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Thrombin Activatable Fibrinolysis Inhibitor binds to *Streptococcus pyogenes* by interacting with collagen-like proteins A and B¹

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Running title: Binding of TAFI to SclA and SclB from *S. pyogenes*

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Regulation of proteolysis is a critical element of the host immune system and plays an important role in the induction of pro- and anti-inflammatory reactions in response to infection. Some bacterial species take advantage of these processes and recruit host proteinases to their surface in order to counteract the host attack. Here we show that Thrombin Activatable Fibrinolysis Inhibitor (TAFI)², a zinc-dependent procarboxypeptidase, binds to the surface of group A streptococci of an M41 serotype. The interaction is mediated by the streptococcal collagen-like surface proteins A and B (SclA and SclB), and the streptococcal-associated TAFI is then processed at the bacterial surface via plasmin and thrombin/thrombomodulin. These findings suggest an important role for TAFI in the modulation of host responses by streptococci.

Streptococcus pyogenes is an important human Gram-positive pathogen that mainly causes throat and skin infections. Although these conditions are normally superficial and self-limiting, they can occasionally turn into invasive and life-threatening diseases such as sepsis and necrotizing fasciitis (1). In order to colonize the human host, *S. pyogenes* expresses so-called adhesins, which allow the bacterium to attach to the extra-cellular matrix or to cell surface

structures. So far, at least 20 adhesins have been described in *S. pyogenes*, including for instance M proteins and protein F1 (for a review see (2)). The two related streptococcal collagen-like surface proteins SclA and SclB, that were first described in 2000 and 2001, also belong to this family (3-7). Although their extra-cellular parts differ in size and primary sequence, SclA and SclB are organized into a similar “lollipop”-like structure. The stalk is made up of a collagen-like (CL) region with varying numbers of GXY repeats, whereas the globular head consists of a non-collagenous amino-terminal variable (V) region. Both proteins have a conserved signal peptide and a carboxy-terminal region that is attached to the cell wall via an LPATG anchor. The CL regions of Scls have been shown to mediate adhesion to human lung epithelial cells (5) and fibroblasts (4). It has also been reported that SclA from M type 41 activates the collagen receptor $\alpha_2\beta_1$ integrin on fibroblasts (8), and interacts with the low density lipoprotein (LDL) in human plasma (9).

Proteolysis plays an important role in host parasite interactions. While some immune defence mechanisms such as complement, coagulation, and fibrinolysis are dependent on their activation by limited proteolysis, bacteria have evolved strategies to benefit from these host systems by assembling host proteinases at their surface. Probably the best studied

interaction in this respect is the binding of plasmin(ogen) to the bacterial surface, which is thought to be a mechanism for the bacteria to trigger their dissemination in the human host (10).

Thrombin Activatable Fibrinolysis Inhibitor (TAFI), also known as procarboxypeptidase B, procarboxypeptidase R, and procarboxypeptidase U, is an arginine and lysine specific procarboxypeptidase. The protein is synthesized in the liver and secreted into plasma, where it circulates as a zymogen at concentrations between 60 and 275 nM (11). The most potent activator of TAFI is the thrombin/thrombomodulin complex (12), but plasmin, trypsin and neutrophil elastase have also been reported to function as activators (13-15). Active TAFI (TAFIa) has anti-fibrinolytic properties as the enzyme removes carboxy-terminal lysine residues from fibrin, thereby attenuating accelerated plasmin formation. Apart from its role in regulating fibrinolysis, TAFI can also modulate inflammatory responses. For instance, TAFIa has been shown to inactivate the anaphylatoxins C3a and C5a by removing their carboxyterminal arginine residues (16), and it has been suggested that TAFI is involved in the conversion of the fibrinopeptide B (FPB), a potent neutrophil chemoattractant, to its inactive metabolite des-Arg FPB (17). In another study, it was found that the removal of the carboxy-terminal arginine from thrombin-cleaved osteopontin by TAFI impairs the adhesive function of the protein (18). Moreover, TAFI has also pro-inflammatory properties, since it can convert bradykinin into desArg⁹bradykinin, a selective agonist of the kinin B1 receptor, which has an important role in chronic inflammatory processes (16,19). However, the pathophysiological effects of these modifications are not completely understood.

The present study was undertaken to investigate the interaction between TAFI and *S. pyogenes*. Here we find that streptococci of serotype 41 are able to bind TAFI and that activation of TAFI can occur on the bacterial surface, which might be an important mechanism for bacteria to modulate inflammatory reactions of the host.

EXPERIMENTAL PROCEDURES

Reagents - TAFI and PAB were produced and purified as described elsewhere (20,21). Plasminogen and plasmin were purchased from

Sigma Aldrich, St. Louis, MO, and thrombin was from Chemicon, Malmö, Sweden. Oligonucleotides were obtained from DNA Technology A/S, Aarhus, Denmark. Rabbit lung thrombomodulin was purchased from American Diagnostica (Greenwich, CT), hippuryl-Arginine and H-D-Phe-Pro-Arg-Chloromethylketone (PPACK) from Bachem (Bubendorf, Switzerland), and potato carboxypeptidase inhibitor from Calbiochem (La Jolla, CA). For the TAFI activity assays, thrombin was used that was a generous gift from Dr W. Kisiel (University of New Mexico, Albuquerque, NM), while plasmin and t-PA (actilyse) were purchased from Roche (Germany).

Bacteria and culturing conditions - *S. pyogenes* AP strains, obtained from the Institute of Hygiene and Epidemiology (Prague, Czech Republic), were grown in Todd-Hewitt broth (BD, Franklin Lakes, NJ) in 5% CO₂ at 37°C. To express SclA and SclB recombinantly, the *E. coli* strain BL21 Star (DE3) was used. Bacteria were grown in LB broth (1% (w/v) Tryptone (BD, Franklin Lakes, NJ), 0.5% (w/v) Yeast extract (Oxoid Ltd, Basingstoke, Hampshire, UK), and 1% (w/v) NaCl (Merck, Darmstadt, Germany), supplemented with 1% (w/v) Glucose (Prolabo, Fontenay sous bois Cedex, France) and Kanamycin 50 µg/ml (Sigma Aldrich, St. Louis, MO).

Radio-labelling - Proteins were labelled with ¹²⁵I with IodoBeads (Pierce, Rockford, IL) according to the manufacturer's instructions.

Binding assays - Overnight cultures of bacteria were washed three times in PBS with 0.02% Na-azide and 0.05% Tween, adjusted to 0.02-2 x 10⁹ CFU/ml and incubated with 40,000 cpm of ¹²⁵I-labelled TAFI, plasmin, or thrombin for 1 h at room temperature. Bacteria were washed once in PBS, 0.02% Na-azide and 0.05% Tween, and the radioactivity of the cell pellets were detected with a gamma counter (Perkin Elmer). For competition experiments, AP41 bacteria (1 x 10⁷ CFU/ml) were incubated with 50,000 cpm of ¹²⁵I-TAFI, in the presence or absence of 40 µg/ml rSclA/AP/M41, rSclB/AP/M41, or protein PAB. Alternatively, bacteria were incubated with 50,000 cpm of ¹²⁵I-plasmin or ¹²⁵I-thrombin in the absence or presence of 12.5-100 µg/ml of unlabeled plasmin or 100-800 µg/ml of unlabeled thrombin, respectively. Bacteria were washed and bound ¹²⁵I-labeled ligand was determined as above.

Protein Sequence analysis - TAFI was coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech, Sollentuna, Sweden) according to the protocol provided by the manufacturer. Surface proteins of *S. pyogenes* AP41 were removed by cleavage with CNBr as described (22). Briefly, bacteria were washed and resuspended in PBS (2 ml PBS/g bacteria, wet weight), and mixed with an equal volume of CNBr 30 mg/ml in 0.2 M HCl. After incubation for 15 h on rotation at room temperature, cells were pelleted by centrifugation. The supernatant was filter sterilized, dialyzed against 0.1 M HCl, neutralized with 1.5 M Tris-HCl pH 8.8, and finally incubated with TAFI-coupled Sepharose at 4°C overnight. After extensive washing, bound proteins were eluted with 0.1 M glycine-HCl pH 2.0. Eluted fractions were examined by SDS-PAGE, protein bands were excised and sent to Eurosequence (Groningen, The Netherlands) for identification by internal sequencing. The peptide sequences generated by internal sequencing were matched against streptococcal proteins in a BLAST search.

PCR and Sequencing - Genomic DNA was obtained by boiling bacteria in H₂O for 5 min. PCR was performed in a Mastercycler (Eppendorf) using *Taq*-polymerase and buffers from Saveen & Werner AB (Malmö, Sweden). dNTPs were from Fermentas (Burlington, Ontario, Canada). Sequencing was performed using BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Primers with the following sequences were used: *SclA* forward: CAA CAT ATG TTG ACA TCA AAG CAC, *SclA* reverse: GGG TTG GAT CCC TAA CCA GTT GCT G, *SclB* forward 1: ATA CAA AAA GAA CTT TAC AAT CAT TC, *SclB* forward 2: CTT AAA GAG TTA AAT ATG TTT G, and *SclB* reverse: GAT TGG ATC CTT AGC CTG TTG CTG GC.

Recombinant protein expression - *SclA* and *SclB* from the AP41 strain were PCR-amplified with the following primer pairs: *SclA* for: CAC CGA TAT CTG GGA CCA GGA GC, *SclA* rev: GCT CTC AAG TTG CTG GTA GAC GTC, *SclB* for: CAC CGA TGG CGA AGG TAT CAG AAA G, and *SclB* rev: GTT TCT CAT GTT GCT GGC AAT TG. The PCR-products were cloned into the pET200 TOPO vector (Invitrogen, Paisley, UK), resulting in His-tagged fusion proteins. The vector was amplified in *E. coli* TOP 10 cells and then transformed into *E. coli* of the BL21 Star (DE3) strain for protein

expression. Recombinant proteins were purified on Ni-NTA His-Bind Sepharose (Novagen, Madison, WI). Recombinant *SclA* from the M41 strain MGAS6183 and r*Scls* from the M28 strain MGAS6274 were produced and purified as described previously (23).

Slot Blot analysis - Different amounts of recombinant *SclA* and B from different strains were applied to the wells of a MilliBlot (Millipore Corporation, Billerica, MA), and transferred to a methanol-activated Immobulone Transfer Membrane (Millipore Corporation, Billerica, MA) by gentle suction. After blocking in VBS buffer (140 mM NaCl, 5 mM Na-5,5-diethylbarbiturate, pH 7.35) supplemented with 0.25% (w/v) gelatine and 0.25% (v/v) Tween20 for 4 x 10 min, the membrane was incubated with ¹²⁵I-labelled TAFI (2 x 10⁵ cpm/ml) in VBS with 0.1% (w/v) gelatine overnight at 4°C. The membrane was then washed in PBS 0.25% (w/v) gelatine and 0.25% (v/v) Tween 20, and exposed to an X-ray film (AGFA, Mortsel, Belgium) for 10 days.

Binding studies using a BIAcore 2000 biosensor system - Recombinant AP41 *SclA* and *SclB* were immobilized to a CM5 sensor chip using the amine coupling kit according to the supplier's recommendation (Biacore AB). *SclA* and *SclB* were applied in 10 mM NaAc (pH 3.1). Immobilization of *SclA* on the chip resulted in an increase of the resonance signal by about 400 resonance units, and with *SclB* of approximately 440 resonance units. Binding studies were done using 10 mM Hepes, 150 mM NaCl, 0.005% P20, pH 7.4, at a flow rate of 30 µl/min at 25°C. Different concentrations of TAFI (0 – 2 µM) were injected for 3 minutes. The K_D values were calculated using the steady-state model in the BiaEvaluation software.

TAFI cleavage - ¹²⁵I labelled TAFI (2 x 10⁶ cpm/ml) was incubated in the absence or presence of *S. pyogenes* AP41 bacteria (1 x 10⁸ CFU/ml in PBS containing 0.25% (v/v) Tween20, final concentration) together with different concentrations of thrombin and thrombomodulin (5-100 nM of each protein) at 37°C for 10 min, or with plasmin (0.5-10 µg/ml) at 37°C for 20 min. Bacteria were then washed once in PBS with 0.25% (v/v) Tween20, resuspended in SDS-sample buffer (125 mM Tris, 4% (w/v) SDS, 10% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 2% (w/v) bromophenolblue) and boiled for 5 min. Cells were removed by centrifugation and the supernatants were separated on an SDS-PAGE

gel alongside with the samples incubated without bacteria. The gels were dried and exposed to X-ray film. Alternatively, AP41 bacteria (1×10^8 CFU/ml in PBS containing 0.25% (v/v) Tween20, final concentration) were pre-incubated with thrombin/thrombomodulin (100 nM of each protein) or plasmin 10 μ g/ml for 1 h on ice. Unbound proteins were removed by a washing step. Bacteria were then incubated with 125 I-TAFI (200,000 cpm) at 37°C for 15 min (thrombin/thrombomodulin) or 30 min (plasmin) followed by a centrifugation step. Supernatants were collected and cell pellets were subsequently washed and resuspended in SDS sample buffer as described above. Supernatants and proteins recovered from the bacterial pellets were separated on SDS-PAGE gels and analysed as described above.

Negative Staining Transmission Electron Microscopy - SclA, SclB, and TAFI, as well as TAFI in complex with SclA or SclB were analyzed by negative staining and electron microscopy as described previously (24). TAFI and rScls were mixed in TBS buffer and allowed to react for 30 min at 4°C. Final sample concentrations were 20 nM in 50 mM Tris-HCl, 0.15 M NaCl, pH 7.4 (TBS). Subsequently, 5 μ l aliquots were adsorbed onto carbon-coated grids for 1 min, washed with two drops of water, and stained on two drops of 0.75% uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air. Specimens were observed in a Jeol JEM 1230 electron microscope operated at 60 kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 CCD camera.

Determination of the stability of TAFIa in the presence of rScls - The assay was basically done as described (25). To determine the effect of the presence of rSclA/AP/M41 and rSclB/AP/M41 on the rate of TAFIa inactivation, TAFI (12.5 nM, all concentrations are final concentrations) and rScls (0-0.18 μ M) were added to a premix of thrombin (16 nM) and thrombomodulin (32 nM) in the presence of CaCl_2 (5 mM), in 20 mM Hepes, pH 8.0/0.1% BSA. This mixture was incubated for 1 minute at 37°C before the thrombin activity was stopped by adding PPACK, while the mixture remained at 37°C to allow spontaneous TAFIa inactivation. At several time points, 20 μ l samples were withdrawn and added to 50 μ l of the substrate hippuryl-Arginine (21.4 mM). After 30 min incubation with the substrate at 37°C, substrate

conversion was stopped by adding 50 μ l of 1 M HCl. Then, o-methyl hippuric acid (1.7 μ M) was added as an internal standard, and both o-methyl hippuric acid and hippuric acid were extracted using a Solid Phase Extraction unit according to the supplier's recommendation (Waters Oasis, Wexford, Ireland) and samples were analyzed by HPLC as described (25). The TAFIa activity is expressed in units per liter (U/L). One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μ mol of substrate per minute at 37°C under the conditions described (25).

Influence of Scls on TAFI activation - The assay was done as described (26) with minor modifications. In a microtiterplate, 40 μ l of reaction mixture, 20 μ l TAFI (100 nM) and 20 μ l of rSclA/AP/M41 or rSclB/AP/M41 (0-0.45 μ M) were mixed and prewarmed to 37°C. The reaction mixture was composed of PEP (2 mM, all concentrations are final concentrations in the assay), ATP (2.7 mM), NADH (0.5 mM), arginine kinase (21 U/L), pyruvate kinase/dehydrogenase and hippuryl-arginine (5 mM). The activation mixture contained thrombin (8 nM), thrombomodulin (16 nM) and CaCl_2 (5 mM) or plasmin (0.36 U/ml). Volumes were adjusted with 20 mM Hepes pH7.4/0.01%Tween20. The reactions were started by adding 20 μ l of TAFI (100 nM). TAFI activation was followed over time as a loss of NADH absorbance at 340 nm in a Thermomax microplate reader (Molecular Devices Corporation, Menlo Park, CA).

Clot-lysis assay - The clot lysis assay was performed essentially as described previously (27). Briefly, 47 μ l of citrated, pooled, human plasma (pooled normal plasma) was mixed with various concentrations of TAFI and rSclA/AP/M41 or rSclB/AP/M41 (0-0.45 μ M, all concentrations are final concentrations in the assay) in a 96-well microtiter plate. The experiments were done in the presence or absence of carboxypeptidase inhibitor (12 μ M). The volumes were adjusted to 65 μ l with HBS (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, pH 7.4) containing 0.1% (w/v) bovine serum albumin. A mixture (35 μ l) of a thousand-fold dilution of Innovin, CaCl_2 (17 mM) and recombinant tissue-type plasminogen activator (actilyse, 0.3 ng/ml) in HBS/0.1% (w/v) bovine serum albumin, was added to the plasma and turbidity was measured in time at 37°C at 405 nm in a Thermomax microplate reader

(Molecular Devices Corporation, Menlo Park, CA). The clot-lysis time was defined as the time difference between half-maximal lysis and half-maximal clotting.

RESULTS

TAFI binds to collagen-like surface proteins from S. pyogenes - To investigate the interaction between TAFI and streptococci, the binding of ^{125}I -TAFI to twelve different streptococcal serotypes was tested. Figure 1 shows that the strains displayed varying degrees of TAFI binding, with the AP41 strain being the most efficient. Based on these results, the AP41 strain was chosen for further characterization. To identify the streptococcal receptor(s) involved in TAFI binding, surface proteins of the AP41 strain were solubilized with CNBr and incubated with TAFI immobilized to Sepharose. Following a washing step, bound proteins were eluted from the column, separated on SDS-PAGE, and visualized by Coomassie staining. Figure 2A depicts that a protein with an apparent molecular weight of approximately 35 kDa was recovered. The band was excised from the gel and further processed by internal sequencing. Two peptides were generated, of which both shared 90% homology with streptococcal collagen-like surface protein A (SclA) from an M41 strain (Table 1). All group A streptococcal serotypes tested so far, carry the genes coding for SclA and SclB. As previous studies have shown that the extracellular domains of the two proteins differ significantly from each other and also vary within serotypes, we decided to sequence both genes from the AP41 strain. As expected, the results show that the deduced nucleotide sequences of the two peptides (Table 1) were found in SclA, but not in SclB.

To study the interaction between Scls and TAFI at the protein level, the extracellular part of SclA (rSclA/AP/M41) and SclB (rSclB/AP/M41) from the AP41 strain were recombinantly expressed in *E. coli* and purified as described in *Experimental Procedures*. Figure 2B shows that the apparent molecular weights of the two proteins were slightly higher than expected, which was also reported when recombinantly expressed Scls from other serotypes were examined by SDS-electrophoresis (3). Binding of TAFI to purified Scls was studied using the BIAcore biosensor system. Analysis of the binding curves (figure 3A and B) showed that the interactions did not

follow a one to one stoichiometry model. The K_D for steady state binding of TAFI to SclA was 215 ± 31 nM and to SclB 253 ± 37 nM. For further binding studies, rSclA/AP/M41 and rSclB/AP/M41 were immobilized on a PVDF-membrane and probed with ^{125}I -TAFI. Moreover, SclA from the M41 strain 6183 (rSclA/176/M41) and the M28 strain 6274 (rSclA/161/M28) as well as SclB from that M28 strain (rSclB/163/M28) were also applied to the membrane. After a washing step, bound ^{125}I -TAFI was detected by exposing the membrane to an X-ray film. Figure 3C shows that rSclA/AP/M41 binds more efficiently to TAFI than rSclB/AP/M41. Interestingly, rSclA/176/M41 derived from another M41 strain bound TAFI to a similar degree as rSclA/AP/M41, while rSclA/161/M28 and rSclB/163/M28 showed no interaction. The latter observation is in accordance with the finding that streptococci of the AP28 strain displayed only poor TAFI-binding properties (Fig.1). To further establish the role of Scls in the binding of TAFI to *S. pyogenes*, AP41 bacteria were incubated with ^{125}I -TAFI in the absence or presence of recombinant Scls. As a control, the surface protein PAB from *Finnegoldia magna* was used. PAB had no influence on TAFI binding, whereas rSclA/AP/M41 and rSclB/AP/M41 reduced TAFI binding with 84 and 42%, respectively (Fig 3D). No additional effect was seen with both proteins in combination (data not shown).

To visualize the interaction of rSclA/AP/M41 and rSclB/AP/M41 with TAFI, negative staining and transmission electron microscopy was employed. Figure 4A and C show that rSclA/AP/M41 displayed a characteristic lollipop-like structure with a globular head and a tail as also has been described for other Scls (9,23), while TAFI had a horseshoe-like shape (Fig. 4 B and E). When incubating TAFI with rSclA/AP/M41, the carboxypeptidase was frequently ($> 95\%$) localized close to the heads of SclA (Fig. 4D and F), and this was also found when the interaction between rSclB/AP/M41 and TAFI was investigated (data not shown). Interestingly, similar findings were recently reported when the binding of ApoB100 to SclA was analyzed (9).

S. pyogenes strain AP41 binds plasmin and thrombin to its surface - Under physiological conditions, TAFI can be activated by plasmin or the thrombin/thrombomodulin complex (12,13). The binding of plasmin/ogen

to various streptococcal strains, including the AP41 strain (28), is well established and has been described earlier by many other groups (29-31). However, the interaction between Streptococci and (pro)thrombin has been less investigated and to our knowledge all binding studies conducted so far showed that (pro)thrombin had no affinity for the streptococcal strains tested (31,32). Thus, to investigate whether activation of TAFI at the bacterial surface can be induced by recruited plasmin or thrombin/thrombomodulin, we studied the interaction of ^{125}I -plasmin or ^{125}I -thrombin with AP41 bacteria. Figure 5A shows that ^{125}I -plasmin binds avidly to the bacteria. In addition, also an interaction with ^{125}I -thrombin was observed. In both cases the binding was displaceable by the addition of unlabeled plasmin and thrombin, respectively (Fig. 5B and C). 50% displacement occurred at concentrations around 30 $\mu\text{g/ml}$ for plasmin and 300 $\mu\text{g/ml}$ for thrombin, which would correlate to K_D values of approximately 0.3 μM and 8 μM , respectively. Taken together, the results demonstrate that not only TAFI, but also two TAFI activators, namely plasmin and thrombin, bind to the surface of AP41 bacteria.

TAFI is cleaved by plasmin and thrombin/thrombomodulin at the bacterial surface - TAFI activation by plasmin or the thrombin/thrombomodulin complex occurs by the removal of a 19 kDa activation peptide from the amino-terminal part of the protein. The resulting 36 kDa active carboxypeptidase (TAFIa) has a very short half-life time (~5 min) because of a conformational reorganization that drives the enzyme into its inactive form (TAFIai). Further processing by plasmin or the thrombin/thrombomodulin complex leads then to the generation of smaller inactive fragments (13,20). To analyze whether streptococcal-bound TAFI can be cleaved by a similar mechanism as described above, AP41 bacteria were incubated with ^{125}I -TAFI in the presence of increasing concentrations of plasmin or thrombin/thrombomodulin. After a short incubation time, bacteria were washed and proteins recovered from the bacterial surface were separated on SDS-PAGE. As control, ^{125}I -TAFI was incubated by plasmin or thrombin/thrombomodulin in the absence of bacteria. Figures 6A + B show that treatment of bacteria-bound TAFI with plasmin or thrombin/thrombomodulin leads to the generation of a 36 kDa fragment that is also seen when

TAFI was cleaved in solution. To verify that cleavage occurs at the bacterial surface, AP41 bacteria were pre-incubated with plasmin or thrombin/thrombomodulin. Unbound proteins were removed by a washing step and bacteria were incubated with ^{125}I -TAFI for 30 min (plasmin) and 15 min (thrombin/thrombomodulin). Thereafter, ^{125}I -TAFI that was recovered from the bacterial surface or remained in solution, was analyzed by SDS-PAGE. Figure 6C shows that TAFI processing occurred at the bacterial surface, but not in solution. These findings implicate that bacteria-bound TAFI is susceptible to cleavage by its two most important activators.

The physiologic activity of TAFI is not inhibited by SclA or SclB - To investigate the influence of rScls on the activation profile of TAFI, TAFI activation by thrombin/thrombomodulin or plasmin was followed over time. At the concentrations tested (0-0.45 μM), neither rSclA/AP/M41 nor rSclB/AP/M41 had any influence on the activation profile (data not shown). In addition, the TAFIa half-life was not changed by the presence of rScls (0-0.18 μM) (data not shown). Based on these findings we next wanted to investigate whether the rScls affected the fibrinolytic action of TAFI. To this end, the effect of rScls was studied in a clot-lysis test. In these assays, clotting of normal pooled plasma supplemented with various concentrations of rSclA/AP/M41 or rSclB/AP/M41 was initiated by tissue factor, and fibrinolysis was initiated by recombinant t-PA. Clot-lysis times were determined in the presence or absence of a carboxypeptidase inhibitor (CPI) to visualize the TAFI-dependent prolongation. The results show that the clot-lysis times of plasma were not influenced by the presence of rScls (Fig. 7). Taken together, these experiments demonstrate that binding of TAFI to rSclA/AP/M41 and rSclB/AP/M41 do not affect TAFI activation, TAFIa stability or TAFI's anti-fibrinolytic potential, which may point to an important function of bacteria-bound TAFI in the regulation of the inflammatory host response.

DISCUSSION

Over the years, a number of host-pathogen interactions have been described to be triggered by proteolytic-driven cascades (33,34). Based on the intensity of activation, these systems can either clear an infection or promote

pathological inflammatory reactions (35). Typical examples are complement, coagulation, and fibrinolysis, which are normally involved in host defence during infection, but can also cause deleterious and sometimes life-threatening complications once they are activated in a systemic manner. Thus, the study of these systems in relation to their activation by invading pathogens may lead to a better understanding of how a normally harmless infection can evoke serious conditions in the human host. For instance, many bacterial pathogens have been shown to utilize the host's own proteolytic systems to establish an infection. Probably the best-studied human proteinase in this respect is plasminogen, which apart from *S. pyogenes* also interacts with many other bacterial species, including *Borrelia burgdorferi*, *Escherichia coli*, *Salmonella enteritidis*, *Yersinia pestis*, and the *Neisseria* species *gonorrhoeae* and *meningitidis* (34). Activation of plasminogen is a critical step in the progression of an infection, and several modes of actions for different bacteria have been described. While species such as *S. pyogenes* and *Y. pestis* express plasminogen activating proteins (streptokinase and protein Pla, respectively), others such as *B. burgdorferi*, *Salmonella enteritidis*, and *E. coli* recruit tissue plasminogen activator or urokinase-type plasminogen activator from the host for this purpose (34). Importantly, studies with *S. pyogenes*, *B. burgdorferi*, and *S. aureus* have demonstrated that plasmin, mobilized to the bacterial surface, is protected from inhibition by host proteinase inhibitors, in particular α_2 -antiplasmin, resulting in a long-lasting surface-associated enzymatic activity (29,36,37). The binding of plasmin/ogen to the streptococcal surface is well documented and involves M and M-like proteins (30,38), α -enolase (39), and glyceraldehyde-3-phosphate dehydrogenase (40). While some M proteins bind plasminogen indirectly via fibrinogen (38), other strains express an M-like protein called PAM (plasminogen-binding group A streptococcal M-like protein) that binds plasminogen directly with high affinity (30). Notably, the strains that displayed the highest TAFI binding (M-type 41

and 53) were also PAM-expressing strains (28). Bacterial interactions with pro/thrombin on the other hand, are less investigated, but it is well established that *S. aureus* secretes a staphylocoagulase which interacts with thrombin without proteolysis (41). Both plasmin and thrombin have in common that they are important physiologic activators of TAFI and, of note for the present study, they assemble in their active forms at the surface of AP41 bacteria. In the present study, we described for the first time that TAFI binds to *S. pyogenes* by interacting with the streptococcal collagen-like surface proteins SclA and SclB. Importantly, bacteria do not have the machinery to activate surface-bound TAFI, and they therefore have to recruit TAFI's natural activators. The finding that TAFI is assembled with its activators plasmin and thrombin at the streptococcal surface implies that potentially high concentrations of activated TAFI may be obtained locally at the site of infection. They also suggest that TAFI activation can be regulated by bacteria via for instance streptokinase-activated, bacteria-bound plasmin. Activated TAFI is mostly known as an inhibitor of fibrinolysis. Notably, previous studies have shown that *S. pyogenes* bacteria adhering to epithelial cells are coated with a fibrin network (32). It can therefore be speculated that the blood clot forms a shield that protects the bacterium from the host's immune defence systems, and that the recruitment and activation of TAFI on the bacterial surface would help stabilize the clot. Moreover, activation of complement, coagulation, and fibrinolysis occurs by limited proteolysis and often involves the cleavage of an Arg-Ile or a Lys-Ile bond. Thus, many peptides involved in these processes, such as the anaphylatoxins C3a and C5a, bradykinin and kallidin, as well as fibrinopeptide B have an arginine at their carboxy-terminus. As the generation of these peptides is induced in response to infection, it is tempting to speculate that bacteria-bound TAFI is used as a tool to modulate the host response. Taken together, the present study shows that TAFI is bound to AP41 bacteria via SclA and SclB, and can be converted into its active fragment by recruited plasmin and thrombin.

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FOOTNOTES

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The nucleotide sequences reported in this paper have been submitted to the GenBankTM/EMBL Data Bank with accession numbers EF042101 and EF042102.

² The abbreviations used are: CFU, colony forming unit; cpm, counts per minute; FPB, fibrinopeptide B; LDL, low density lipoprotein; PAB, Peptostreptococcal Albumin Binding; PAM, plasminogen-binding group A streptococcal M-like protein; PBS, Phosphate-Buffered Saline; PCR, polymerase chain reaction; PPACK, H-D-Phe-Pro-Arg-Chloromethylketone; Scl, streptococcal collagen-like surface protein; SDS-PAGE; TAFI, Thrombin Activatable Fibrinolysis Inhibitor; TBS, Tris-Buffered Saline; t-PA, tissue plasminogen activator.

FIGURE LEGEND

Fig. 1. Binding of TAFI to *S. pyogenes*. 12 different strains of *S. pyogenes*, 2×10^9 CFU/ml, were incubated with ^{125}I -TAFI for 1 h at room temperature. Cells were washed, and the amount of radio-labeled protein that remained bound to the bacteria was determined in a gamma counter.

Fig. 2. Isolation of a TAFI-binding protein from *S. pyogenes*. (A) Surface proteins from the AP41 strain of *S. pyogenes* were solubilized with CNBr as described in *Experimental procedures* and incubated with TAFI-coupled Sepharose. After washing the Sepharose with PBS, bound proteins were eluted and analysed on SDS-PAGE. The figure shows a Coomassie-stained SDS-PAGE gel with solubilized surface proteins from AP41 (lane 1) and eluted proteins from the TAFI-coupled Sepharose (lane 2). The arrow indicates the protein band that was subjected to internal sequencing. (B) SclA and SclB from the AP41 strain were cloned and expressed in *E. coli* and analyzed by SDS-PAGE followed by Coomassie staining.

Fig. 3. TAFI binds to SclA and SclB. (A and B) Overlay plots of the binding of TAFI to immobilized SclA (A) and SclB (B) using plasmon resonance spectroscopy. (C) Different concentrations of recombinant Scls from two M41 strains (rSclA/AP/M41, rSclB/AP/M41, and rSclA/176/M41), and rScls from one M28 strain (rSclA/161/M28 and rSclB/163/M28) were immobilized on a PVDF-membrane, and incubated with ^{125}I -TAFI. Unbound TAFI was removed by a washing step, and the membrane was exposed to an X-ray film. (D) Bacteria were incubated with ^{125}I -TAFI in the presence or absence of 40 $\mu\text{g/ml}$ SclA, SclB, or protein PAB. Statistical analysis ($n = 3$) was performed using Student's *T*-test (un-paired, two tailed). *** indicates $p < 0.001$.

Fig. 4. Electron microscopy of rSclA/AP/M41 and TAFI. Representative fields of rSclA/AP/M41 (A) and TAFI (B) are visible after negative staining. Selected molecules of rSclA/AP/M41 (C) and TAFI (E) are shown at higher magnification. (D and F), TAFI binds in proximity to the globular domain of rSclA/AP/M41. Arrowheads indicate globular domains of rSclA/AP/M41 and arrows point to TAFI molecules. (E and F right panels), interpretative presentations in pseudocolors of rSclA/AP/M41 (green) and TAFI (red) are shown. Scale bars represent 50 nm (A and B), 30 nm (C and D), and 10 nm (E and F).

Fig. 5. AP41 bacteria bind plasmin and thrombin. (A) Serial dilutions of AP41 bacteria were incubated with ^{125}I -plasmin (black line) or ^{125}I -thrombin (grey line) for 1 h, followed by a washing step to remove unbound ligand. Bacteria were centrifuged and the amount of radio-labeled protein in the cell pellet was determined with a gamma counter. The figure shows the means and standard deviations of three independently performed experiments. (B and C) AP41 bacteria, were incubated for 1 h with ^{125}I -plasmin (B) or ^{125}I -thrombin (C) in the presence of varying concentrations of the corresponding unlabeled ligand. Cells were washed and the radioactivity of the cell pellets were thereafter determined as described above. The figure shows a representative experiment out of four, each done in duplicates.

Fig. 6. TAFI is cleaved by plasmin and thrombin/thrombomodulin at the bacterial surface. AP41 bacteria were incubated with ^{125}I -TAFI in the absence or presence of plasmin (A) or thrombin/thrombomodulin (B) for 20 or 10 min, respectively. Bacteria were then washed, and proteins bound to the bacterial surface were eluted and separated on SDS-PAGE. ^{125}I -TAFI was visualized by exposing the gel to an X-ray film. White arrowheads indicate intact TAFI, whereas black arrowheads point to cleaved TAFI of 36, 25, and 11 kDa. The figure shows representative results from at least three independently performed experiments. (C) AP41 bacteria were pre-incubated with plasmin, thrombin/thrombomodulin or left untreated. After a washing step to remove unbound protein, bacteria were incubated with ^{125}I -TAFI for 30 min (plasmin) and 15 min (thrombin/thrombomodulin), respectively. Bacteria bound ^{125}I -TAFI (B) and ^{125}I -TAFI in solution (S) were analyzed by SDS-PAGE. As a control, ^{125}I -TAFI before treatment was used (far left panel).

Fig. 7. Effect of rScls on the anti-fibrinolytic potential of TAFI. Clot-lysis times were determined in pooled normal plasma by measuring the turbidity of a thrombin-induced fibrin clot and tissue-type

plasminogen activator-mediated fibrinolysis. The plasma was supplemented with various concentrations of rSclA/AP/M41 or rSclB/AP/M41, as indicated in the figure (SclA, solid bars; SclB, open bars). The experiments were done in the absence or presence of CPI (+) to visualize the TAFIa dependent prolongation of the clot-lysis time. Each bar represents the mean of 3 independently performed experiments $\pm$ SEM.

Table 1. Internal sequence analysis of TAFI-binding protein

Amino acid sequence	Data base match [*]
Internal peptide 1 DTGAQGPVGPQ	SclA from <i>S. pyogenes</i> (serotype M41) ETGAQGPVGPQ (positions 159-169)
Internal peptide 2 GLPGLPGLPG	SclA from <i>S. pyogenes</i> (serotype M41) GLTGLPGLPG (positions 134-143)

^{*}Solubilized surface proteins from AP41 bacteria were incubated with TAFI-coupled Sepharose, followed by extensive washing and elution of bound proteins. A 35-kDa protein was identified and analyzed by internal sequencing. Two peptide sequences were generated and matched against streptococcal proteins in a BLAST search.

Fig. 1

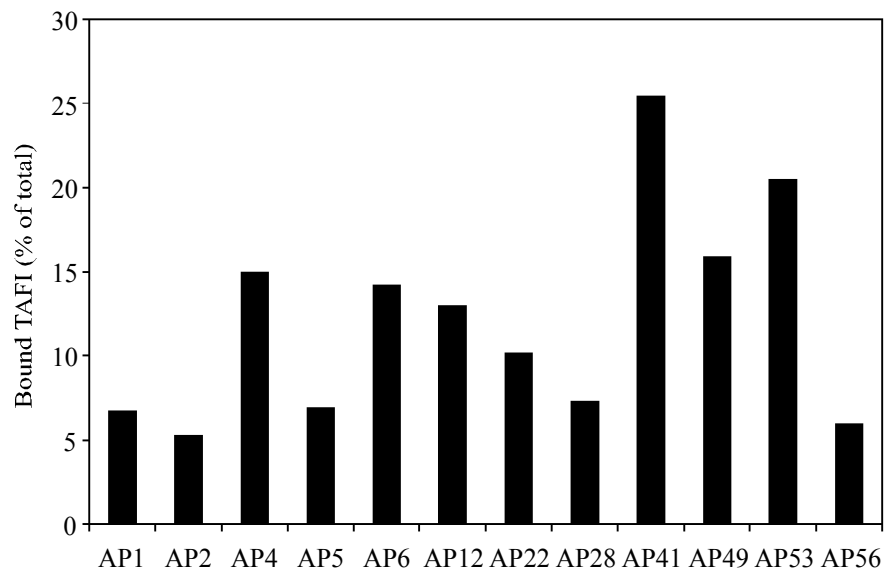


Fig. 2

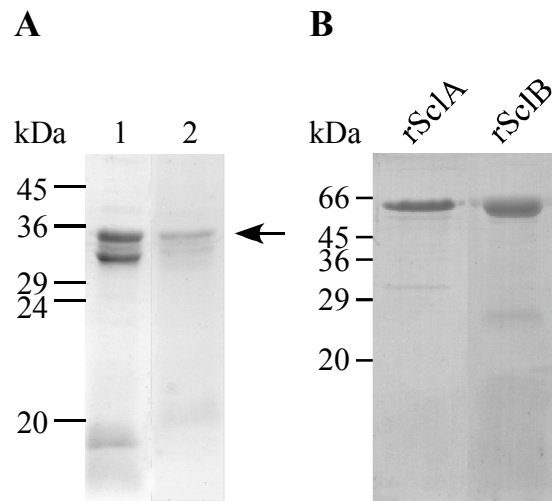


Fig. 3

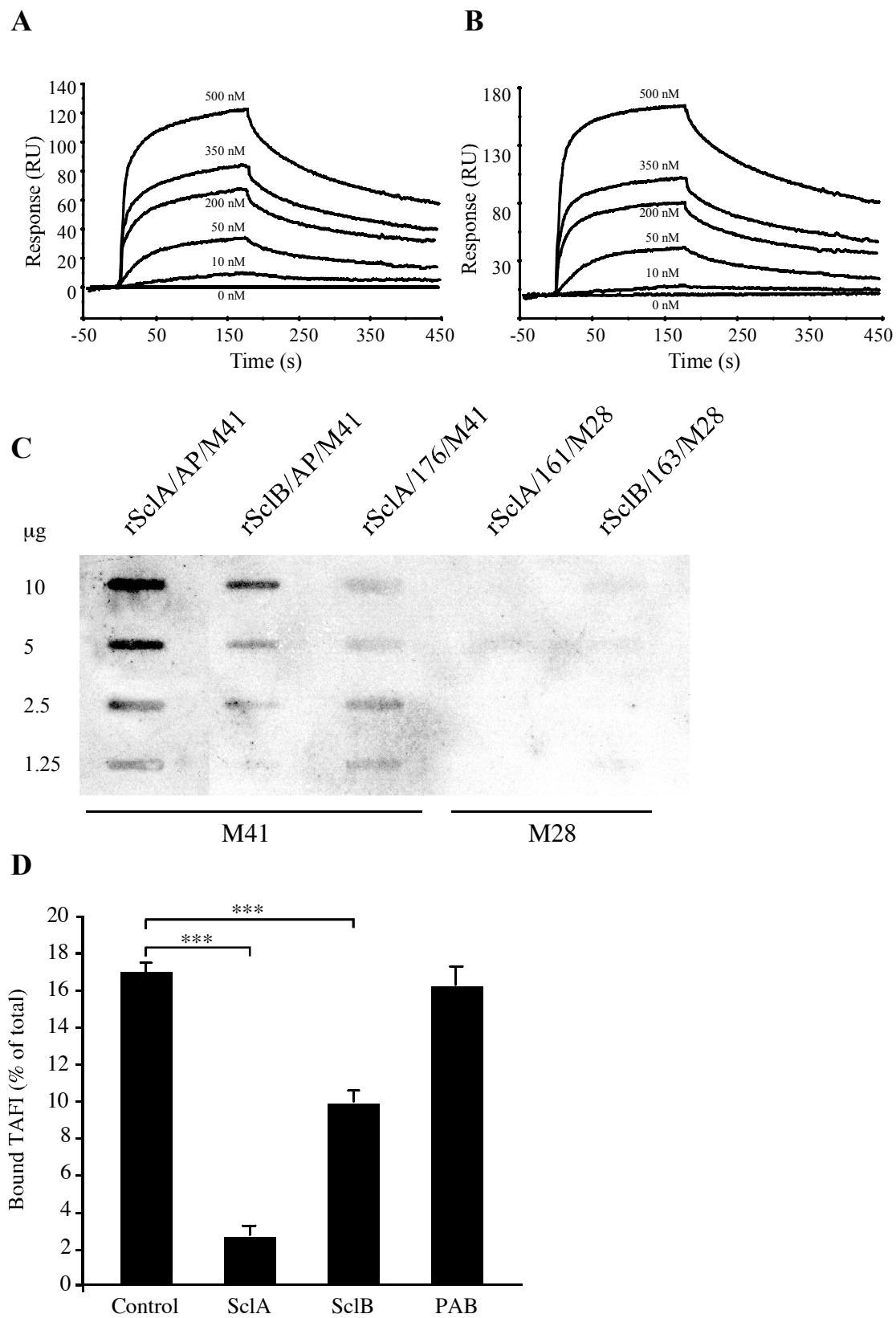


Fig. 4

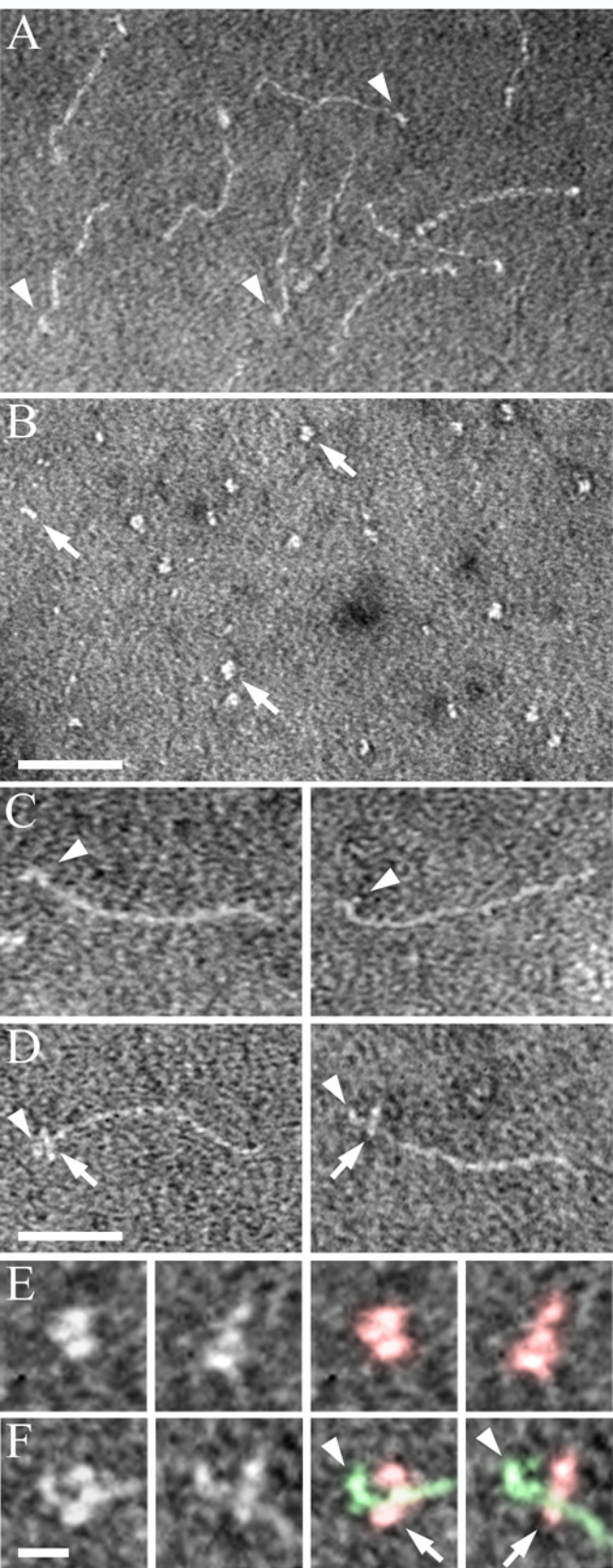


Fig. 5

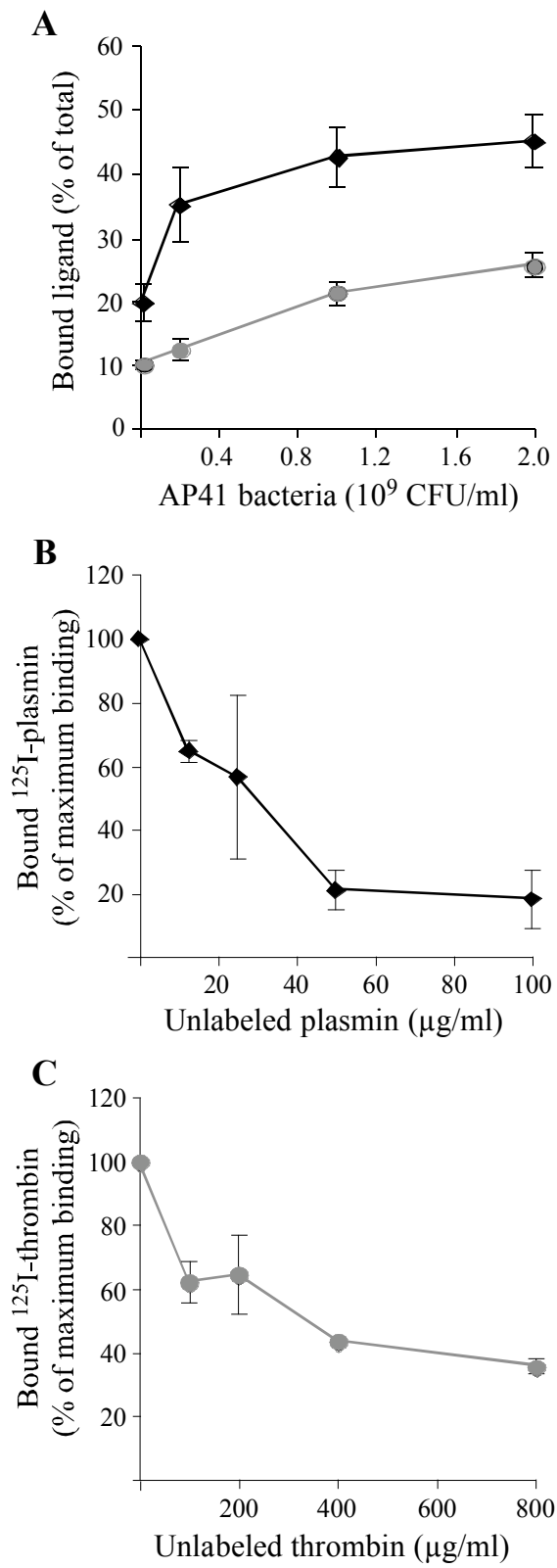
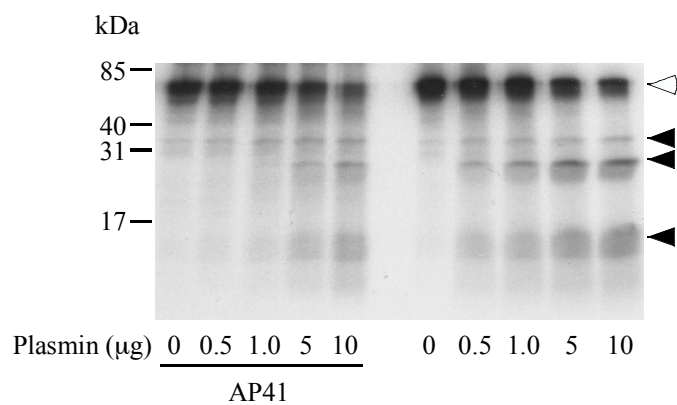
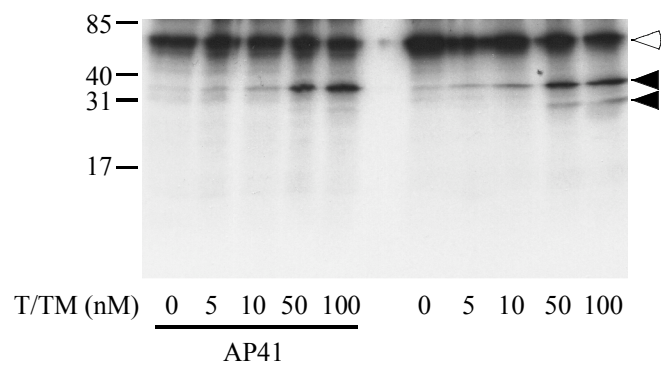


Fig. 6

A



B



C

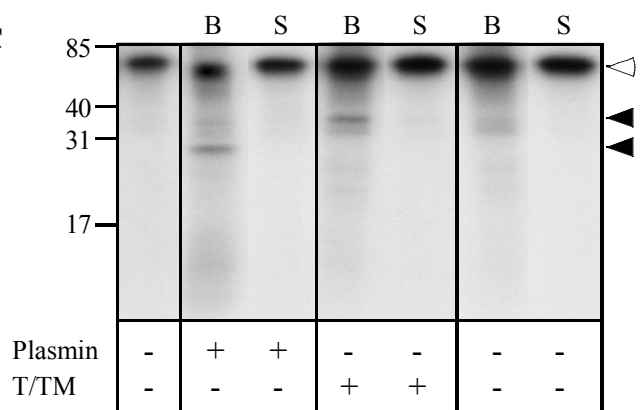


Fig. 7

