Thrombin Activatable Fibrinolysis Inhibitor, a Potential Regulator of Vascular Inflammation*

Received for publication, July 1, 2003, and in revised form, October 2, 2003
Published, JBC Papers in Press, October 2, 2003, DOI 10.1074/jbc.M306977200

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The latent plasma carboxypeptidase thrombin-activatable fibrinolysis inhibitor (TAFI) is activated by thrombin/thrombomodulin on the endothelial cell surface, and functions in dampening fibrinolysis. In this study, we examined the effect of activated TAFI (TAFIa) in modulating the proinflammatory functions of bradykinin, complement C5a, and thrombin-cleaved osteopontin. Hydrolysis of bradykinin and C5a and thrombin-cleaved osteopontin peptides by TAFIa was as efficient as that of plasmin-cleaved fibrin peptides, indicating that these are also good substrates for TAFIa. Plasma carboxypeptidase N, generally regarded as the physiological regulator of kinins, was much less efficient than TAFIa. TAFIa abrogated C5a-induced neutrophil activation in vitro. Jurkat cell adhesion to osteopontin was markedly enhanced by thrombin cleavage of osteopontin. This was abolished by TAFIa treatment due to the removal of the C-terminal Arg by TAFIa from the exposed SVVGLR αβ integrin-binding site in thrombin-cleaved osteopontin. Thus, thrombin cleavage of osteopontin followed by TAFIa treatment may sequentially up- and down-modulate the pro-inflammatory properties of osteopontin. An engineered anticoagulant thrombin, E229K, was able to activate endogenous plasma TAFI in mice, and E229K thrombin infusion effectively blocked bradykinin-induced hypotension in wild-type, but not in TAFI-deficient, mice in vitro. Our data suggest that TAFIa may have a broad anti-inflammatory role, and its function is not restricted to fibrinolysis.

Thrombin has long been regarded as a multifunctional procoagulant enzyme important in hemostasis. At sites of vascular injury it converts fibrinogen to fibrin, amplifies the clotting cascade by activating factor XI (FXI) and the cofactors FV and FVIII, stabilizes the fibrin clot by activating FXIII, and activating platelets via the protease-activated receptors (PAR). It can also function as an anticoagulant by binding to thrombomodulin (TM) on the surface of endothelial cells and activating protein C, which inhibits FVa and FVIIa, effectively localizing clot formation to the site of vascular injury (1). TM-bound thrombin can also activate thrombin-activatable fibrinolysis inhibitor (TAFI; Refs. 2 and 3), a latent plasma procarboxypeptidase also known as carboxypeptidase R, carboxypeptidase U, and procarboxypeptidase B (4–6). TAFI is now recognized as the second physiological substrate for the thrombin/TM complex. It is important for dampening fibrinolysis by removal of plasmin-exposed lysines on partially digested fibrin clots thereby restricting tissue plasminogen activator binding and further activation of plasminogen (2, 7). Thus, activated protein C (aPC) and TAFIa may play complementary roles in hemostasis, with aPC dampening the clotting cascade and preventing excessive thrombin generation, while TAFIa serves to protect the clot already formed at the site of injury.

Thrombin can also act as a proinflammatory molecule by the activation of PAR on monocytes, smooth muscle cells and endothelial cells, thus providing a direct link between coagulation and inflammation (8). On the other hand, thrombin/TM activation of PC could also function as a negative regulator of inflammation along with its defined role as an anticoagulant (1). aPC suppressed the expression of proinflammatory cell adhesion molecules and augmented the expression of anti-apoptotic molecules in cultured endothelial cells (9, 10). This anti-inflammatory role of aPC may contribute to its clinical efficacy in the treatment of severe sepsis (11). It has been proposed that TAFIa could also play a role in regulating inflammation by inactivation of kinins and anaphylatoxins through its arginine/lysine specific carboxypeptidase activity (12, 13). However, the efficiency of TAFIa in inactivating these biologically active mediators in comparison with carboxypeptidase N, the constitutively active plasma anaphylatoxin inhibitor, has not been defined.

Recently, a new mechanism by which thrombin can regulate inflammation and tissue repair is revealed by its interaction with osteopontin (OPN, 14–16). OPN is a multifunctional RGD-containing phosphoprotein with adhesive and cell-signaling functions involved in cell-cell and cell-matrix interactions important in inflammatory responses (17). It is present as an extracellular matrix component in mineralized tissues and in the subendothelial matrix of blood vessels involved by atherosclerosis. OPN also circulates as a soluble proinflammatory cytokine and is widely expressed by many inflammatory cells in culture, including T cells, macrophages, and NK cells. Its expression is enhanced in response to inflammation, tissue injury...
and stress. It interacts with many cells via RGD-dependent (αβ1, αβ3, or αβ5) and RGD-independent integrins (αβ1, αβ2, αβ3, or αβ5) and also CD44. It is chemotactic for various cell types, notably monocytes and macrophages and stimulates cell motility and cell survival (17). Of interest, thrombin cleavage of OPN increases the adhesion, spreading, and migration of cell motility and cell survival (17). Of interest, thrombin cleavage of OPN generates a N-terminal fragment that exposes a cryptic integrin-binding motif 148IVYGGLR158 on its C terminus, allowing the specific interaction to cells bearing the integrins αβ1 (19–22) or αβ2 (23, 24). In certain cell types, such as melanoma cells, cell binding only occurs with the thrombin-cleaved, but not the intact OPN, suggesting that thrombin cleavage is critical for certain OPN-cell interactions (15, 24).

Here we address the potential role of TM-dependent thrombin activation of TAFI, and the subsequent TAFIa inactivation of bradykinin, C3a, and thrombin-cleaved OPN. Our data suggest that along with aPC, TAFIa serves to counterbalance the prothrombotic and proinflammatory effects of thrombin generation and thus its biological role is not limited to fibrin clot stabilization.

EXPERIMENTAL PROCEDURES

Materials—Synthetic peptides were synthesized and purified by the peptide synthesis core facility at the Stanford University Beckman Center (Stanford, CA). The peptides were based on residues 160–168 of the C terminus of thrombin-cleaved osteopontin (OPN160–168), residues 69–77 of activated complement C3a (C3a69–77), residues 64–74 of C5a (C5a64–74) and peptides at the fibrin plasmin cleavage sites on α-chain α-Arg160 (Fβa-Arg160), β-chain β-Lys133 (Fββ-Lys133), and γ-chain γ-Lys66 (Fβγ-Lys66) and γ-Lys77 (Fβγ-Lys77). The same peptides were synthesized as their des-Arg or des-Lys forms for calibration curves. The anti-integrin antibodies HP2/1 (α1, CD49d), AM7/4 (α2, CD51), SAM1 (α4, CD49e), 7E4 (β2, CD18), 25.3.1 (α5, CD11a), BEAR1 (α7, CD11b), and 4B4 (β2, CD29) were from Beckman Coulter (Brea, CA). The sheep anti-human TAFI antibody SATAFI-AP was from Affinity Biosciences (Ontario, Canada) and horseradish peroxidase-conjugated rabbit anti-sheep antibody was from Pierce. Recombinant soluble human TM and human carboxypeptidase N (CPN) are from Berlex Biosciences (Richmond, CA). Human TAFI was purchased from Hematological Technologies (Essex Junction, VT). Bradykinin (BK), benzamidine, S-phenylalaninyl-propyl-arginylchloromethylketone (PPACK), C5a, cytchalasin B, o-dianisidine, potato carboxypeptidase inhibitor, glutathione, and Thrombomax-HS reagent were purchased from Sigma Chemicals. The protease inhibitor mixture set III and Bugbuster reagent were purchased from Novagen (Madison, WI). Recombinant human wild-type and E229K thrombins were expressed and purified in E. coli as inclusion bodies. The thrombin sequence for mature full-length human OPN (OPN-FL: amino acids 17–314, N-terminal OPN mimicking thrombin-cleaved OPN (OPN-Arg168: amino acids 17–168) and the N-terminal OPN mimicking thrombin-cleaved and TAFIa-treated OPN (OPN-Leu150: amino acids 17–167) were inserted as C-terminal in-frame fusions to GST using the E. coli expression vector pGEX-3P (Amersham Biosciences). The constructs of OPN-FL, pOPN-Arg168, and pOPN-Leu150 also incorporated the 159RGD161 to 159RAA161 substitutions by site-directed mutagenesis using the Quick-change site directed mutagenesis kit (Stratagene, La Jolla, CA) to form the constructs pRAA/OPN-FL, pRAA/OPN-Arg168, and pRAA/OPN-Leu150. Constructs were sequenced and then transformed into E. coli BL21 Gold (Stratagene, La Jolla, CA). For large scale expression of the constructs, transformed cells were grown in 1 l of LB broth supplemented with ampicillin (100 μg/ml) at 37 °C until an OD600 of 0.6–0.7. Isopropyl-1-thio-b-D-galactopyranoside (0.2 mM) was added and grown for a further 2 h at 37 °C. Cells were pelleted and washed twice with PBS, pH 7.2. Cells were lysed with Bugbuster reagent supplemented with the protease inhibitor III mixture set and 2 mM PPACK. The cell lystate was clarified, diluted 5-fold (50 mM Tris, 50 mM NaCl, 2 mM DTT, pH 7.5, and the protease inhibitors) and loaded onto a hiPrep QXL column utilizing an FPLC system (Amersham Biosciences) at 4 °C. The column was washed extensively with wash buffer (50 mM Tris, 200 mM NaCl, 2 mM DTT, pH 7.5, the protease inhibitors), then eluted with high salt buffer (50 mM Tris, 600 mM NaCl, 2 mM DTT, pH 7.5) containing 1 mM benzamidine. Fractions containing GST-OPN fusion proteins were pooled and loaded onto a 10-m glutathione-Sepharose column equilibrated with low salt buffer with 2 mM DTT and 1 mM benzamidine. The column was washed with low salt buffer and OPN was eluted with low salt buffer containing 10 mM glutathione. Fractions containing GST-OPN fusion proteins were pooled, and dialyzed against 100 mM NaCl, pH 7.5. The recombinant proteins were greater than 95% pure as judged by SDS-PAGE and Coomassie Blue staining.

Adhesion Assays—Jurtak cells were grown in RPMI 1640 media supplemented with 10% fetal calf serum. Cells with greater than 95% viability as demonstrated by Trypan Blue exclusion assay were washed with Hanks balanced salt solution (HBSS) with 50 mM Hepes, pH 7.5 and adjusted to a concentration of 2 × 106 cells/ml in buffer containing 0.2 mM MnCl2 (21). Recombinant wild-type and mutant OPN fusion proteins were diluted in PBS (concentrations ranging from 0, 0.1, 1, 10, 100 μM) and coated onto high protein binding 96-well microtiter plates (Greiner Labortechnik, Ocala, FL) with a final volume of 100 μl per well. The wells were incubated overnight at 4 °C. For experiments studying the effect of thrombin and TAFIa on OPN-FL mediated Jurtak cell adhesion, the plates were washed three times with thrombin assay buffer (25 mM Hepes, 150 mM, 5 mM CaCl2, 0.1% PEG7000, pH 7.5). To some of the wells, 100 μl of thrombin (100 nM final concentration) was added, and incubated for 1 h at 37 °C. Wells were washed three times with thrombin assay buffer. To some of the wells that had been treated with thrombin, 100 μl of TAFIa (1.7 nM final concentration) was added and incubated for 60 min at room temperature (RT). The plates were washed three times with PBS, then blocked for 1 h with 3% BSA in PBS. The wells were then washed three times with HBSS-Hepes buffer with 0.2 mM MnCl2. To each well, 100 μl of 2 × 105 cells/ml were added in the same HBSS-Hepes buffer and wells were allowed to adhere for 60 min at 37 °C. Cells were washed twice with PBS, once with absolute ethanol and fixed for 20 min with absolute ethanol. Wells were then washed three times with PBS, stained with 0.1% crystal violet, and again washed three times with PBS. Cells were then lysed with 0.5% Triton X-100. Lysates were read at 570 nm using a SpectroMAX plate reader (Molecular Dynamics, Sunnyvale, CA). For studies using recombinant GST-OPN fusion proteins (OPN-Arg168 and OPN-Leu150 with or without the RGD → RRA substitution), protein adsorption to the microtiter wells was carried out and Jurkat cell binding determined as described above. For antibody inhibition of Jurkat cell binding to OPN-Arg168 and OPN-Leu150, cells were preincubated with the integrin antibodies: 25G2 (aα5, CD49d), 7E4, 25.3.1, 4B4, and BEAR1 for 10 min on ice. Cells were then allowed to attach for 60 min and the amount of cell binding determined.

Neutrophil Myeloperoxidase Release Assay—Neutrophils were prepared from buffy coat concentrates obtained from Stanford Blood Bank following a published protocol (28). Neutrophils (4 × 106 cells/ml) were suspended with 0.2% bovine serum albumin (BSA) and stimulated with 0.25% calcium ionophore A23187. To test the effect of TAFIa on C5α-mediated myeloperoxidase release, 60 nm recombinant soluble TM was complexed with 3 nm thrombin for 10 min. TM-complexed thrombin was used to activate TAFI (200 nM). Human C5a (10 μM) was hydrolyzed by 0, 1, 10, 100 nM TAFIa at RT. 10 μl aliquots were removed at 1, 5, 10, 30, and 60 min and the TAFIa inactivation was performed with potato carboxypeptidase inhibitor, which were then dialyzed in 0.2 mM MnCl2. Following dialysis, each sample was then diluted 10-fold such that the concentration of C5α/C5αLeu150 was 1.0 μM. 1 μl of neutrophils (4 × 106 cells/ml) was treated with 5 μM cytchalasin B for 5 min at 37 °C. 1 μl of each diluted C5α/TAFIa reaction was added to the cell suspension and incubated for 15 min at
Peptides were digested with either TAFIa or carboxypeptidase N (CPN) as described under “Experimental Procedures.” Cleaved peptides were resolved by HPLO, and the areas of the cleaved peptide peaks calculated using a calibration curve with purified des-Arg or des-Lys forms of the peptides. Values for $K_m$ and $k_{cat}$ were determined (mean ± S.M., $n = 4$).

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\begin{array}{cccc}
\text{Substrate} & \text{Enzyme} & K_m & k_{cat} \\
\hline
\text{BK} & \text{TAFIa} & 70.6 ± 4.8 & 19.7 ± 4.8 & 2.8 \times 10^5 \\
\text{OPN}^{159–168} & \text{TAFIa} & 57.7 ± 5.3 & 23.6 ± 0.6 & 4.1 \times 10^5 \\
\text{C5a}^{6–74} & \text{TAFIa} & 219.0 ± 16.2 & 29.5 ± 0.7 & 1.3 \times 10^5 \\
\text{C3a}^{8–72} & \text{TAFIa} & 35.9 ± 6.6 & 8.4 ± 0.6 & 2.3 \times 10^5 \\
\text{FBa-Arg}^{96–104} & \text{TAFIa} & 361.4 ± 20.3 & 1.5 ± 0.1 & 4.2 \times 10^4 \\
\text{FBa-Lys}^{125–133} & \text{TAFIa} & 14.3 ± 0.7 & 13.6 ± 0.2 & 9.5 \times 10^3 \\
\text{FBa-Lys}^{125–133} & \text{TAFIa} & 34.0 ± 4.1 & 2.6 ± 0.1 & 7.6 \times 10^3 \\
\text{FBa-Lys}^{125–133} & \text{TAFIa} & 238.9 ± 24.2 & 5.9 ± 0.3 & 2.5 \times 10^3 \\
\text{BK} & \text{CPN} & 302.7 ± 28.1 & 9.1 ± 0.2 & 3.0 \times 10^4 \\
\text{OPN}^{159–168} & \text{CPN} & 141.6 ± 11.6 & 2.3 ± 0.1 & 1.6 \times 10^4 \\
\text{C5a}^{6–74} & \text{CPN} & 602.2 ± 74.3 & 9.3 ± 0.4 & 1.5 \times 10^4 \\
\text{C3a}^{8–72} & \text{CPN} & 77.1 ± 11.2 & 57.9 ± 2.1 & 7.5 \times 10^3 \\
\text{FBa-Arg}^{96–104} & \text{CPN} & 448.9 ± 43.8 & 2.9 ± 0.1 & 6.5 \times 10^3 \\
\text{FBa-Lys}^{125–133} & \text{CPN} & 53.2 ± 4.9 & 109.1 ± 3.6 & 2.1 \times 10^4 \\
\text{FBa-Lys}^{125–133} & \text{CPN} & 3727.0 ± 408.6 & 11.8 ± 0.8 & 3.2 \times 10^5 \\
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37°C, then centrifuged. Myeloperoxidase released to the supernatant was measured by adding 10 μl of PBS to 130 μl of 33 mM phosphate buffer pH 6.2, 0.002% H2O2, 6.7% 0.1% diisodinated-HCl. Reaction velocities were followed at A405 for 30 min.

Measurement of Mouse aPTT—Mice were anesthetized with isoflurane. A midline incision was made and the internal jugular vein cannulated. Saline, wild-type, or E229K thrombin solutions were infused through the cannulated jugular vein at rates ranging from 0 to 50 μl of thrombin/kg/min. Heart and respiratory rates were monitored and blood samples drawn after a 10-min infusion time. Blood was taken through a syringe inserted into the left ventricle and collected into 0.3% citrate. Blood samples were spun down for 1 min, 100 μl of plasma was warmed to 37°C then mixed with 200 μl of prewarmed Thrombomax-HS reagent, and the aPTT determined using a BBL fibrinometer. Fibrinogen levels were taken from similarly processed plasma while platelets counts were performed on whole blood collected into citrate anticoagulant tubes by the Stanford Animal Laboratory Facility (Stanford, CA). At each E229K thrombin dose, at least three mice were used. Results are presented as the mean aPTT ± S.E. (n = 5 for each data point in E229K, n = 3 for each data point in wild type thrombin). The study was approved by the Stanford Panel on Laboratory Animal Care and was conducted in compliance with university regulations.

The Effect of E229K on Bradykinin-induced Hypotension in Vivo—Male C57BL/6 mice (8–12 weeks old, 25–32 g) or TAFI-deficient mice and their wild-type littersates (in a mixed background of C57BL/6 and 129/Sv) were anesthetized with isoflurane 2%. A midline incision was made in the upper thorax to expose the carotid artery and jugular vein. The carotid artery was cannulated with a pressure transduction catheter connected to a computerized pressure monitor (PowerLab, Colorado Springs, CO) to record blood pressure. The transduction system was calibrated using a sphygmomanometer. Another catheter (PE-10) was inserted into the left jugular vein serving as a route of delivery for experimental treatments. After the level of isoflurane was reduced to 1%, the blood pressure and respiratory rate of the mouse was allowed to stabilize for several minutes. Then 100 μl of either E229K thrombin (40 μg/kg) or a saline control was administered to the jugular vein. Immediately afterward, 50 μl of saline was injected to flush out the catheter, then 100 μl of BK in saline (10 nmol/kg) or the equivalent molar dosage of des-ArgBK was given to the mouse through the same jugular vein catheter. Blood pressure tracings were monitored and recorded. Data are presented as maximum drops in mean arterial pressure after administration of BK or des-Arg BK.

RESULTS

Bradykinin, C5a and Thrombin-claved Osteopontin Were Good Substrates for TAFIa—The established view on TAFIa function is that it modulates clot fibrinolysis by removing exposed C-terminal lysines from partially plasmin-degraded clots. Removal of the lysines reduces t-PA and plasminogen binding thereby reducing t-PA enhanced activation of plasmin (2, 3, 5–7). Recent studies also imply the possible role of TAFIa inactivation of kinins and anaphylatoxins (4, 12, 13). To show that kinins such as BK, anaphylatoxins such as complement C3a and C5a, and the thrombin-claved cytokine, OPN, are potential substrates of TAFIa, we determined their Michaelis-Menten constants for hydrolysis by TAFIa. These were compared with the hydrolysis of peptides based on four major plasmin cleavage sites on fibrin clots (27), the fibrin ω-chain Arg104–Asp105, the β-chain Lys133–Asp134, and the γ-chain Lys82–Ala86 and Lys85–Ser86 sites (Table I). TAFIa is thermolabile (5); however, using reaction conditions in the presence of excess substrate with short incubation times at 37°C, no significant TAFIa thermal instability and inactivation was observed (data not shown). The $K_m$ and $k_{cat}$ values for hydrolysis of the fibrin peptides ranged from 14.3 μM and 13.6 s−1 for FBaLys125–133 to 361.4 μM and 1.5 s−1 for FBaArg96–104. Differences were both in $K_m$ and $k_{cat}$ for the fibrin peptides with FBaLys125–133 and FBaLys125–133 being the best substrates for TAFIa while FBaArg96–104 and FBaLys85–86 were the worst. By comparison, the overall specificity constants for BK (2.8 × 106 M−1 s−1), OPN159–168 (4.1 × 106 M−1 s−1), C5a66–74 (1.3 × 106 M−1 s−1) and C3a69–77 (2.3 × 106 M−1 s−1) were similar to the two best fibrin substrates FBaLys125–133 (9.5 × 105 M−1 s−1) and FBaLys125–133 (7.6 × 105 M−1 s−1). The in vitro data suggests that BK, C5a, C3a, thrombin-claved OPN and the surface exposed basic residues on partially degraded clots are all good substrates for TAFIa in vivo. It is notable that CPN, commonly regarded as the physiological inactivator of kinins and anaphylatoxins, was less efficient for hydrolysis of BK (3.0 × 104 M−1 s−1), OPN159–168 (1.6 × 106 M−1 s−1) and C5a66–74 (1.5 × 104 M−1 s−1) with $k_{cat}/K_m$ values ~9- and 26-fold lower. The peptides C3a69–77, FBaArg96–104, and FBaLys125–133 appeared to be slightly better substrates for CPN ($k_{cat}/K_m$ varied from 7.5 to 6.0 × 105 M−1 s−1).

TAFIa Reduced Jurkat Cell Adhesion to Thrombin-claved Osteopontin—Recombinant full-length OPN (OPN-FL) was adsorbed to 96-well microtiter plates at various concentrations and then treated with either thrombin or thrombin followed by TAFIa and compared with untreated OPN-FL for Jurkat cell binding (Fig. 1A). Thrombin-treated OPN-FL at a concentration of 1.0 μg/ml supported a 4.7-fold increase in Jurkat cell adhesion (A570 = 0.47 ± 0.12, n = 6) compared with untreated OPN-FL (A570 = 0.10 ± 0.03, n = 13) (Fig. 1, A and B). These results are consistent with previous studies showing that the
increased cell adhesion is due to exposure of a cryptic integrin-binding motif SVVYGLR at the C terminus of the thrombin-cleaved OPN fragment (15–18). TAFIa treatment of thrombin-cleaved OPN-FL reduced Jurkat cell adhesion to levels seen with OPN-FL (A_{570} = 0.12 \pm 0.03, n = 6), suggesting a potential role for TAFIa regulating integrin-mediated cell adhesion to thrombin-cleaved OPN. At higher concentrations of OPN (>10.0 \mu g/ml), cell adhesion between the differently treated OPN were similar to OPN-FL, suggesting saturation of secondary lower affinity binding sites.

To confirm that the TAFIa effect was mediated by hydrolysis of the C-terminal arginine from the newly exposed SVVYGLR motif and not due to some other effects of TAFIa, we expressed and purified recombinant GST-OPN-Arg^{168} (which mimicked the thrombin-cleaved N-terminal fragment of OPN) and GST-OPN-Leu^{\Delta 168} (which mimicked the TAFIa treatment). Dose response studies showed that the EC_{50} for Jurkat cell binding was 2.3 \pm 0.5 \mu g/ml for OPN-FL while OPN-Arg^{168} showed higher affinity at 0.5 \pm 0.1 \mu g/ml (Fig. 2A). OPN-Leu^{\Delta 168} mimicked TAFIa treatment of OPN-Arg^{168} as seen by 7-fold decrease in cell adhesion with an EC_{50} value of 3.4 \pm 0.6 \mu g/ml. The differences in cell adhesion between OPN-FL, OPN-Arg^{168} and OPN-Leu^{\Delta 168} closely paralleled the differences in adhesion between thrombin-treated and thrombin/TAFIa-treated OPN at 1.0 \mu g/ml (Figs. 1B and 2B).

**The Roles of RGD and SVVYGLR in Supporting Cell Binding**

to Thrombin-cleaved Osteopontin—The RGD site in OPN has been shown to have enhanced RGD-dependent cell binding following thrombin cleavage, presumably due to either exposure and/or increased affinity at the RGD site to RGD-dependent integrins (16). There is also evidence of a third integrin-binding site, ELVTDFPTDLPA at 13 (22). In order to delineate the relative importance of the RGD site and the SVVYGLR site in OPN-cell interaction, we mutated the RGD motif to RAA in OPN-FL (OPN-RAA-FL), OPN-Arg^{168} (OPN-RAA-Arg^{168}), and OPN-Leu^{\Delta 168} (OPN-RAA-Leu^{\Delta 168}) and expressed and purified the GST-OPN fusion proteins. Jurkat cell binding studies revealed minimal cell adhesion to OPN-RAA-FL (A_{570} = 0.01 \pm 0.01, n = 10) as compared with OPN-FL (A_{570} = 0.10 \pm 0.03, n = 13) indicating that the RGD site in intact OPN is the major functional binding site and the SVVYGLR site is normally cryptic. OPN-RAA-Leu^{\Delta 168}, where both the RGD and SVVYGLR are functionally inactive, also showed minimal cell binding (A_{570} = 0.04 \pm 0.02, n = 14), indicating that the reported ELVTDFPTDLPA binding site is not functionally active, at least in this Jurkat cell binding assay (22).

Interestingly, while the single SVVYGLR site in the cleaved OPN (OPN-RAA-Arg^{168}) was able to support substantial cell binding (A_{570} = 0.29 \pm 0.04, n = 6), the magnitude of cell binding is clearly less than that when both sites were exposed in OPN-Arg^{168} (A_{570} = 0.53 \pm 0.04, n = 14). Previous studies have shown that different integrins bind the RGD motif (\alpha_\text{v}\beta_1, \alpha_\text{v}\beta_2, \alpha_\text{v}\beta_3, \alpha_\beta_\text{I}, or \alpha_\beta_\text{II}I) and SVVYGLR motif (\alpha_\text{v}\beta_1, \alpha_\text{v}\beta_2, \alpha_\beta_\text{I}I, and \alpha_\beta_\text{II}I). The close proximity of the RGD and SVVYGLR sites suggests that these two sites cannot bind different integrins simultaneously (26). Therefore the observed enhanced Jurkat cell binding in OPN-Arg^{168} may be due to binding of the different OPN molecules to different integrins, resulting in increased overall cell adhesion (Fig. 3). Alternatively it is possible that the RAA mutation or the des-Arg mutation alters the conformation of the other binding site, leading to decreased cell binding affinity. Antibody blocking studies indicate that the anti-\alpha_\text{v} and anti-\beta_\text{I} antibodies blocked the cell binding to OPN-Arg^{168} by ~75% (Fig. 4), while anti-\alpha_\text{v}, anti-\alpha_\text{v}, anti-\alpha_\text{v}, anti-\alpha_\text{v}, and anti-\beta_\text{I} antibody had no effect, consistent with the hypothesis that the binding is partly mediated by \alpha_\text{v}\beta_1 integrin (on Jurkat cells) binding to the exposed SVVYGLR site on cleaved OPN. The data indicate that in thrombin-cleaved OPN, both the RGD site and the exposed SVVYGLR site are functionally active, capable of supporting Jurkat cell adhesion.

**TAFIa Inactivates C5a-mediated Myeloperoxidase Release from Cytochalasin-primed Neutrophils—**Kinetic studies on TAFIa hydrolysis of peptides based on the C termini of C3a and C5a revealed that both anaphylotoxins were potentially good substrates for TAFIa (Table 1). The effect of TAFIa on C5a-induced myeloperoxidase release from cytochalasin-primed neutrophils was investigated using purified human neutrophils in a functional assay. TAFIa inactivation of C5a was dose-dependent and effectively inhibited the ability of C5a to activate neutrophils at a molar ratio of 1:1000 within 30 min (Fig. 5). This suggests that TAFIa could potentially regulate the proinflammatory effects of anaphylatoxins in vivo at sites of inflammation.

**The Anticoagulant Thrombin E229K Activated Mouse Protein C and TAFI in Vivo—**In order to investigate the inactivation of BK by TAFIa in mice, we assessed the ability of the human anticoagulant thrombin E229K to activate mouse TAFI in vivo. Previous studies have shown that E229K thrombin has largely lost all its procoagulant functions but maintains ~50% wild-type activity to activate protein C and TAFI when bound.

![Fig. 1. Effect of TAFIa treatment on thrombin-cleaved GST-OPN-mediated adhesion of Jurkat cells.](image)
to TM (25; data not shown). Intravenous infusion of E229K thrombin produced a dose-dependent reversible anticoagulation in mice as monitored by prolongation of aPTT, an indirect measure of protein C activation (Fig. 6). A 2-fold prolongation of aPTT was achieved with E229K thrombin infused at 20 μg/kg/min for 10 min, without any decrease in plasma fibrinogen level or platelet count (Table II). Higher doses of E229K thrombin caused a progressive shortening of the aPTT. It is possible that with such a large dose of E229K thrombin, even with a small residual fibrinogen clotting activity, disseminated intravascular coagulation (DIC) occurred. In contrast to E229K, wild-type thrombin did not prolong the aPTT and at 20 μg/kg/min, caused severe depletion of platelets and fibrinogen, consistent with widespread DIC (Table II). The E229K thrombin also activated TAFI in mice in vivo, as demonstrated by TAFIa activity in the mouse plasma as well as by Western blot analysis of the mouse serum (Fig. 7). It is notable that there appeared to be >90% conversion of plasma TAFI to TAFIa after 10 min of infusion of E229K at 5 μg/kg/min. At this infusion rate of E229K, the aPTT was only modestly prolonged (~13 s versus ~9 s baseline, Fig. 6), suggesting that E229K thrombin was more efficient in activating TAFI than PC in mice in vivo.

The data indicate that human E229K thrombin is a useful tool for investigating the functional role of TAFIa since it can bind mouse TM and activate mouse TAFI in vivo.

The Effect of E229K Thrombin on Bradykinin-induced Hypotension in Mice in Vivo—To investigate the role of TAFIa inactivation of BK in vivo, we used the E229K thrombin to activate mouse TAFI and determine if TAFIa could modulate BK-induced hypotension. Administration of BK caused a mean decrease of arterial blood pressure of 19.2 ± 2.7 mm Hg (n = 5) in mice while E229K thrombin infusion effectively blocked BK-induced hypotension with a decrease in blood pressure of only 6.4 ± 4.1 mm Hg (n = 6) (67% inhibition, p < 0.001, Fig. 8A). Des-Arg BK did not produce similar acute hypotension in mice (2.7 ± 2.0 mmHg, n = 5), strongly suggesting that the E229K thrombin blockage effect is mediated by generation of TAFIa in the mouse plasma, with its subsequent inactivation of BK. To confirm this, we treated TAFI-deficient mice and their wild-type littermates with E229K thrombin, followed by BK. E229K thrombin reduced BK-induced hypotension in the wild-type littermates, with a decrease in mean arterial blood pressure of 5.8 ± 1.0 mm Hg (n = 4, Fig. 8B), similar to the wild-type mouse response above. In contrast, E229K thrombin

Fig. 2. Jurkat cell adhesion to OPN-Arg<sup>168</sup> and OPN-Leu<sup>AArg</sup>. Jurkat cell binding to GST-OPN fusion proteins mimicking thrombin-cleaved OPN (OPN-Arg<sup>168</sup>) and thrombin-cleaved TAFIa-treated GST-OPN (OPN-Leu<sup>AArg</sup>) along with the full-length GST-OPN (OPN-FL) was performed. Panel A shows cell binding over a range of OPN concentrations, and panel B shows cell binding at 1.0 μg/ml OPN. Results represent the mean ± S.E. (n = 6).
had no effect on the BK-induced hypotension in TAFI-deficient mice where the decrease of mean arterial blood pressure of 13.9 ± 4.8 mmHg (n = 6, p < 0.01, Fig. 8B) was similar to wild-type mice infused with saline followed by treatment with BK. The data indicate that TAFIa is more effective than the endogenous CPN in inactivating BK in this experimental setting.

**Discussion**

A major premise of this study is that TAFIa has broad substrate specificity, and by cleaving C-terminal arginine or lysine from a number of biologically active peptides, it functions as an anti-inflammatory molecule. Initial studies by Campbell and co-workers (4, 12, 13) showed that TAFIa could have a role as a regulator of inflammation by inactivation of kinins and anaphylatoxins. To test this, we studied the kinetics of TAFIa hydrolysis of BK and peptides based on the thrombin-cleaved OPN SVVYGLR motif and the C-terminal residues of C3a, C5a and compared these to peptides based on exposed C-terminal arginine or lysine sites on plasmin-degraded fibrin clots (27). In general, BK, C3a Arg168–Leu168, and the thrombin-cleaved OPN motif SVVYGLR (OPN160–168) were equally good substrates for TAFIa as those based on the fibrin peptides (FBαArg164, FBβLys133, FBγLys65, FBβLys85), which are commonly regarded as the physiological substrates for TAFIa in vivo. It is interesting that plasma CPN, generally thought to be the physiological inactivator of the kinins and anaphylatoxins, hydrolyzed the fibrin peptides quite efficiently, suggesting a possible role of CPN in the maintenance of fibrin clot stability. CPN is more efficient in hydrolyzing C3a than C5a, while TAFIa shows the opposite preference, consistent with previous findings (13). On the other hand, in addition to C5a, TAFIa is also much more efficient than CPN in cleaving BK and OPN160–168. Our data confirm and extend the initial observations by Campbell and co-workers (4, 12, 13). The thrombin-cleaved OPN fragment demonstrated in this study, in addition to kinins such as BK and kallidin, the anaphylatoxins C3a and C5a, can all be hydrolyzed by TAFIa efficiently and are thus potential substrates for TAFIa in vivo.

To further investigate the functional role of TAFIa on OPN, kinins (BK), and anaphylatoxins (C5a), we employed several in vitro and in vivo assays. The role of TAFIa on integrin binding by thrombin-cleaved OPN was assessed using a Jurkat cell adhesion assay. Previous studies have shown that several T-lymphocyte derived cell lines, including Jurkat, HL-60, and

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**FIG. 3.** Jurkat cell binding to OPN-FL, OPN-Arg168, and OPN-Leu168, and the effect of mutating the RGD motif. GST-OPN fusion proteins OPN-Arg168 and OPN-Leu168 had the RGD motif mutated to RAA and purified as described under “Experimental Procedures.” Binding of Jurkat cells to OPN fusion proteins was performed. Results represent the mean ± S.E. from at least two separate experiments performed in triplicate. (OPN-FL versus OPN-Arg168 p = 0.001; OPN-Arg168 versus OPN-Leu168 p = 0.04; and OPN-Arg168 versus OPN-RAA-Arg168 p = 0.01).

**FIG. 4.** Jurkat cell adhesion to OPN-Arg168 is mediated by αβ1. OPN-Arg168 was adsorbed to the wells at 1.0 μg/ml. Jurkat cells were preincubated with 10 μg/ml antibodies for 10 min on ice. Jurkat cell binding was then carried out and results represent mean ± S.E. (n = 6).

**FIG. 5.** Inactivation of C5a by TAFIa. Human C5a (10 nM) was cleaved by 0, 1, 10, 100 nM TAFIa at room temperature as described under “Experimental Procedures.” Aliquots were removed at indicated time points, and TAFIa inhibited with potato carboxypeptidase inhibitor. The samples were then diluted and added to cytochalasin B-primed purified human neutrophils. Myeloperoxidase release was measured (mean ± S.E., n = 6).
Ramos cells that express /H9251 4 /H9252 1 integrin, have increased adhesion to OPN following thrombin cleavage and this is mediated by binding to the exposed integrin-binding motif SVVYGLR (19–22). We confirmed that thrombin cleavage of OPN enhanced Jurkat cell binding to the SVVYGLR motif and this was mediated by 4 1 and was independent of the RGD motif (Figs. 1–4). TAFIa treatment of thrombin-cleaved OPN and recombinant proteins mimicking the des-Arg N-terminal thrombin-cleaved OPN fragment had markedly reduced adhesion indicating the critical importance of the exposed C-terminal arginine on SVVYGLR. Of interest, both the RGD motif and the SVVYGLR motif, which are contiguous and capable of binding to different integrins, appear to be functional in the thrombin-cleaved OPN fragment and both contribute to the enhanced cell binding (Fig. 4) with minimal contribution by the ELVTDFPT-DLPAT binding site (22). It has been previously suggested that due to their close proximity, the RGD and SVVYGLR binding sites on a thrombin-cleaved OPN fragment may not bind to two integrins simultaneously (28). One interpretation of our data is that, while each thrombin-cleaved OPN fragment can bind only one type of integrin via either binding motif, Jurkat cells utilized different integrin molecules to bind to different OPN fragments, resulting in enhanced cell adhesion. Alternatively it is possible that the RAA mutation alters the conformation of

![FIG. 6. Anticoagulant effect of E229K thrombin in vivo](image)

Saline, wild-type, or E229K thrombin solutions were infused through the cannulated jugular veins of anesthetized mice at the indicated infusion rates for 10 min. At the end of each infusion, blood was taken through a syringe inserted into the left ventricle and aPTT determined. Results represent the mean ± S.E. (n = 5 for each data point in E229K, n = 3 for each data point in wild-type thrombin).

**TABLE II**

| Treatment          | n | Fibrinogen | Platelet count |
|--------------------|---|------------|----------------|
| Saline control     | 3 | 350        | 965            |
| E229K (20 μg/kg/min) | 3 | 307        | 1208           |
| Wild type (20 μg/kg/min) | 2 | 78         | 139            |

![FIG. 7. Activation of TAFI in vivo by E229K thrombin](image)

Mice were infused with E229K thrombin, wild-type thrombin, or saline control at 20 μg/kg/min for 10 min. Fibrinogen level and platelet counts were determined.

![FIG. 8. The effect of E229K on bradykinin-induced hypotension in vivo](image)

Each mouse was anesthetized and the carotid artery was cannulated with a pressure transduction catheter connected to a computerized pressure monitor to record blood pressure. Either E229K thrombin (40 μg/kg) or a saline control was administered via the jugular vein. Saline was injected to flush out the catheter then BK or des-Arg BK (10 nmol/kg) was administered. Blood pressure tracings were monitored and recorded. Panel A shows the results for maximum drops in mean arterial pressure after administration of BK or des-Arg BK (BK (n = 5) versus E229K followed by BK (n = 6), p < 0.001; BK (n = 5) versus des-Arg BK (n = 4), p < 0.0001). Panel B shows the drop in mean arterial pressure for wild-type mice (littermates for knockout mice) treated with E229K followed by BK (n = 4) versus TAFI knockout mice treated with E229K followed by BK (n = 6) p < 0.01.
either or both the SVVYGLR and ELVTDFPPTDLPAT binding sites thus reducing its cell binding affinity. Functional deletion of both sites resulted in minimal Jurkat cell binding, indicating that the third integrin binding site ELVTDFPPTDLPAT does not play a significant functional role, at least in this in vitro assay system (22).

\( \alpha_4 \) and \( \alpha_9 \) are sole members of a subfamily of integrin \( \alpha \) subunits (29). The \( \alpha_4\beta_1 \) integrin (VLA-4) is predominantly expressed on leukocytes, especially lymphocytes, monocytes and eosinophils (30). On the other hand, \( \alpha_6\beta_1 \) is predominantly expressed on neutrophils and is also expressed on epithelial cells, smooth muscle cells and skeletal muscle. Both \( \alpha_4\beta_1 \) and \( \alpha_6\beta_1 \) can bind to the SVVYGLR site on thrombin-cleaved OPN (31). TAFIa cleavage of the C-terminal arginine largely abolished \( \alpha_4\beta_1 \)-mediated Jurkat cell binding (Figs. 1, 3, and 4), while its effect on the OPN fragment binding to \( \alpha_6\beta_1 \)-bearing cells has not been tested. Since both thrombin and OPN are present at high concentrations at sites of tissue injury and tissue repair, thrombin cleavage of OPN with the subsequent exposure of the SVVYGLR site for \( \alpha_4\beta_1 \) and \( \alpha_6\beta_1 \) binding is likely of physiological significance. Thus thrombin cleavage of OPN followed by TAFIa treatment may sequentially up- and down-modulate the pro-inflammatory properties of OPN.

An important part of the inflammatory response is the generation of BK that leads to an increase in vascular permeability and vasodilatation (32). CPN is generally regarded to be the physiological inactivator of BK (33). However cleavage studies showed that TAFIa is in fact 10-fold more efficient than CPN in hydrolyzing BK in vitro (Table I). To investigate the role of TAFIa inactivating BK in vivo, we used a mouse model where we could activate endogenous TAFI by infusing the mice with the anticoagulant thrombin E229K (25). Infusion with E229K thrombin rapidly ablated the BK-induced hypotension, presumably by activating TAFI leading to the conversion of BK to its des-Arg form (Fig. 8). This is supported by our findings that E229K thrombin infusion effectively converted TAFI to TAFIa in mice (Fig. 7) and that des-Arg BK did not produce a significant hypotensive effect (Fig. 8). Thus thrombin E229K demonstrated using a variety of acute or subacute clot lysis models (34). On the other hand, enhanced pulmonary clot lysis was found in TAFI-deficient mice (35). It is interesting that in these compound deficient mice, increased number of leukocytes was demonstrated in thioglycollate-induced peritoneal inflammation. While the increased leukocyte influx could be due to enhanced cell migration secondary to unimpeded cell surface fibrinolysis, it is also possible that it is the result of increased inflammation because of TAFI deficiency. These two possibilities are not mutually exclusive. Since E229K thrombin can effectively activate TAFI in mice, it becomes a powerful tool to study the role of TAFIa in vivo by comparing its effect in wild-type and TAFI-deficient mice.

Taken together, our data suggest that TAFIa has broad substrate specificity and its function may not be restricted to inhibition of fibrinolysis. At sites of inflammation, thrombin not only has proinflammatory effects but also can act as an indirect anti-inflammatory molecule by binding TM and activating PC and TAFI. TAFIa then inactivates kinins, anaphylatoxins and thrombin-cleaved OPN. Hence, thrombin plays a key role in regulating coagulation and inflammation through the intricate interplay between thrombin activation of proinflammatory molecules and thrombin/TM-dependent activation of anticoagulant and anti-inflammatory effectors.

Acknowledgment—We thank Dr. Dean Sheppard (UCSF) for useful discussions during the course of this work.

REFERENCES

1. Esmon, C. T. (2002) J. Exp. Med. 196, 561–564
2. Bajzar, L., Manuel, R., and Nesheim, M. E. (1995) J. Biol. Chem. 270, 11477–1484
3. Bajzar, L. (2000) Arterioscler. Thromb. Vasc. Biol. 20, 2511–2518
4. Campbell, W., and Okada, H. (1989) Biochem. Biophys. Res. Commun. 162, 933–939
5. Hendriks, D., Scharpé, S., van Sande, M., and Lammerda, M. P. (1989) J. Clin. Chem. Clin. Biochem. 27, 275–285
6. Eaton, D. L., Malloy, B. E., Tsai, S. P., Henzel, W., and Dratna, D. (1991) J. Biol. Chem. 266, 21833–21838
7. Beditz, A., Tan, A., Eton, D. L., and Plow, E. F. (1995) J. Clin. Investig. 96, 2534–2538
8. Coughlin, S. R. (2000) Nature 407, 258–264
9. Joyce, R. E., Gelbert, L., Guevara, A., DeHoff, B., and Grinnell, B. W. (2001) J. Biol. Chem. 276, 11999–12003
10. Biewald, M., Petrovian, R. J., Donner, A., Mueller, B. M., and Ruf, W. (2002) Science 296, 1880–1882
11. Bernard, G. R., Vincent, J. L., Laterre, P. F., LaRosa, S. P., Dhainaut, J. F., Lopez-Rodriguez, A., Steingrub, J. S., Garber, G. E., Helterbrand, J. D., Ely, E. W., and Fisher, C. J. Jr. (2001) N. Engl. J. Med. 344, 699–709
12. Shinohara, T., Sakurada, C., Suzuki, T., Takeuchi, O., Ikeda, E., and Fisher, C. J. Jr. (2001) Nature 411, 699–709
13. Campbell, W. D, Lazcura, E., Okada, N., and Okada, H. (2002) Microbiol. Immunol. 46, 131–134
14. Senger, D. R., Perruzzi, C. A., Papadopoulos, A., and Tenen, D. G. (1989)
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Biochim. Biophys. Acta 996, 43–48
15. Senger, D. R., Perruzzi, C. A., Papadopoulos-Sergiou, A., Van de Water, L. (1994) Mol. Biol. Cell 5, 565–574
16. Xuan, J. W., Hota, C and Chambers, A. F. (1994) J. Cell. Biochem. 54, 247–255
17. Denhardt, D. T., Noda, M., O’Regan, A. W., Pavlin, D., and Berman, J. S. (2001) J. Clin. Investig. 107, 1055–1061
18. Senger, D. R., Perruzzi, C. A., Gracey, C. F., Papadopoulos, A., and Tenen, D. G. (1988) Cancer Res. 48, 5770–5774
19. Bayless, K. J., Meininger, G. A., Scholtz, J. M., and Davis, G. E. (1998) J. Cell Sci. 111, 1163–1174
20. Barry, S. T., Ludbrook, S. B., Morrison, E., and Horgan, C. M. T. (2000) Exp. Cell Res. 258, 342–351
21. Green, P. M., Ludbrook, S. B., Miller, D. D., Horgan, C. M. T., and Barry, S. T. (2001) FEBS Lett. 503, 75–79
22. Bayless, K. J., and Davis, G. E. (2001) J. Biol. Chem. 276, 13483–13489
23. Smith, L. L., Cheung, H-K., Ling, L. E., Chen, J., Sheppard, D., Pytela, R., and Giachelli, C. M. (1996) J. Biol. Chem. 271, 28485–28491
24. Smith, L. L., and Giachelli, C. M. (1998) Exp. Cell Res. 242, 351–360
25. Tsiang, M., Paborsky, L. R., Li, W. X., Jain, A. K., Mao, C. T., Dunn, K. E., Lee, D. W., Matsunura, S. Y., Matteucci, M. D., Coutre, S. E., Leung, L. L., and Gibbs, C. S. (1996) Biochemistry 35, 16449–16457
26. Henson, P. M., Zanolari, B., Schwartzman, N. A., and Hong, S. R. (1978) J. Immunol. 121, 851–855
27. Collen, D., Kudryk, B. J., Hessel, B., and Blomback, B. (1975) J. Biol. Chem. 250, 5808–5817
28. Yokosaki, Y., and Sheppard, D. (2000) Trends Cardio. Med. 10, 155–159
29. Palmer, E. L., Ruegg, C., Ferrando, R., Pytela, R., Sheppard D. (1993) J. Cell Biol. 123, 1289–1297
30. Taoka, Y., Chen, J., Yednock, T., and Sheppard, D. (1999) J. Cell Biol. 1145, 413–420
31. Yokosaki, Y., Matsunura, N., Sasaki, T., Murakami, I., Schneider, H., Higashiyama, S., Saitoh, Y., Yamakido, M., Taoka, Y., and Sheppard, D. (1999) J. Biol. Chem. 274, 36328–36334
32. Collen, B. W. (1999) Thromb. Haemost. 82, 1568–1577
33. Huey, R., Bloor, C. M., Kawahara, M. S., and Hugli, T. E. (1983) Am. J. Pathol. 112, 48–60
34. Nagashima, M., Yin, Z. F., Zhao, L., White, K., Zhu, Y., Lasky, N., Halks-Miller, M., Broze, G. J., Jr., Fay, W. P., Morser, J. (2002) J. Clin. Investig. 109, 101–110
35. Kochinsky, M. L., Beffa, M. B., Nesheim, M. E., Zinman, B., Hasley, A. J. G., Harris, S. B., Cao, H., and Hegele, R. A. (2001) Clin. Genet. 60, 345–349
36. Tsama, B. J, Sarma, V., Pierson, C. L., Warner, R. L., Huber-Lang, M., Bless, N. M., Schmal, H., Friedl, H. P., and Ward, PA. (1999) Nat. Med. 5, 788–792
37. Swaingood, C. M., Schmitt, D., Eaton, D., and Plow, E. F. (2002) J. Clin. Investig. 110, 1275–1282