Thrombin-activatable fibrinolysis inhibitor and bacterial infections

Valls Serón, M.

Citation for published version (APA):

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 3

*Thrombin-activatable fibrinolysis inhibitor is degraded by Salmonella enterica and Yersinia pestis*

Mercedes Valls Serón, Johanna Haiko, Philip G. de Groot, Timo K. Korhonen, and Joost C.M. Meijers

Journal of Thrombosis and Haemostasis, 2010; 8: 2232-40
Abstract
Pathogenic bacteria modulate the host coagulation system to evade immune responses or to facilitate dissemination through extravascular tissues. In particular, the important bacterial pathogens *Salmonella enterica* and *Yersinia pestis* intervene with the plasminogen/fibrinolytic system. Thrombin-Activatable Fibrinolysis Inhibitor (TAFI) has anti-fibrinolytic properties as the active enzyme (TAFIa) removes C-terminal lysine residues from fibrin, thereby attenuating accelerated plasmin formation. Here, we demonstrate inactivation and cleavage of TAFI by homologous surface proteases, the omptins Pla of *Y. pestis* and PgtE of *S. enterica*. We show that omptin-expressing bacteria decrease TAFI activatability by thrombin-thrombomodulin and that the anti-fibrinolytic potential of TAFIa was reduced by recombinant *Escherichia coli* expressing Pla or PgtE. The functional impairment resulted from C-terminal cleavage of TAFI by the omptins. Our results indicate that TAFI is degraded by the omptins PgtE of *S. enterica* and Pla of *Y. pestis*. This may contribute to the ability of PgtE and Pla to damage tissue barriers, such as fibrin, and thereby to enhance spread of *S. enterica* and *Y. pestis* during infection.
Introduction

Several bacterial infectious diseases are associated with an imbalance of the mammalian fibrinolytic/coagulation systems, however, the mechanistic details and pathogenetic roles of these processes have remained largely unexplored. Fibrinogen-binding proteins have been mainly identified in Gram-positive bacterial pathogens, such as *Staphylococcus aureus*, *Staphylococcus epidermis*, and group A, C, and G Streptococci [1]. They interact with fibrinogen and are thought to protect invading bacteria against host immune response and to enhance survival of bacteria inside the host [2]. On the other hand, enhanced fibrinolytic activity has been associated with several bacterial infectious diseases [3-6].

Plasmin is a key player in fibrinolysis, and bacterial pathogens have been observed to enhance fibrinolysis either by directly activating plasminogen, by inactivating the main plasmin inhibitor α₂-antiplasmin, or by immobilizing plasminogen/plasmin on the bacterial surface [6]. The relationships between bacteria and the coagulation/fibrinolysis pathways appear complex, as recent reports have identified that some serious bacterial pathogens, such as *S. aureus*, *Yersinia pestis* and *Salmonella enterica* exhibit in vitro capacities which promote either procoagulant or anticoagulant activities that lead to enhanced or decreased formation of fibrin clots [5,7-10].

*Y. pestis* is the causative agent of plague, a highly fatal zoonotic infection transmitted to humans through an intradermal bite by an infected flea in bubonic plague, or via respiratory tract in aerosols from individuals with pneumonic plague. The importance of plasminogen activation in pathogenesis of plague is well established. The plague bacterium harnesses the plasminogen system for migration from intradermal infection sites at the early stages of bubonic plague [5]. Altered fibrinolysis and importance of plasminogen activation have been detected in both bubonic and pneumonic plague [10-12] and plasminogen-deficient mice are more resistant than normal mice to infections by *Y. pestis* [5].

Plasminogen activation by *Y. pestis* is mediated by the transmembrane β-barrel surface protease Pla, which also inactivates α₂-antiplasmin, thus creating uncontrolled plasmin activity at the infection site [12,13]. Deletion or inactivation of the *pla* gene attenuates *Y. pestis* both in bubonic [11,12] and pneumonic plague [10], and the *pla* gene is highly transcribed by *Y. pestis* from buboes in infected rats [14].

Pla belongs to the omptin family of bacterial outer membrane proteases whose members occur in several Gram-negative bacterial pathogens [15-17]. PgtE is the Pla ortholog in *S. enterica* serovar Typhimurium, an intracellular pathogen that causes gastroenteritis. PgtE is highly expressed by *S. enterica* inside infected mouse macrophages [4] and its genetic deletion attenuates *S. enterica* in infected mice [18]. Fibrinolysis has not been earlier associated with salmonellosis, and PgtE is a poor plasminogen activator [8] but it efficiently inactivates α₂-antiplasmin and activates procollagenases and progelatinases [4,18,19] which can increase proteolysis and motility of macrophages and bacteria during salmonellosis [17].
Thombin-activatable fibrinolysis inhibitor (TAFI) is a regulatory, anti-fibrinolytic protein linking the coagulation and fibrinolytic systems. The protein is synthesized in the liver and secreted into plasma as a 56-kDa procarboxypeptidase with 2 domains: the first 92 amino acids form the activation peptide; the next 309 amino acids form the catalytic domain. During coagulation, the proenzyme is activated to TAFIa by thrombin. TAFIa has anti-fibrinolytic properties as it inhibits plasmin-mediated blood clot lysis by removing C-terminal lysine residues from partially degraded fibrin that are required for positive feedback in tissue-type plasminogen activator dependent plasmin generation. Apart from thrombin, the serine proteases plasmin, trypsin and neutrophil elastase have been reported to function as TAFI activators [20-22]. TAFI activation by thrombin is inefficient but the process can be stimulated approximately 1250-fold by the cofactor thrombomodulin [23]. TAFIa is unstable at 37 °C and upon activation by thrombin it is inactivated (TAFIai) by a conformational change in a temperature-dependent manner [24]. In contrast, in the presence of plasmin, TAFIa is inactivated by plasmin proteolysis [20].

No direct physiological inhibitors of TAFIa have been identified to date. The cleavage of TAFI by human proteinases has been well described biochemically and functionally [20,25], whereas its possible activation or inactivation by bacteria has not been described. As TAFIa regulates plasmin formation, invasive microorganisms that use the plasminogen system to spread may target TAFI to enhance fibrinolysis. Therefore the aim of this study was to investigate the effects of Y. pestis and S. enterica proteases Pla and PgtE on TAFI in order to provide novel insights in the complex relationships between bacteria, coagulation and fibrinolysis.

Materials and methods

Reagents

Hippuryl-arginine and H-D-Phe-Pro-Arg-chloromethylketone (PPACK) were purchased from Bachem (Bubendorf, Switzerland), and ε-amino-n-caproic acid was purchased from Sigma-Aldrich (St Louis, MO, USA). Rabbit lung thrombomodulin was purchased from American Diagnostica (Stamford, CT, USA). Phosphoenolpyruvate (PEP), ATP, NADH, pyruvate kinase/dehydrogenase (PK/LDH), and recombinant tissue-type Plasminogen Activator (t-PA, Actilyse) were purchased from Biopool AB (Umeå, Sweden). Thrombin was a generous gift from Dr. W. Kisiel (University of New Mexico, Albuquerque, NM, USA). TAFI was purified as previously described [25], except that proteins were eluted from the Nik-9H10-Sepharose column with 0.1 M glycine (pH 4.0) and that the fractions were collected in 20 mM Tris, 200 mM NaCl (pH 7.4). TAFI was stored at -80 °C after addition of Tween-20 (0.01%). Fibrinogen was purchased from Kordia (Leiden, The Netherlands). A polyclonal antibody specific for TAFI was obtained in rabbits as described elsewhere [27]. Plasminogen was purified from frozen human plasma as previously described [28]. Alkaline-phosphatase-conjugated anti-rabbit immunoglobulin G (IgG) was purchased from Dako (Glostrup, Denmark).
Bacteria and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *pla, pgtE* and their derivatives have been cloned in the inducible pSE380 vector in *Escherichia coli* XL1-Blue MRF’ and described before [13,29]. The recombinant *E. coli* strains were cultivated overnight at 37 °C in Luria broth (10 ml) supplemented with glucose (0.2% wt/vol), ampicillin (100 µg/ml) and tetracycline (12.5 µg/ml). For protein expression, the culture was pelleted, suspended in 150 µl of phosphate-buffered saline (PBS, pH 7.1) and plated on Luria plates containing 5 µM isopropyl-β-D-thiogalactopyranoside (IPTG) and antibiotics as above. *Y. pestis* strains were cultivated over two nights at 37 °C on brain-heart infusion (BHI) plates and then inoculated in 10 ml BHI broth supplemented with hemin (40 µg/ml) and cultivated twice over two nights at 37 °C. *S. enterica* 14028R and 14028R-1 were cultivated overnight at 37 °C in 10 ml PhoP/Q-inducing medium (N-minimal medium, pH 7.4, supplemented with 38 mM glycerol, 0.1% casamino acids, 2 mg/ml thiamine, and 8 mM MgCl₂). *S. enterica* 14028R-1 complemented with pSE380 or pMRK3 were cultivated overnight at 37 °C in 10 ml Luria broth supplemented with 5 µM IPTG and ampicillin (100 µg/ml). For the assays, bacteria were collected in PBS or in Tris-buffered saline (TBS, pH 7.4), pelleted, and adjusted to OD₆₀₀ value of 2.0 (corresponding to c. 2 × 10⁹ cells/ml).

TAFIa activation and activity assay

TAFI (54 nM for *Y. pestis* and *S. enterica*; for *E. coli*, 179 nM or 714 nM in dose- and time-dependent assays; all concentrations are final concentrations) was incubated with the bacteria (2 × 10⁷ or 4 × 10⁷ in TBS) in 100 mM Hepes/0.01% Tween-20, pH 8.0. In some experiments, ε-ACA (2.5 mM) was added. Incubations were performed at 37 °C shaking. After incubation, the samples were centrifuged, and the supernatants were further analyzed. TAFIa activity was measured as previously described [26,30]. TAFI (40 nM) was added to a premix of thrombin (8 nM) and thrombomodulin (16 nM) in the presence of CaCl₂ (5 mM) in 100 mM Hepes/0.01% Tween-20, pH 8.0 for 15 min at room temperature. Subsequently, a reaction mixture containing MgSO₄ (2.7 mM), KCl (10.9 mM), PPACK (30 µM), PEP (2.4 mM), NADH (0.5 mM), ATP (2.7 mM), hippuryl-arginine (6 mM), PK (45 µg/ml), LDH (15 µg/ml), and excess of arginine kinase was added in 100 mM Hepes/0.01% Tween-20, pH 8.0. When plasma was used, 20 µl TAFI-deficient plasma (three times diluted) was reconstituted with TAFI to a final concentration of 165 nM and incubated with 20 µl bacteria (4 × 10⁹). Incubations and TAFIa activity measurement were performed as described above. Prewarmed reaction mixture (90 µl) was transferred to a prewarmed microtiter plate, and 10 µl of TAFI activated as described above was added. TAFI activation was followed over time as a loss of NADH absorbance at 340 nm in a Thermomax microplate reader (MolecularDevices Corp., Menlo Park, CA, USA) or in a Multiskan EX reader (Thermo, Waltham, MA, USA).
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> XL1 Blue MRF’</td>
<td>Δ(mcrA) 183 Δ(mcrCB-hsdSMR-mrr) endA1 supe44 thi-1 recA1 gyrA96 relA1 lac [F’ proAB lacO52 Δ M15 Tn 10 (tet)]</td>
<td>Stratagene</td>
</tr>
<tr>
<td><em>Yersinia pestis</em> KIM D27</td>
<td>pPCP1* Δpgm pYV* derivative of <em>Y. pestis</em> KIM-10</td>
<td>[52,53]</td>
</tr>
<tr>
<td><em>Yersinia pestis</em> KIM D34</td>
<td>pPCP1*, otherwise identical to KIM D27</td>
<td>[52,53]</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> serovar Typhimurium 14028R</td>
<td>Rough lipopolysaccharide derivative of 14028</td>
<td>[54]</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> serovar Typhimurium 14028R-1</td>
<td>ΔpgtE derivative of 14028R</td>
<td>[4]</td>
</tr>
<tr>
<td>pMRK1</td>
<td>pla in pSE380</td>
<td>[13]</td>
</tr>
<tr>
<td>pMRK3</td>
<td>pgtE in pSE380</td>
<td>[29]</td>
</tr>
<tr>
<td>pMRK31</td>
<td>PgtE D206A</td>
<td>[29]</td>
</tr>
</tbody>
</table>

**Clot-lysis assay**

TAFI (179 nM or 714 nM in dose- and time-dependent assays) was incubated with the bacteria (2 × 10⁷ or 4 × 10⁷ in TBS) in 100 mM Hepes/0.01% Tween-20, pH 8.0, at 37 °C with continuous shaking. After incubation, the samples were centrifuged and the supernatants were further analysed. The clot-lysis times were determined in a purified system. Briefly, 10 nM thrombin, 0.3 μg/ml recombinant t-PA (Actilyse), 20 mM CaCl₂ and 5 nM thrombomodulin were mixed with 40 nM preincubated TAFI (all concentrations are final concentrations in the assay) in a 96-well microtiter plate. The volumes were adjusted to 30 μl with HBS (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, pH 7.4) containing 0.1% (wt/vol) bovine serum albumin. A mixture (20 μl) of fibrinogen (4.5 μM), plasminogen (90 nM) in HBS/0.1% bovine serum albumin was added and turbidity was measured in time at 37 °C at 405 nm in a Thermomax microplate reader. The clot-lysis time was defined as the time difference between half-maximal lysis and half-maximal clotting.

**Degradation of TAFI**

For detecting the degradation of TAFI with anti-TAFI antibody, TAFI (820 nM) was incubated with the bacteria (4 × 10⁷ in PBS) in a volume of 12.5 μl for 2 h at 37 °C shaking. The bacteria were pelleted, and the supernatant was boiled with sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing β-mercaptoethanol. The samples were run in a 12% (wt/vol) SDS-PAGE gel, transferred onto a nitrocellulose membrane and detected
with polyclonal anti-TAFI antibody (1:2000), followed by alkaline-phosphatase-conjugated anti-rabbit IgG (1:1000), and phosphatase substrate. To detect the degradation of TAFI by SDS-PAGE, TAFI (1.9 µM) was incubated with bacteria (4×10^7 in TBS) in 100 mM Hepes/0.01% Tween-20, pH 8.0. In some experiments, ε-ACA (5 mM) was added. The samples were prepared as above, subjected to SDS-PAGE (10%) and stained with Coomassie Brilliant Blue.

**Determination of TAFI cleavage site**

Degraded TAFI samples with *E. coli* expressing Pla or PgtE were prepared as for degradation assay by Western blotting with the exception that Pla-expressing bacteria were incubated for 5 h. For N-terminal sequencing, the samples were run in a 12 % SDS-PAGE, blotted and sequenced by Edman degradation. For MALDI-TOF-MS, the samples were run on a 12 % SDS-PAGE, cut from the gel, digested with trypsin, and exposed to matrix-assisted laser desorption ionization - time of flight mass spectrometry (MALDI-TOF-MS). The obtained peptides were analyzed with Mascot search engine (http://www.matrixscience.com).

**Results**

**Decreased activatability of TAFI by bacteria expressing PgtE or Pla.**

To determine the effect of omptins on TAFIa activity, TAFI was pre-incubated with *S. enterica* and *Y. pestis* strains differing in possession of *pgtE* or *pla* genes, and subsequently activated with the thrombin-thrombomodulin complex. The PgtE-positive *S. enterica* 14028R and the Pla-positive *Y. pestis* KIM D27 decreased the activatability of TAFI to TAFIa (by 85% and 97%, respectively) (Fig. 1A). In contrast, the *pgtE* deletion mutant *S. enterica* 14028R-1 had no detectable effect on TAFIa activity, and *Y. pestis* KIM D34, cured of the pla-encoding plasmid pPCP1, reduced TAFIa activity by 59% (Fig. 1A). Inhibition of TAFIa activity was seen with *S. enterica* 14028R-1 (pMRK3) complemented with the *pgtE*-encoding plasmid but not after complementation with the expression vector, *S. enterica* 14028R-1 (pSE380), further indicating that reduction of TAFIa activity by 14028R resulted from the expression of PgtE.

To further confirm the role of PgtE and Pla on TAFI activity, we studied the activatability of TAFI with recombinant *E. coli* XL1 expressing PgtE or Pla. *E. coli* XL1 (pMRK3) with PgtE and *E. coli* XL1 (pMRK1) with Pla reduced TAFI activatability (by 80% and 61%, respectively). *E. coli* expressing PgtE or Pla with the catalytic site substitution D206A (pMRK31 or pMRK111, respectively) decreased TAFI activatability by approximately 32% and 31%, respectively (Fig. 1B).

The recombinant *E. coli* expressing Pla or PgtE attenuated TAFIa activity in a dose-dependent manner (Fig. 1C). Moreover, when the bacteria were pre-incubated with TAFI for different time periods (0.5-2 h), we observed a progressive reduction in TAFIa activity (Fig. 1D).
Figure 1. Effect of PgtE- or Pla-expressing bacteria on TAFIa activity. (A, B) Loss of TAFIa activity with S. enterica, Y. pestis (A) and recombinant E. coli (B). Purified TAFI was incubated for 2 hours with the bacterial strains indicated below the columns. After incubation, TAFI was activated by the thrombin-thrombomodulin complex and TAFIa activity was measured. (C, D) Dose-dependent (C) and time-dependent (D) reduction in TAFIa activity. TAFI was incubated in buffer (▲) or with recombinant E. coli XL1 expressing PgtE (pMRK3) (■), Pla (pMRK1) (●), or the catalytic-site mutants PgtE D206A (pMRK31) (□) and Pla D206A (pMRK111) (○). Data are expressed as a percentage of the maximal TAFIa activity in buffer. For S. enterica and Y. pestis strains, a representative experiment is shown, and for recombinant bacteria, mean ± SD, n = 3 is shown.

Next, we investigated the effect of recombinant E. coli XL1 expressing PgtE or Pla on TAFI in plasma. TAFI was added to TAFI-depleted plasma and incubated for 2 hours at 37°C with recombinant E. coli XL1 expressing PgtE, Pla or the catalytic-site mutants PgtE D206A or Pla D206A. After incubation, TAFI was activated with the thrombin-thrombomodulin complex and TAFIa activity was measured. Our results indicated that E. coli XL1 (pMRK3) with PgtE and E. coli XL1 (pMRK1) with Pla reduced TAFI activatability by 49% and 60%, respectively. E. coli expressing PgtE or Pla with the catalytic site substitution D206A (pMRK31 or pMRK111, respectively) both decreased TAFI activatability by 31%. The omptins did not activate TAFI directly. Also, the omptins had no effect on the half-life of activated TAFI (data not shown).
The reduction in TAFIa activity by PgtE and Pla expressing bacteria could be due to an effect of omptins on the TAFI activator thrombin-thrombomodulin. However, we could exclude this possibility since the activation of another substrate of thrombin-thrombomodulin, protein C, was not affected by the omptins (data not shown).

Taken together, these results indicated that PgtE of *S. enterica* and Pla of *Y. pestis* prevented TAFI to become activated by thrombin-thrombomodulin and that the proteolytic activity of these omptins was essential for the inhibition.

**Reduction of the anti-fibrinolytic potential of TAFIa by recombinant E. coli.**

The anti-fibrinolytic function of TAFIa was studied in a clot-lysis assay using purified components and recombinant *E. coli*. TAFI-dependent prolongation of clot-lysis time was reduced when TAFI was incubated with PgtE- or Pla-expressing *E. coli* XL1, in comparison to bacteria expressing proteolytically inactive PgtE or Pla (Fig. 2A). These data indicate that proteolytically active PgtE and Pla are responsible for functional inactivation of TAFI. Incubation of TAFI with *E. coli* XL1 (pMRK3) or *E. coli* XL1 (pMRK1) showed that the TAFI-dependent prolongation of clot-lysis time was dependent on bacterial numbers (Fig. 2B) and on the incubation time of TAFI with the bacteria (Fig. 2C). Thus, PgtE- and Pla-mediated proteolysis had negative effect on TAFIa function.

**Proteolytic cleavage of TAFI by PgtE of S. enterica and by Pla of Y. pestis.**

To determine why the activatability of TAFI was diminished by PgtE- or Pla-expressing bacteria, we incubated TAFI with the bacteria and analyzed the supernatants by Western blotting with an anti-TAFI antibody (Fig. 3A) and SDS-PAGE (Fig. 3B). TAFI was degraded by PgtE- or Pla-expressing *S. enterica* and *Y. pestis* but not by bacteria lacking the omptins (Fig. 3A). *E. coli* XL1 (pMRK3) with PgtE and XL1 (pMRK1) with Pla degraded TAFI into smaller molecular weight peptides in a 2-h incubation, whereas XL1 (pMRK31) and XL1 (pMRK111) with mutated omptins were much weaker in TAFI degradation (Fig. 3B). These results suggest that the decreased activatability of TAFI after its incubation with omptin-expressing bacteria is a result of proteolytic degradation of TAFI by these bacterial proteases.
Figure 2. Effect of PgtE- or Pla-expressing bacteria on the anti-fibrinolytic potential of TAFIa. (A) Loss of TAFIa anti-fibrinolytic potential. TAFI was incubated for 2 hours with the *E. coli* XL1 strains indicated below the columns. (B, C) Dose-dependent (B) and time-dependent (C) reduction in the fibrinolytic potential of TAFIa. TAFI was incubated in the presence or absence of recombinant bacteria, after incubation, bacteria were removed and the anti-fibrinolytic potential of TAFIa was determined in a clot-lysis assay using purified components. TAFI was incubated in buffer (▲) or in the presence of XL1 (pMRK3) (●), XL1 (pMRK1) (●), XL1 (pMRK31) (○) or XL1 (p MRK111) (○). The TAFIa-dependent prolongation was defined as the difference in clot-lysis time in the presence or absence of TAFI. Data are expressed as a percentage (mean ± SD, n = 3) of TAFIa dependent prolongation of TAFIa in buffer.
S. enterica and Y. pestis proteases degrade TAFI

Figure 3. Degradation of TAFI by bacteria expressing PgtE or Pla. TAFI degradation by PgtE-expressing S. enterica and Pla-expressing Y. pestis (A) and PgtE- or Pla-expressing E. coli XL1 (B) is shown. TAFI was incubated for two hours with the bacterial strains indicated above the lanes. Samples were analyzed by Western blot with anti-TAFI antibody (A) or subjected to SDS-PAGE and Coomassie staining (B). Migration distance of the molecular weight standard (in kilodaltons) is indicated on the left. For plasmid constructs, see Table 1.

The fuzzy migration of the TAFI-degradation product suggested that the activation peptide, where all the four N-linked glycosylation sites are located, was still present [31], suggesting a C-terminal cleavage of TAFI. N-terminal sequencing and peptide mass fingerprint analysis of trypsin-digested omptin-cleaved TAFI confirmed C-terminal cleavage of TAFI (data not shown).

The lysine analogue ε-ACA is considered a TAFIa inhibitor [32], but it also stabilizes TAFIa activity and prevents the formation of the no longer activatable 44.3-kDa TAFI fragment generated after C-terminal cleavage by plasmin [20]. In order to assess if the C-terminal TAFI cleavage by omptins was prevented by ε-ACA, the lysine analogue was added during incubation of TAFI with recombinant E. coli XL1 expressing PgtE or Pla. Addition of ε-ACA prevented the proteolytic cleavage of TAFI (Fig. 4A) and led to full TAFIa activity even in the presence of proteolytically active PgtE and Pla (Fig. 4B). TAFIa activity in the presence of ε-ACA was increased by < 10% (data not shown). This indicated that TAFI cleavage by omptins is dependent on lysine residues, since ε-ACA acted as a competitive inhibitor of TAFI degradation by omptins.
Figure 4. Influence of ε-ACA on TAFI proteolysis and on TAFIa activity by PgtE-expressing (A) or Pla-expressing (B) E. coli XL1. TAFI was incubated with recombinant E. coli XL1 in the presence or absence of ε-ACA. After incubation, bacteria were removed and samples subjected to SDS-PAGE and Coomassie staining. Migration distance of the molecular weight standards (in kilodaltons) is indicated on the left. TAFI incubated with the bacteria was activated by the thrombin-thrombomodulin complex and TAFIa activity was measured (lower panel). Data are expressed as a percentage (mean ± SD, n = 2) of maximal TAFIa activity of TAFI incubated without bacteria. For plasmid constructs, see Table 1.

Discussion

TAFI is an important regulator that participates in maintaining the balance of coagulation and fibrinolysis. Activated TAFI removes lysine residues from partially degraded fibrin, thus reducing the binding of t-PA and plasminogen to the fibrin clot and subsequently diminishing plasmin generation and fibrinolysis. TAFIa is regulated by its intrinsic, temperature-dependent instability but no physiological inhibitors are known to date. Several inhibitory molecules for TAFIa have however been characterized: the carboxypeptidase inhibitor from potato tubers (CPI) [33], chelating and reducing agents [22,23], arginine and lysine analogues [34,35], synthetic inhibitors [36-41], monoclonal antibodies [42-44] and nanobodies [45] against TAFI. This is the first report in which two bacterial proteases, PgtE of S. enterica serovar Typhimurium and Pla of Y. pestis, interfere with the activation of TAFI to TAFIa. Both bacterial species are highly invasive pathogens, and PgtE and Pla have been identified as virulence factors [5,10-12,18] Here we show that PgtE and Pla degrade TAFI via proteolytic breakdown. Degraded TAFI can no longer be activated by the thrombin-thrombomodulin complex and
S. enterica and Y. pestis proteases degrade TAFI

therefore the ability of TAFIa to remove C-terminal lysine residues from partially degraded fibrin is abrogated.

The biological significance of the TAFI degradation remains to be elucidated but it is important to note that decrease in TAFIa activity by PgtE and Pla also occurred in a plasma environment where several putative omptin substrates, such as plasminogen, serpins, and complement proteins, are present [13,46,47].

Plasmin can activate TAFI and subsequently inactivate TAFIa. However, plasmin can also cleave TAFI to a form that cannot be activated anymore, and the TAFIa activity in the presence of plasmin is low. Besides the direct effect of the omptins on TAFI degradation, the plasmin generated by Pla or PgtE may indirectly cause TAFI degradation. The functional heterogeneity of omptins also concerns TAFI degradation as we have observed that OmpT, an omptin of E. coli, does not interact with TAFI (M. Valls Serón, J. Haiko, unpublished observations). As a pathogen, E. coli is less invasive than Y. pestis and S. enterica.

The observation that the S. enterica pgtE-deletion mutant was completely inactive in decreasing TAFIa activity whereas KIM D34 caused an approximately 59% inhibition, indicate that Y. pestis might have other so far unidentified protease(s) involved in the TAFI degradation. The recombinant E. coli XL1 expressing the catalytic-site substitutions D206A in PgtE or Pla were clearly weaker in reducing TAFIa activity but still a low degree of TAFI degradation was detectable with E. coli XL1 expressing the substituted omptins, which indicates that omptin proteolysis is critical for the observed decrease in TAFI activatability. Single substitutions rarely abolish the proteolytic activity completely, which may explain the activity remaining with the substituted Pla and PgtE. On the other hand, our results do not rule out the possibility that additional proteases in E. coli may target TAFI.

We observed that lysine analogue ε-ACA also inhibits the C-terminal cleavage of TAFI by PgtE and Pla, thus indicating that lysine residues are critical for the cleavage of TAFI by these omptins. However, it is also possible that ε-ACA prevents the interaction of the bacterial proteases with TAFI. OmpT preferentially cleaves its substrates after arginine or lysine [48], and Pla prefers Arg-Val bond present in its plasminogen substrate, so it is possible that the cleavage occurs at arginine or lysine residues.

Many invasive bacterial pathogens interact with the haemostatic system in order to disseminate within the host or avoid the host inflammatory immune response. In particular, the Gram positive Streptococcus pyogenes have developed a variety of strategies to circumvent the host defense such as the expression of streptokinase, a plasminogen-activating protein. The streptokinase-plasmin(ogen) complex is protected against enzymatic inactivation by the plasmin inhibitor α2-antiplasmin [49]. Recent studies have shown that TAFI binds to the bacterial surface by interacting with the streptococcal collagen-like surface proteins A and B (ScIA and ScIB) [50]. Bacteria-bound TAFI was activated by its natural activators, plasmin and thrombin, which are also recruited to the streptococcal surface. Other studies revealed that TAFI was used to redirect inflammation from a transient to a chronic state by modulation of the kallikrein/kinin system [51]. Our results thus show, for the first
time, an interaction of TAFI with Gram negative bacteria, indicating that TAFI might be a general target for bacteria.

Pla, PgtE, and OmpT have been recently shown to inhibit tissue-factor pathway inhibitor (TFPI), which inhibits coagulation [9]. This function is seemingly contrary to our present and other previously described results [4,8,12,13] showing enhanced plasminogen activation and fibrinolysis in vitro and in vivo. The in vivo significance of the procoagulation effects of omptins remain to be determined, these observations however indicate that the interactions of omptin proteases with the haemostatic system are complex and perhaps lead to opposite directions at different stages of the infectious processes. S. enterica and Y. pestis employ omptins to advance fibrinolysis by plasminogen activation and α2-antiplasmin degradation, and inhibition of TAFIa function is still another way for these pathogens to influence fibrinolysis and inflammation and promote their spread during infection.
Reference List


28 Sangrar W, Gabel BR, Boffa MB, Walker JB, Hancock MA, Marcovina SM, Horrevoets AJ, Nesheim ME, Koschinsky ML. The solution phase interaction between apolipoprotein(a) and plasminogen inhibits the binding of plasminogen to a plasmin-modified fibrinogen surface. *Biochemistry* 1997; 36: 10353-63.


S. enterica and Y. pestis proteases degrade TAFI


