packard, 1600 TR). Spontaneous release of degradation products in assays with incubation buffer only was not detected (data not shown). The total radioactivity of the radiolabeled ECM or Matrigel was defined as the 100% value. The degradation assay was performed at least three times in triplicate wells.

**Degradation of laminin and fibrinogen**

Pneumococci were preincubated with PLG as described, and 1 x 10⁶ cells were incubated for 3 h (or in kinetic experiments up to 6 h) at 37°C with 4 µg human fibrinogen or 10 µg laminin-1 prepared from Engelbreth Holm Swarva sarcoma (from EHS, Roche, Penzberg, Germany) in a final volume of 125 µl in PBS supplemented with 10 mM EDTA. Degradation was followed in the presence of 0.06 KIU uPA or 0.24 KIU tPA, and the reaction was stopped with 50 µl of SDS-containing sample buffer. Bacteria were sedimented, the supernatant was boiled for 3 min and 15 µl samples were separated by SDS-PAGE followed by transfer of proteins to polyvinylidene difluoride membrane (PVDF; Immobilon-P, Millipore) using a semi-dry blotting system. The membranes were blocked by incubation in 10% fat-free milk in 10 mM PBS prior to incubation with goat antiserum to human fibrinogen (1:1250, ICN, Ohio, USA) or rabbit antimouse laminin polyclonal antibody AB2034 (1:1250, Chemicon, Temecula, Canada). Detection of fibrinogen or laminin peptides was carried using a HRP-conjugated anti-goat antibody (1:1250) or HRP-conjugated anti-rabbit antibody, followed by incubation with the substrate solution containing 1 mg/ml 4-chloro-1-naphthol and 0.1% H2O2 in PBS or by enhanced chemiluminescence detection (ECL; Santa Cruz).

**Degradation of fibrin and transmigration of pneumococci through fibrin**

Fibrin matrix was produced in transwell cell culture inserts (polycarbonate membranes with 3 µm pore size; Costar) by incubating 1 mg PLG depleted human fibrinogen (Calbiochem) with 25 U thrombin (from bovine plasma, MP Biomedicals, Ohio, USA) for 10 h at 37°C in 100 µl PBS. S. pneumoniae D39 and corresponding enolase mutants D39enolInt, D39enolDel and D39enoIntDel, pretreated with PLG as described, were applied with a dose of 1 x 10⁷ in 100 µl PBS-EDTA to the fibrin matrix. Bound PLG was activated with uPA (0.06 KIU). Aprotinin (500 KIU) was used as a serine protease inhibitor in control experiments. Bacterial transmigration from the upper to the lower chamber was quantified by plating serial dilutions of the lower chamber solution on blood agar plates. Experiments were carried out for up to 7 h and samples were plated in intervals of 30 min. After each time point the transwell inserts were replaced into a new well containing PBS-EDTA-buffer. Data represent the means ± SD of three independent experiments.

**Statistical analysis**

The differences in degradation and transmigration were analyzed by the two-tailed unpaired Student t-test. A value of P < 0.05 was considered statistically significant.
Results

Impact of plasminogen-binding site 2 of enolase on pneumococcal cell surface plasmin activity

Plasminogen (PLG) bound to the cell-surface of *S. pneumoniae* D39 was time- and cell-number-dependently activated by uPA and tPA (Fig. 1A and data not shown) as measured by a plasmin specific chromogenic substrate S-2251. Plasmin remained bound to the bacterial cell surface because no activity was measured in the supernatant of the samples (data not shown). No activity was measured in the absence of PAs indicating that pneumococci did not produce plasminogen activators. Both isogenic mutants D39*eno*mut/del and D39*eno*int, which exhibit reduced PLG-binding activity due to mutations in surface-bound enolase as depicted in Table 1 (13), had a substantially reduced plasmin conversion capacity as compared to D39 wild-type or mutant D39*eno*del (Fig. 1A). Activation of pneumococcal-bound plasmin by uPA and tPA (Fig. 1A) or uPA or tPA on recombinant enolase protein pretreated with PLG. P: synthetic peptide FYDKERVYD representing the PLG-BS2 of enolase; mP: synthetic peptide FYDGLGRLYVYD; Apr: aprotinin; EACA: ε-aminocaproic acid.

Table 2: Degradation of NCI-H292 ECM and Matrigel by *S. pneumoniae* D39 and isogenic mutants. Release of radioactivity in the supernatant was measured and the percentage of degradation of total radioactivity used in the assay was calculated. Values are shown after incubation periods of 3.5 h for ECM and 4 h for Matrigel. P values of < 0.05 were regarded as significant.

<table>
<thead>
<tr>
<th>Strain</th>
<th>NCI-H292-ECM</th>
<th>Matrigel</th>
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<tr>
<td></td>
<td>% degradation</td>
<td>% degradation</td>
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<tr>
<td>D39</td>
<td>1.52 ± 0.78</td>
<td>3.83 ± 1.56</td>
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<tr>
<td>D39+PLG+PA</td>
<td>7.11 ± 3.01</td>
<td>7.88 ± 0.58</td>
</tr>
<tr>
<td>D39*PLG+PA+aprotinin</td>
<td>0.58 ± 0.3</td>
<td>4.17 ± 0.83</td>
</tr>
<tr>
<td>D39<em>eno</em>del*</td>
<td>1.02 ± 1.7</td>
<td>4.38 ± 1.00</td>
</tr>
<tr>
<td>D39<em>eno</em>mut/del+PLG+PA</td>
<td>3.77 ± 1.47</td>
<td>5.13 ± 1.19</td>
</tr>
<tr>
<td>D39<em>eno</em>int+PLG+aprotinin</td>
<td>0.08 ± 0.11</td>
<td>3.36 ± 2.17</td>
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*As compared to D39, †as compared to D39+PLG+PA, ‡as compared to D39*eno*mut/del+PLG+PA

Plasminogen acquisition by *S. pneumoniae* enolase PLG-BS2 mediates efficient degradation of reconstituted basement membrane and NCI-H292 ECM

To determine the impact of enolase for plasmin-dependent invasion of various pneumococcal strains, degradation of radiolabeled reconstituted basement membrane (Matrigel™) or ECM derived from human lung epithelial cell line NCI-H292 was studied. In the presence of PLG and PAs, D39 wild-type strain-dependent degradation of ECM or Matrigel was substantially higher than degradation caused by the D39*eno*mut/del mutant (Fig. 2, Table 2). Degradation of ECM or Matrigel by wild-type pneumococci or the D39*eno*mut/del mutant employed in the absence of plasminogen was substantially reduced as compared to PLG-pretreated D39 wild-type. Furthermore, relative differences in the degradation of ECM or Matrigel by PLG pretreated D39*eno*mut/del compared to untreated wildtype D39 or D39*eno*int/del was seen (Fig. 2, Table 2). As expected, the presence of aprotinin in the degradation assays inhibited plasmin and reduced degradation of matrix material to basal levels (Table 2). These data indicate that the BS2 of enolase represents a key cofactor in
the recruitment of surface plasmin activity by pneumococci leading to substantial proteolysis of host basement membrane material.

Degradation of Matrigel and NCI-H292 ECM by plasmin(ogen) bound to enolase

To determine the role of pneumococcal enolase and the impact of the plasminogen-binding sites BS1 (43KK46) and BS2 (248FYDKERKVY256) of enolase in host matrix proteolysis, degradation assays were performed with recombinant enolase proteins Eno (wild-type) and Eno\textsuperscript{int/del} immobilized on inert beads. Enolase wild-type protein Eno and mutated Eno\textsuperscript{int/del} protein were immobilized on inert beads and pretreated with PLG. Radiolabeled S-ECM (A) and D39-Matrigel (B) were incubated with PLG-pretreated pneumococci in the absence or presence of tPA (ECM) or uPA (Matrigel). Degradation was determined by counting released radioactivity (CPM) into the medium in time intervals of 0.5 h for up to 4 h. Degradation of Matrigel in the absence of PLG, activator and bacteria increased to 2.6±0.8% after 4 h. Results are expressed as percentage of total radioactivity (defined as 100%) employed per well of a 96-well cell culture plate. The data represent the mean ± SD of three independent experiments.

Degradation of plasmin specific substrates laminin and fibrinogen by S. pneumoniae D39 and isogenic mutant D39\textsuperscript{eno\textsuperscript{int/del}}

Immunoblot analysis of degradation products of mouse sarcoma laminin (A; lane Lm) and human fibrinogen (B; lane Fg) after incubation with PLG pretreated wild-type S. pneumoniae D39 or isogenic mutant D39\textsuperscript{eno\textsuperscript{int/del}} in the presence of a plasminogen activator. As a control soluble plasmin was used (lane 2 in A). Degradation of laminin by plasmin resulted in two peptides of approximately 121 kDa and 81 kDa. For laminin, degradation by PLG pretreated pneumococci is shown after 4 h. For fibrinogen (Fg), degradation products are shown for different time points. In the absence of PLG the wild-type neither did degrade laminin (not shown) nor fibrinogen. The mutants D39\textsuperscript{eno\textsuperscript{int/del}} did not degrade laminin and fibrinogen indicating the key role of pneumococcal α-enolase and PLG-BS2 of enolase in plasmin formation on the outer-cell surface of pneumococci.

Plasminogen binding to BS2 of pneumococcal enolase promotes degradation of laminin and human fibrinogen

Laminin together with collagen type IV and elastin are major components of Matrigel and the ECM of NCI-H292 cells as indicated by immunoblot analysis (data not shown). Since laminin is a proteolytic target for plasmin (25), the soluble enzyme as well as wild-type D39 or the D39\textsuperscript{eno\textsuperscript{int/del}} mutant (data not shown) in the presence of PLG and uPA or tPA promoted laminin degradation with fragments of approximately 121 kDa and 81 kDa, respectively (Fig. 4).

In contrast, mutants D39\textsuperscript{eno\textsuperscript{int}} (data not shown) and D39\textsuperscript{eno\textsuperscript{int/del}} did not degrade laminin in the presence of PLG and PAs (Fig. 4A), indicative for the critical role of the PLG-BS2 of enolase. Since plasmin is a key enzyme in fibrinolysis (4), we also monitored degradation of fibrinogen (ogen) by PLG-coated wild-type pneumococci and isogenic enolase mutants. While wild-type D39 promoted a time-dependent degradation of human fibrinogen (Fig. 4B), D39\textsuperscript{eno\textsuperscript{int/del}} was ineffective (Fig. 4B). These
results underline the role of enolase and its PLG-BS2 in plasmin-mediated degradation of individual ECM components.

**Role of pneumococcal enolase and PLG-binding sites in fibrin dissolution and transmigration of pneumococci**

A fibrin matrix was generated on membranes of transwell cell culture inserts to investigate pneumococcal enolase-dependent plasmin-mediated fibrinolysis and bacterial transmigration.

In the presence of PLG and PAs the number of transmigrated wild-type D39 and isogenic mutant D39enod del bacteria increased up to 1.5 h and remained constant for up to 5 h (Fig. 5A). In contrast, transmigration of D39enom int and D39enod del was significantly decreased and the number of transmigrated pneumococcal mutants was in the range of PLG-free wild-type D39 (Fig. 5A). Likewise, partial dissolution of the turbid fibrin matrix by D39 and D39enod del was visible after 3.5 h and the fibrin matrix was completely dissolved after 6 h (Fig. 5B), whereas no visible dissolution of fibrin was seen by PLG-free wild-type D39 and PLG-treated mutants D39enom int (data not shown) or D39enod del (Fig. 5B). Electron microscopic studies illustrated the dissolution of the thick fibrin bundles in the presence of PLG and PA, but only a minor dissolution of the thinner fibrils in the absence of a PA (Fig. 6). Aprotinin inhibited plasmin-mediated degradation of fibrin (Fig. 6B) and transmigration of pneumococci decorated with plasmin activity (data not shown). In conclusion, these results indicate a key role of surface bound pneumococcal enolase for pneumococcal transmigration and highlight the function of the enolase BS2 for the recruitment of surface-bound plasmin activity to promote dissolution of the fibrin matrix.

**Discussion**

For infectious process *S. pneumoniae* recruits proteolytic activity to the bacterial cell-outer surface by binding host plasmin(ogen). The glycolytic enzyme α-enolase was previously identified as one of the pneumococcal PLG-binding proteins (12). In this study we specified the mechanisms by which pneumococci-bound enolase served as key cofactor for the efficient degradation of radiolabeled ECM and Matrigel in the presence of host-derived PA and PLG. Plasmin specific substrates laminin and fibrinogen, but not collagen (data not shown), were degraded by PLG-coated pneumococci. On the molecular level we demonstrated the pivotal role of PLG-BS2 FYDKERKVY of
Pneumococcal enolase for degradation of ECM and Matrigel or for fibrinolysis and transmigration through host ECM. Employing mutated pneumococci which lack the PLG-binding activity of BS2 or mutated enolase protein resulted in substantially reduced degradation and dissolution of fibrin, thereby reducing pneumococci transmigration to basal levels.

Plasmin is a key enzyme of intravascular fibrinolysis, but is also known to have a broad spectrum of extravascular functions related to cell-invasive processes where the degradation of the ECM is pivotal to allow cell migration to occur (26). Moreover, degradation of the complex ECM structure is an important prerequisite for vessel formation and sprouting during processes of angiogenesis (9, 27). In analogy, the recruitment of PLG to bacterial cell surfaces endows microorganisms with proteolytic activity and may enhance bacterial virulence by facilitating invasion and dissemination within the infected host (28, 29). Several grampositive and gram-negative invasive bacterial pathogens express PLG receptors (29) including α-enolase of group A streptococci (30). As a common denominator, plasmin recruited by pathogens thereby promotes degradation of soluble and insoluble components of the ECM as shown for Borellia burgdorferi and Salmonella enterica serovar Typhimurium (31, 32) including laminin as the major non-collagenous glycoprotein of basement membranes (33) and major constituent of Matrigel and NCi-H292 ECM used in this study. The impact of pneumococcal bound proteolytic activity to invasion was indicated by degradation of radiolabeled Matrigel or ECM as well as soluble laminin or fibrinogen, and the here characterized enolase-dependent mechanism provides new insights into bacterial invasion strategies.

Previous analysis of the pneumococcal α-enolase revealed an octameric structure (consisting of four dimers), with binding sites BS1 and BS2 located on the surface of each pneumococcal α-enolase dimer (19). Within the octamer, BS1 is located in an inter-dimer groove, whereby the BS2 of each monomer is exposed to the octamer surface. Structural and molecular analysis revealed that BS2 is the primary PLG binding site (13, 19). In the present study, the pivotal role of the α-enolase and its BS2 was demonstrated by employing the PLG-treated mutant D39eno<sup>int/del</sup> or mutated enolase<sup>int/del</sup> protein in degradation and transmigration assays. Although the D39eno<sup>int/del</sup> mutant has a residual PLG-binding activity, degradation of ECM or soluble ECM components and transmigration were reduced to basal level as measured for the PLG-untreated wild-type D39.

Pneumococci express several other proteases such as the IgA-protease (34), the surface-associated subtilisin-like serine protease PtaA (35), the heat shock-induced serine protease HtrA (36), and putative zinc metalloproteinas ZmpB and ZmpC (37, 38). The gene encoding ZmpC, which cleaves MMP-9, is not present in D39 and its derivatives (38). It is therefore likely that the basal level of degradation measured in the absence of PLG but in the presence of bacteria is probably caused by surface-associated or secreted proteases such as PtaA. All plasmin-mediated degradations were inhibited in the presence of the low-molecular-weight plasmin inhibitor aprotinin.

The mechanisms by which different bacteria can generate surface bound plasmin differs in many ways, related to the presence of bacterial PAs and cofactors: Immobilized PLG is activated by host PAs which hydrolyze the peptide bond between Arg561 and Val562 thereby creating active plasmin (3). Moreover, the catalytic domain of plasmin can recruit adapter molecules or cofactors, such as the bacterial PAs staphylokinase and streptokinase, which in turn convert other plasminogen molecules to active plasmin (39, 40). The PLG activator Pla of Yersinia pestis harbours proteolytic activity and cleaves PLG at the same Arg-Val bond like tPA and uPA (41). Pneumococci did not activate PLG in the absence of a host PA suggesting that they lack a functional PA molecule. Plasmin activity of mutant D39eno<sup>del</sup>, expressing enolase with a deleted BS1, was comparable with plasmin activity of the wild-type strain. In contrast, pneumococcal mutants D39eno<sup>int</sup> and D39eno<sup>int/del</sup>, both expressing enolase with a mutated BS2, exhibit a substantially reduced plasmin activity. Plasmin formation was also inhibited by the synthetic peptide FYDKERKIVD and EACA, indicating the key role of PLG-BS2 of enolase and its lysine residues.

Immobilization of PLG onto surfaces is associated with conformational changes in the molecule (42), thereby enhancing the susceptibility of the proenzyme to activation and leading to significant protection against physiological inhibitors like α2-antiplasmin and α2-macroglobulin. This principle is also fulfilled in intravascular fibrinolysis, where only tPA binds to fibrin serves as an efficient plasminogen activator, as well as by concentrating uPA on cell surfaces via the uPA receptor (uPAR). Here, binding of uPA to uPAR is responsible for pericellular and ECM proteolysis (43) as well as promotes cellular invasion. For pathogens such as Staphylococcus aureus, group A, C, and G streptococci, B. burgdorferi and S. enterica serovar Typhimurium it was shown that formation of bacterium-bound plasmin activity was not inhibited by α2-antiplasmin. Pla of Y. pestis inactivates α2-antiplasmin by proteolytic cleavage (32, 44–46). Pneumococcal-bound plasmin was inhibited by aprotinin but the serine protease activity was partially resistant to inhibition by α2-antiplasmin (data not shown). This observation indicates that enolase-bound plasminogen behaves similar like the fibrin-bound enzyme, such that under (patho-)physiological conditions, invasion of pneumococci will not be interfered by natural inhibitors of the host. The use of PAI-1 gene deficient mice indicated that PAI-1 did not influence the clearance of pneumococci from the lungs (47).

Several indicators are available to associate bacteria-mediated plasmin activity with a pathological phenotype: Elevation of cross-linked fibrin degradation products was found in patients with Gram-negative bacteremia (48), and the enhancement of tPA-catalyzed PLG activation by Escherichia coli S fimbiae has been associated with neonatal septicemia and meningitis (49). Group A streptococci engage the host fibrinolytic system to facilitate bacterial access to the vasculature. One of the key bacterial factors for this invasive strategy is the streptokinase, which activates species-specifically human PLG (50). In a mouse model of group A streptococcal skin infection using transgenic mice expressing human PLG, susceptibility has been shown to be dependent on streptokinase expression (51). In addition, mice infections with strains expressing low levels of streptokinase but expressing PLG-binding proteins PAM and enolase indicated additional mechanisms of S. pyogenes, which can be used to exploit the host fibrinolytic system contributing to invasion (51).
Since components of the plasminogen activation system are expressed by airway epithelium and involved in lung tissue remodelling during infectious diseases, their participation in the enolase-dependent mechanism of \textit{S. pneumoniae} is expected. Although an intervening strategy that would decrease the host or bacteria-dependent plasmin activity may sound beneficial, it may also increase fibrosis formation. Thus, an enolase-related therapeutic approach could circumvent such unwanted side effects and may prove to be feasible in ongoing studies.

**Abbreviations**

BS, binding site; CPM, counts per minute; EACA, ε-aminocaproic acid; ECM, extracellular matrix; PA, plasminogen activator; PLG, plasminogen; PBS, phosphate-buffered saline; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

**References**