Evidence That Both Exosites on Thrombin Participate in Its High Affinity Interaction with Fibrin*

Exosite 1 on thrombomediates low affinity binding to sites on the NH₂ terminus of the α- and β-chains of fibrin. A subpopulation of fibrin molecules (γA/γγ-fibrin) has an alternate COOH terminus of the normal γ-chain (γA/γA-fibrin) that binds thrombin with high affinity. To determine the roles of exosites 1 and 2 in the high affinity interaction of thrombin with γA/γγ-fibrin, binding studies were done with thrombin variants and exosite 1- or 2-directed ligands. α-Thrombin bound γA/γγ-fibrin via high and low affinity binding sites. A peptide analog of the COOH terminus of the γ-chain that binds α-thrombin via exosite 2 blocked the high affinity binding of α-thrombin to γA/γγ-fibrin, suggesting that the interaction of α-thrombin with the γ-chain is exosite 2-mediated. In support of this concept, (a) γ-thrombin, which lacks an functional exosite 1, bound to γA/γγ-fibrin, but not to γA/γA-fibrin; (b) thrombin R98A/R97A/R101A, an exosite 2-defective variant, bound only to γA/γγ-fibrin via low affinity sites; and (c) exosite 2-directed ligands reduced α-thrombin binding to γA/γγ-fibrin. However, several lines of evidence indicate that exosite 1 contributes to the high affinity interaction of thrombin with γA/γγ-fibrin. First, the affinity of γ-thrombin for γA/γγ-fibrin was lower than that of α-thrombin. Second, removal of a low affinity binding site on the β-chain of γA/γγ-fibrin reduced its affinity for α-thrombin. Third, exosite 1-directed ligands reduced α-thrombin binding to γA/γγ-fibrin. Taken together, these data suggest that, although exosite 2 mediates the interaction of thrombin with the γ-chain of γA/γγ-fibrin, simultaneous ligation of exosite 1 by low affinity binding sites is essential for the high affinity interaction of thrombin with γA/γγ-fibrin.

Thrombin is a serine protease that plays an essential role in hemostasis, effecting procoagulant, anticoagulant, and profibrinolytic responses. As a procoagulant, thrombin activates platelets, converts fibrinogen to fibrin, and promotes its own generation by activating factors V, VIII, and XI. After binding to thrombomodulin, its cellular receptor, thrombin triggers anticoagulant and antifibrinolytic pathways by activating protein C and thrombin-activable fibrinolysis inhibitor, respectively (1, 2).

The activity of thrombin is primarily regulated through inhibition by antithrombin in a reaction that is enhanced by heparin (3). Although the heparin-antithrombin complex readily inhibits fluid-phase thrombin, thrombin that remains bound to fibrin after clotting is protected from inactivation by the heparin-antithrombin complex. This protection reflects, at least in part, heparin-mediated bridging of thrombin onto fibrin to form a ternary thrombin-heparin-fibrin complex (4, 5).

Fibrin-bound thrombin retains its ability to cleave fibrinogen (4, 6), a phenomenon that provides a plausible explanation for the in vitro observation that thrombi induce activation of platelets and trigger coagulation (7). The concept that fibrin-bound thrombin contributes to the procoagulant activity of thrombi is supported by the recent demonstration that human thrombin obtained from pathological specimens harbor active thrombin (8). Sequestered thrombin may be generated by activated factor X (factor Xa) bound to platelets within the thrombus. Like fibrin-bound thrombin, factor Xa within the platelet-bound prothrombinase complex also is protected from inactivation by the heparin-antithrombin complex (9, 10). Consequently, thrombus-associated factor Xa may further contribute to the procoagulant activity of thrombi by triggering local thrombin generation (11).

Thrombin binds to fibrin via a domain distinct from the active site of the enzyme. Thrombin possesses two electropositive domains (termed exosites) that bracket the catalytic site. Exosite 1 serves as the initial docking site that orients substrates and inhibitors within the active site clef (12). The second electropositive domain (exosite 2) binds heparin, other glycosaminoglycans, and prothrombin fragment 2 (13). In addition to acting as binding sites, the two exosites on thrombin may also modulate thrombin enzymatic activity. Thus, ligand binding to either exosite alters the active site environment (14), effecting conformational changes that alter thrombin substrate specificity (2, 6). Moreover, there is evidence for allosteric linkage between the two exosites because ligand binding to one exosite can influence the binding properties of the other (15).

Thrombin utilizes exosite 1 to dock fibrinogen as it is converted to fibrin. Fibrinogen is composed of duplicated αA-, ββ-, and γ-chains held together by disulfide bonds. Two chromatographically distinct forms of fibrinogen, distinguished by the structure of their γ-chains, can be isolated from human plasma (16). Most fibrinogen molecules contain two γA-chains, each composed of 411 amino acids, and are thus designated γA/γA. About 10% of circulating fibrinogen molecules contain a variant of the native γ-chain (termed γ') composed of 427 amino acids. This longer γ'-chain results from alternative mRNA polyadenylation and has an acidic hirudin-like 20-amino acid sequence at its COOH terminus (17, 18).

Fibrinogen het-
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Conversion of $\gamma$- or $\gamma'$-fibrinogen to fibrin requires sequential thrombin-mediated release of fibrinopeptides A and B from the NH$_2$ termini of the A- and B-chains of fibrinogen, respectively. Current thinking is that once thrombin-mediated fibrinopeptide release is effected, some of the thrombin remains bound to the resultant fibrin via exosite 1 (16). Functionally, $\gamma$- and $\gamma'$-fibrin are distinguished by their thrombin-binding properties (16). Binding of thrombin to $\gamma$- or $\gamma'$-fibrin occurs through a single class of low affinity binding sites that have been localized to the NH$_2$ termini of the $\alpha$- and $\beta$-chains, proximal to the fibrinopeptide cleavage sites (19). Thrombin binds to these low affinity sites with $K_v$ values that range from 1 to 3.4 $\mu M$ (20). In addition to low affinity thrombin binding, $\gamma$- or $\gamma'$-fibrin also displays high affinity thrombin binding that has been attributed to the COOH terminus of the $\gamma'$-chain. The $K_v$ value for this interaction is 0.18 $\mu M$ (16).

To further explore the high affinity interaction of thrombin with $\gamma$- and $\gamma'$-fibrin, we measured the affinity of thrombin for a synthetic 20-amino acid peptide analog of the COOH terminus of the $\gamma'$-chain. Using thrombin variants or DNA aptamers directed against the exosites, we demonstrated that thrombin binding to this $\gamma'$-peptide is mediated by exosite 2. Building on this observation, we then explored the role of exosites 1 and 2 in the interaction of thrombin with $\gamma$- and $\gamma'$-fibrin. These studies suggest that both exosites on thrombin contribute to the high affinity interaction of thrombin with $\gamma$- and $\gamma'$-fibrin. In contrast, the low affinity interaction of thrombin with $\gamma$- or $\gamma'$-fibrin is mediated solely by exosite 1.

EXPERIMENTAL PROCEDURES

Materials

Reagents—Human $\alpha$-thrombin ($\alpha$-IIa), $\gamma$-thrombin ($\gamma$-IIa), and fibrinogen were obtained from Enzyme Research Laboratories Inc. (South Bend, IN). Thrombin R93A/R97A/R101A (RA-IIa), and fibrinogen variants or DNA aptamers directed against the exosites, were obtained from Dr. C. T. Esmon. A 20-amino acid analog of the COOH-terminal portion of the $\gamma'$-chain of fibrinogen (22), $\gamma$-peptide (H-Val-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro) was synthesized by Bachem AG (San Diego, CA), and its integrity was confirmed by quantitative amino acid analysis and reverse-phase HPLC. Although the two Tyr residues in the COOH-terminal domain of the $\gamma'$-chain of native fibrinogen are sulfated, the corresponding residues in the synthetic peptide were phosphorylated. The efficacy of this substitution has been verified with hirudin peptides and the $\gamma$-peptide (23, 24). HD22 (5'-AGTCCGTGGTAGGGCAGGTTGGGGTGACT-3') (25) and HD1 (5'-GGTTGCTGTGGTGGT-3') (26), DNA aptamers that bind exosites 2 and 1 on thrombin, respectively, were synthesized by the Molecular Biology and Biotechnology Institute (McMaster University). The exosite 1-binding hirudin fragment (hirudin 54–65), H-Gly-Asp-Phe-Glu-Glu-Ile-Glu-Glu-Tyr-Glu-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro-Ala-Thr-Glu-Tyr-PO$_2$H$_2$-Asp-Ser-Leu-Arg-Pro-Glu-His-Pro), was phosphorylated with PKA (27). The thrombin-directed substrate tosyl-Gly-Pro-Arg-p-nitroanilide (tGPRA-pNA), fluorescein isothiocyanate, and Crotalus atrox venom were obtained from Sigma. The protease III fraction of C. atrox venom was isolated as previously described (28). p-Phenylacetyl-glutamyl ketone (PFPck) was obtained from Calbiochem. Fluorescein-PFPRck and activated factor XIII (29) that binds to exosite 1 on thrombin was synthesized by Chiron Mimotopes Peptide Systems (San Diego). The integrity of this peptide was confirmed by quantitative amino acid analysis and reverse-phase HPLC. A rabbit antibody raised against the $\gamma$-peptide (30) was a kind gift from Dr. D. H. Farrell (University of Oregon). Human prothrombin fragment 2 (h-F2) was isolated as described (15) with minor modifications. A thiosemicarbazide and 4-chloro-7-nitrobenz-2-oxa-1,3-diazole derivative of this fragment (30) was used in the binding studies. The $\alpha$-, $\beta$-, and $\gamma$-chains are identified on the right.

Isolation of $\gamma$- and $\gamma'$-fibrinogen—Human fibrinogen was fractionated on a DEAE-Sepharose column (1.5 x 11.3 cm) (31). After diluting fibrinogen with water and 39 mM Tris and 5 mM phosphate buffer (pH 8.3) (buffer A) at a 1:1 ratio, 180 mg of fibrinogen was applied to the column at a flow rate of 5 ml/min. A concave pH gradient from buffer A to 500 mM Tris and 500 mM phosphate buffer (pH 4.0) was used to separate $\gamma$- and $\gamma'$-fibrinogen (31). 10-ml fractions were collected, and their absorbance at 280 nm was determined using a Beckman 685 spectrophotometer by measuring absorbance at 280 and using $\epsilon_{280}^{1}%$ values that range from 250 to 500 $\mu M$ NaCl.

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Characterization of fibrinogen—Native $\gamma$- and $\gamma'$-fibrinogen (lanes 1 and 2, respectively) and des-BrB-42-$\gamma$- and des-BrB-42-$\gamma'$-fibrinogen (lanes 3 and 4, respectively) were subjected to SDS-PAGE under reducing conditions (A) and subsequent immunoblots (B) using antibodies against fibrinogen (lanes 1 and 2) and des-BrB peptides (lanes 3 and 4). The $\alpha$-, $\beta$-, $\gamma$-, and $\gamma'$-chains are identified on the right. The molecular weights of the mobility markers are indicated on the left.
Preparation of des-Bβ1-42-γA/γC- and des-Bβ1-42-γA/γFibrinogen—γC/γA (f-γ-fibrinogen) (8.4 μM; in TBS containing 12.5 mM EDTA) was incubated with 2.3 μM protease III fraction from C. atrox venom for 90 min at 23 °C. Reactions were terminated by addition of FPRc to 10 μM. After 10 min, 20% (v/v) ethanol was added, and the solution was maintained on ice for 30 min prior to centrifugation at 14,000 × g for 4 min. Pellets were resuspended in 1 mL of TBS and dialyzed against TBS. Protein concentrations were determined as described above, and the material was stored in aliquots at −70 °C. The integrity of des-Bβ1-42-γA/γC- or des-Bβ1-42-γA/γC-fibrinogen was assessed by SDS-PAGE analysis. Immunoblot analysis using an antibody against the γ-chain was used to ensure that treatment with C. atrox venom had no effect on the COOH-terminal region of the γ-chain of γC-fibrinogen. Open SDS-PAGE, the β-chain lacking β1-42 migrated with the γ-chain (Fig. 1A, lanes 3 and 4). Immunoblot analysis demonstrated that the γ-chain of des-Bβ1-42-γA/γC-fibrinogen was still recognized by the antibody against the γ-chain (Fig. 1B, lane 4). NH₂-terminal sequence analysis of the β-chain of des-Bβ1-42-γA/γC- or des-Bβ1-42-γA/γC-fibrinogen confirmed that the β-chains of both species were cleaved at the β45-45 bond. In contrast, the sequences of the NH₂ termini of the α- and γ-chains corresponded to those of native fibrinogen (data not shown).

Labeling of Proteins—Fluorescent derivatives of α-IIa, γ-IIa, and RA-IIa (f-FPR-α-IIa, f-FPR-γ-IIa, and f-FPR-RA-IIa, respectively) were prepared by incubation with a 5-fold molar excess of fluorescein-FPRc before and after addition of f-FPRc was assessed by SDS-PAGE analysis. Immunoblot analysis using an antibody against the γ-chain was used to ensure that treatment with C. atrox venom had no effect on the COOH-terminal region of the γ-chain of γC-fibrinogen. Open SDS-PAGE, the β-chain lacking β1-42 migrated with the γ-chain (Fig. 1A, lanes 3 and 4). Immunoblot analysis demonstrated that the γ-chain of des-Bβ1-42-γA/γC-fibrinogen was still recognized by the antibody against the γ-chain (Fig. 1B, lane 4). NH₂-terminal sequence analysis of the β-chain of des-Bβ1-42-γA/γC- or des-Bβ1-42-γA/γC-fibrinogen confirmed that the β-chains of both species were cleaved at the β45-45 bond. In contrast, the sequences of the NH₂ termini of the α- and γ-chains corresponded to those of native fibrinogen (data not shown).

Methods

γ-Peptide Binding to f-FPR-IIa—f-FPR-α-IIa, f-FPR-γ-IIa, or f-FPR-RA-IIa (at a concentration of 100 nM in 1 mL of TBS) was added to a semi-micro quartz cuvette, and samples were stirred with a mini-stir plate. The 492 nm and 535 nm, respectively, with a 515-nm cutoff filter in the emission beam and excitation and emission slit widths of 10 nm. Aliquots of supernatant containing 1 M CaCl₂ and 2 M FPRc were added to a series of microcentrifuge tubes containing 2 M CaCl₂ in the absence or presence of 2 M γC/γC-fibrinogen. Clotting was initiated by addition of atroxin to 5% (v/v), and after a 60-min incubation at 23 °C, fibrin was pelleted by centrifugation at 14,000 × g for 4 min, and two 10-μl aliquots of supernatant were removed. The chromogenic activity of thrombin in each of these samples was assessed by their addition to the wells of a 96-well plate prefilled with 200 μl of 200 μM tGPRc.

Results

Interaction of α-IIa with the γ-Peptide—To explore the interaction of α-IIa with the γ-peptide, the fluorescence of 100 nM f-FPR-α-IIa was monitored as the sample was titrated with the γ-peptide. Addition of the γ-peptide elicited a saturable increase in the fluorescence intensity of f-FPR-α-IIa (Fig. 2), consistent with binding, and nonlinear regression analysis of the data revealed a Kᵣ value of 2.2 μM. This compares with the Kᵣ value of 0.68 μM reported for interaction of the fluorescently labeled γ-peptide with α-IIa (24). To identify the exosite mediating the interaction of f-FPR-α-IIa with the γ-peptide, the experiment was repeated using f-FPR-γ-IIa, a thrombin derivative lacking exosite 1, and f-FPR-RA-IIa, a thrombin variant with an impaired exosite 2. The γ-peptide bound to f-FPR-γ-IIa aliquots of HD1 or HD22 to a final concentration of 5.6 or 4.4 μM, respectively. Data were analyzed as described above.

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Effect of Exosite 1- or 2-directed Ligands on Thrombin Binding to Fibrin—Concentrations of hirudin-(54–65) (50 μM), hirudin cofactor II-(54–75) (100 μM), HD1 (1 μM), h-F2 (64 μM), γC/γC-fibrinogen (236 μM), and HD22 (0.25 μM), determined to be saturating in separate experiments were added to a series of microcentrifuge tubes containing 1 M γC/γC-fibrinogen or 0.35 μM γC/γC-fibrinogen, 2 M CaCl₂, and 25 nM f-FPR-α-IIa. Clotting was initiated by addition of atroxin to 5% (v/v), and after a 60-min incubation at 23 °C, fibrin was pelleted by centrifugation at 14,000 × g for 2 min. Duplicate 10-μl aliquots of supernatant were then removed and counted for radioactivity. The amount of 125I-FPR-α-IIa bound to fibrin in the absence or presence of competitor was calculated by subtraction. Data were displayed by plotting the concentration of 125I-FPR-α-IIa bound to fibrin in the presence of each concentration of competitor as a percentage of that bound in the absence of competitor.

Statistical Methods—Unless otherwise indicated, experiments were performed at least three times in duplicate. Results are presented as the mean ± S.E. Student’s t test was used to evaluate significance.

RESULTS

Interaction of α-IIa with the γ-Peptide—To explore the interaction of α-IIa with the γ-peptide, the fluorescence of 100 nM f-FPR-α-IIa was monitored as the sample was titrated with the γ-peptide. Addition of the γ-peptide elicited a saturable increase in the fluorescence intensity of f-FPR-α-IIa (Fig. 2), consistent with binding, and nonlinear regression analysis of the data revealed a Kᵣ value of 2.2 μM. This compares with the Kᵣ value of 0.68 μM reported for interaction of the fluorescently labeled γ-peptide with α-IIa (24). To identify the exosite mediating the interaction of f-FPR-α-IIa with the γ-peptide, the experiment was repeated using f-FPR-γ-IIa, a thrombin derivative lacking exosite 1, and f-FPR-RA-IIa, a thrombin variant with an impaired exosite 2. The γ-peptide bound to f-FPR-γ-IIa
with a $K_d$ of 1.0 μM (Fig. 2), an affinity similar to that for f-FPR-α-IIa. In contrast, the γ’-peptide did not bind to f-FPR-RA-IIa. These data suggest that exosite 2 mediates the interaction of α-IIa with the γ’-peptide, in agreement with a recent report (24).

To further explore this concept, we performed reverse titrations to examine the ability of exosite 1- or 2-directed DNA aptamers to displace the f-γ’-peptide from α-IIa. The fluorescence intensity of 50 nM f-γ’-peptide decreased by 6% after addition of α-IIa (Fig. 3A). Titration with HD22, an aptamer directed against exosite 2, restored the fluorescence intensity to initial background levels, indicating displacement of the f-γ’-peptide from α-IIa. In contrast, HD1, an exosite 1-directed aptamer, had no effect (Fig. 3B). These data support the concept that the interaction of α-IIa with the γ’-peptide is mediated by exosite 2.

Binding of $^{125}$I-α-IIa to γ′/γα- and γα/γ′-Fibrin—The interactions of $^{125}$I-FPR-α-IIa with clots formed from γ′/γα- and γα/γ′-fibrinogen were compared to examine the influence of the γ′-chain on binding. Gel electrophoretic analysis verified that the γ′/γα- and γα/γ′-fibrinogen preparations contained one and two γ-chains, respectively (Fig. 1). $^{125}$I-FPR-α-IIa bound to both forms of fibrin in a concentration-dependent and saturable fashion. Scatchard analysis of the data for the interaction of $^{125}$I-FPR-α-IIa with γα/γ′-fibrinogen (Fig. 4A) reveals a straight line, consistent with a single class of binding sites. The $K_d$ of 2.3 ± 0.32 μM, determined by nonlinear regression analysis of the direct plot, is in good agreement with previously reported results (20). Binding to γ′/γα-fibrin was also saturable, but the direct plot revealed a steeper increase in binding at lower $^{125}$I-FPR-α-IIa concentrations, indicating a higher affinity interaction. The Scatchard plot for the interaction of $^{125}$I-FPR-α-IIa with γ′/γα-fibrin is nonlinear (Fig. 4B), indicating heterogeneous binding sites or negative cooperativity. A double-reciprocal plot of 1/bound versus 1/free yields a straight line, whereas a plot of bound/free versus bound yields a sigmoidal curve (data not shown). These findings indicate the possibility of heterogeneous binding sites by negating cooperative binding. Accordingly, the data were fit to a two-site model (Equation 3) by nonlinear regression analysis, yielding $K_d$ values of 107 ± 36 nM and 1.5 ± 0.24 μM for the high and low affinity binding sites, respectively. These results are consistent with the $K_d$ values of 180 nM and 3.4 μM reported previously (16).

To confirm the role of the γ′-chain in the high affinity interaction of α-IIa with fibrin, we examined the influence of the γ’-peptide on $^{125}$I-FPR-α-IIa binding to γ′/γα-fibrin. 2 μM γ′/γα-fibrinogen was added to the presence of $^{125}$I-FPR-α-IIa (0–8 μM) in the absence and presence of 50 μM γ’-peptide (Fig. 5). The direct plot for the data in the presence of the γ’-peptide closely resembles that for $^{125}$I-α-IIa binding to γ′/γα-fibrin. Scatchard analysis of the binding data in the presence of the γ’-peptide yields a straight line, consistent with a single class of binding sites with a $K_d$ of 2.8 ± 0.12 μM, a value compatible with the data for the low affinity interaction of α-IIa with γ′/γα-fibrin. These findings suggest that the γ’-peptide eliminates the high affinity interaction of α-IIa with γ′/γα-fibrin by competing with its γ′-chain for access to exosite 2 on α-IIa. The data also support the concept that the γ’-peptide serves as a good surrogate for the γ′-chain of intact fibrin. The γ’-peptide had no effect on the $K_d$ of $^{125}$I-FPR-α-IIa binding to γ′/γα-fibrin (data not shown).

Binding of α-IIa, γ-IIa, or RA-IIa to γ′/γα- and γα/γ′-Fibrin—Thrombin and thrombin variants were used to further explore the contribution of exosites 1 and 2 to the interaction of thrombin...
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Fig. 4. Binding of 125I-FPR-α-IIa to γ_{A}/γ_{A}-fibrin. 125I-FPR-α-IIa (0–8 μM) was added to microcentrifuge tubes containing 2 μM γ_{A}/γ_{A}-fibrinogen (A) or γ_{A}/γ_{A}-fibrinogen (B) and 2 mM CaCl2, and clotting was initiated by addition of 10 nM thrombin. A/B-fibrinogen (A) or A/B-fibrinogen (B) and 2 mM CaCl2, and clotting was initiated by addition of 10 nM thrombin. The concentration of 125I-FPR-α-IIa that bound to fibrin was calculated by comparing the radioactivity of clot supernatants with that in control titrations lacking fibrinogen and active thrombin. Free (horizontal axis) refers to the concentration of unbound thrombin, whereas Bound refers to the moles of thrombin bound per mol of input fibrinogen. The solid lines represent nonlinear regression analysis of the data. The insets are Scatchard plots of the binding of thrombin to γ_{A}/γ_{A}- or γ_{A}/γ_{A}-fibrin. The data shown in the inset to A indicate that labeled thrombin bound to γ_{A}/γ_{A}-fibrin via a single class of binding sites with a Kd of 2.3 ± 0.32 μM. In contrast, the data depicted in the inset to B best fit a two-site model with high and low affinity sites that bind thrombin with Kd values of 107 ± 36 nM and 1.5 ± 0.24 μM, respectively (mean ± S.E. of three experiments).

Fig. 5. Effect of the γ-peptide on 125I-FPR-α-IIa binding to γ_{A}/γ_{A}-fibrin. 125I-FPR-α-IIa (0–8 μM) was added to microcentrifuge tubes containing 2 μM γ_{A}/γ_{A}-fibrinogen and 2 mM CaCl2 in the absence (●) or presence (◆) of 50 μM γ-peptide. Clotting was initiated by addition of 10 nM thrombin, and the concentration of 125I-FPR-α-IIa that bound to the resultant fibrin was calculated as described in the legend to Fig. 4. For comparison purposes, the binding of 125I-FPR-α-IIa to γ_{B}/γ_{A}-fibrin is also illustrated (◆). The Scatchard plot (inset) illustrates that, in the presence of the γ-peptide, 125I-FPR-α-IIa bound to γ_{A}/γ_{A}-fibrin through a single class of binding sites with a Kd value of 2.8 ± 0.12 μM, a value similar to that for the binding of 125I-FPR-α-IIa to γ_{A}/γ_{A}-fibrin (Kd value of 2.3 ± 0.65 μM). Results are the mean ± S.E. of three experiments, each done in duplicate.

 binding with γ_{A}/γ_{A} or γ_{A}/γ_{A} fibrin. These experiments were performed with unlabeled active thrombin species where unbound thrombin was detected by measuring chromogenic activity in the absence of γ_{A}/γ_{A}-fibrin. Active α-IIa bound to γ_{A}/γ_{A}-fibrin with a Kd value of 2.25 ± 0.05 μM comparable to that of 125I-FPR-α-IIa (Table I). RA-IIa, whose exosite 1 is intact, bound to γ_{A}/γ_{A}-fibrin with a Kd value of 3.11 ± 0.02 μM, an affinity similar to that of α-IIa. In contrast, γ-IIa, a variant with a cleaved exosite 1, did not bind to γ_{A}/γ_{A}-fibrin. These findings are consistent with the concept that the low affinity interaction of α-IIa with γ_{A}/γ_{A}-fibrin is mediated solely by exosite 1.

Experiments were then repeated using γ_{A}/γ_{A}-fibrin in place of γ_{A}/γ_{A}-fibrin. α-IIa bound to γ_{A}/γ_{A}-fibrin with a Kd of 80 ± 30 nM, reflecting the high affinity interaction with the γ-chain. Because the affinity was measured by titrating α-IIa with fibrinogen, two-site binding of α-IIa to γ_{A}/γ_{A}-fibrin could not be discriminated. RA-IIa bound to γ_{A}/γ_{A}-fibrin with a Kd of 2.02 ± 0.03 μM, an affinity similar to that of α-IIa for γ_{A}/γ_{A}-fibrin. These findings suggest that without a functional exosite 2, RA-IIa cannot bind to the γ-chain and can interact only with low affinity binding sites on γ_{A}/γ_{A}-fibrin.

To further investigate the importance of exosite 2 in the interaction of thrombin with γ_{A}/γ_{A}-fibrin, the binding of γ-IIa to γ_{A}/γ_{A}-fibrin was examined. γ-IIa bound to γ_{A}/γ_{A}-fibrin with a Kd of 5.54 ± 0.02 μM. Because γ-IIa lacks a functional exosite 1, its interaction with γ_{A}/γ_{A}-fibrin must be mediated by exosite 2. Furthermore, γ-IIa must bind to the γ-chain of γ_{A}/γ_{A}-fibrin because γ-IIa does not bind to γ_{A}/γ_{A}-fibrin. These concepts are supported by the observation that the γ-peptide inhibited γ-IIa binding to γ_{A}/γ_{A}-fibrin in a concentration-dependent fashion (Fig. 6).

**Binding of α-IIa to Des-B15–42-γ_{A}/γ_{A}- or Des-B15–42-γ_{A}/γ_{A}-Fibrin**—Although both α-IIa and γ-IIa bound to the γ-chain on γ_{A}/γ_{A}-fibrin, the affinity of α-IIa for γ_{A}/γ_{A}-fibrin was 69-fold higher than that of γ-IIa (Kd values of 0.08 ± 0.03 and 5.54 ± 0.02 μM, respectively). These findings raise the possibility that the high affinity interaction of α-IIa with γ_{A}/γ_{A}-fibrin requires both exosites on thrombin, wherein exosite 2 is ligated by the γ-chain of fibrin and exosite 1 by the NH2 termini of α- and β-chains. To explore this possibility, the binding of α-IIa to fibrin generated from des-B91–125-γ_{A}/γ_{A}-γ_{A}/γ_{A}-fibrinogen was measured. α-IIa bound des-B15–42-γ_{A}/γ_{A}-fibrin with a Kd value of 7.67 ± 0.09 μM (Table I). Thus, the affinity of α-IIa for γ_{A}/γ_{A}-fibrin reduced 3.4-fold when the β15–42 sequence was removed, consistent with the concept that this sequence represents at least part of the low affinity thrombin-binding site on γ_{A}/γ_{A}-fibrin (16, 40). Although the affinity of α-IIa for des-B15–42-γ_{A}/γ_{A}-fibrin was 2.2-fold higher than that for des-B15–42-γ_{A}/γ_{A}-fibrin (Kd values of 3.51 ± 0.07 and 7.67 ± 0.09 μM, respectively), the affinity of α-IIa for des-B15–42-γ_{A}/γ_{A}-fibrin was 44-fold lower than that for γ_{A}/γ_{A}-fibrin. The
30 nm α-IIa, γ-IIa, or RA-IIa was added to microcentrifuge tubes containing γA/γA, γA/γ', des-β15–42-γA/γ', or des-β15–42-γA/γ'-fibrinogen (0–10 μM) and 2 mM CaCl₂. For studies with γ-IIa, 5% (v/v) atroxin was used to induce clotting. The concentration of bound α-IIa, γ-IIa, or RA-IIa was calculated by comparing the thrombin chromogenic activity in the clot supernatants with that in control reactions containing all reagents except the thrombin variant and, in the case of γ-IIa, atroxin. Data were analyzed by nonlinear regression analysis to determine the $K_d$ values. Each value represents the mean ± S.E. of three experiments, each done in duplicate.

| Ila variant | Native fibrin | des-β15–42-fibrin | des-β15–42-fibrin | des-β15–42-fibrin |
|-------------|---------------|--------------------|-------------------|--------------------|
| α-IIa       | γA/γA 2.25 ± 0.05 | γA/γ' 0.08 ± 0.03 | γA/γ' 7.67 ± 0.09 | γA/γ' 3.51 ± 0.07 |
| RA-IIa      | γA/γA 3.11 ± 0.02 | γA/γ' 2.02 ± 0.03 | γA/γ' 11.72 ± 0.05 | γA/γ' 10.93 ± 0.03 |
| γ-IIa       | No binding | 5.54 ± 0.02 | No binding | 5.86 ± 0.02 |

Fig. 6. Effect of the γ'-peptide on the binding of γ-IIa to γA/γ'-fibrin. 100 nm γ-IIa was added to microcentrifuge tubes containing 10 μM γA/γ'-fibrinogen and 2 mM CaCl₂ in the absence or presence of the γ'-peptide at the concentrations indicated. After clotting the fibrinogen with 5% (v/v) atroxin, the concentration of γ-IIa bound to fibrin was calculated by comparing γ-IIa-mediated hydrolysis of tGPR-pNA with that in control fibrinogen without a functional exosite 2, RA-IIa can interact only with low affinity binding sites on fibrin. However, when the affinity of RA-IIa for γA/γA' or γA/γ'-fibrinogen is compared with its affinity for des-β15–42-γA/γA' or des-β15–42-γA/γ'-fibrinogen, it is apparent that removal of the β15–42 sequence from γA/γA' and γA/γ'-fibrinogen reduces their affinity for RA-IIa by 3.8- and 5.4-fold, respectively.

As expected, γ-IIa did not bind to des-β15–42-γA/γA'-fibrinogen. However, γ-IIa bound to des-β15–42-γA/γ'-fibrinogen with an affinity similar to that for γA/γ'-fibrinogen ($K_d$ values of 5.86 ± 0.02 and 5.54 ± 0.02 μM, respectively). Thus, removal of a low affinity thrombin-binding site had no effect on the interaction of γ-IIa with the binding site on the γ'-chain of γA/γ'-fibrinogen or des-β15–42-γA/γ'-fibrinogen. These findings support the concept that binding of γ-IIa to the γ'-chain is independent of exosite 1.

Effect of Exosite 1- or 2-directed Ligands on α-IIa Binding to γA/γA' or γA/γ'-Fibrin—To verify the contribution of the two exosites on α-IIa to its interaction with fibrin, binding assays were performed in the absence or presence of ligands directed at either exosite 1 or 2 of α-IIa, and the ability of these ligands to block α-IIa binding was determined. When ligands directed to exosite 1, hirudin-(54–65), HD1, and heparin cofactor II-(54–75), were present at saturating concentrations (50, 1, and 100 μM, respectively), α-IIa binding to γA/γA' or γA/γ'-fibrinogen was reduced by 70–80% (Fig. 8). These findings highlight the importance of α-IIa exosite 1 binding to either form of fibrin. To address the role of exosite 2, the experiment was then repeated with saturating concentrations of γ'-peptide, h-F2, and HD22 (236, 64, and 0.25 μM, respectively). The γ'-peptide produced almost 100% reduction in α-IIa binding to γA/γ'-fibrinogen. In contrast, the γ'-peptide reduced α-IIa binding to γA/γ'-fibrinogen by 50%. Because the interaction of α-IIa with γA/γ'-fibrinogen is not expected to involve exosite 2 directly, these results suggest an allosteric linkage between the two exosites. Similar results were obtained with h-F2 and HD22, although the extent of reduction was less with both forms of fibrin.

DISCUSSION

Fibrin-bound thrombin has been implicated as an important trigger of thrombus growth (11, 37). Consequently, it is of interest to define the mode of thrombin interaction with fibrin. Two classes of thrombin-binding sites have been identified on...
Both Thrombin Exosites Mediate High Affinity Fibrin Binding

The concept that both exosites on thrombin are necessary for its high affinity interaction with $\gamma_\alpha/\gamma_\gamma$-fibrin provides an explanation for the results of thrombin binding studies with dysfibrinogen Naples I. When clefted, fibrinogen Naples I (B8 A68T) exhibits reduced affinity of both high and low affinity binding interactions (40). These findings suggest that attenuation of exosite 1-mediated interaction with the mutant NH$_2$ terminus of the $\beta$-chain impairs high affinity binding mediated by the $\gamma$-chain.

The proposal that both exosites are necessary for the high affinity interaction of $\alpha$-IIa with fibrin is supported by studies using synthetic exosite 1- or 2-directed ligands. Exosite 1-directed ligands reduce $\alpha$-IIa binding to $\gamma_\alpha/\gamma_\gamma$-fibrin by 80–100%, thereby confirming that this exosite is important for both the high and low affinity interaction of $\alpha$-IIa with fibrin. Exosite 2-directed ligands reduce $\alpha$-IIa binding to $\gamma_\alpha/\gamma_\gamma$-fibrin by 60–100%, supporting the concept that exosite 2 is important for the interaction of $\alpha$-IIa with $\gamma_\alpha/\gamma_\gamma$-fibrin. However, exosite 2 ligands also produce modest reductions in $\alpha$-IIa binding to $\gamma_\alpha/\gamma_\gamma$-fibrin, despite the fact that this interaction is mediated by exosite 1. These findings can be rationalized by our previous observation that there is allosteric linkage between the two exosites such that binding of a ligand to one exosite alters the affinity of the opposite exosite for its ligands (15). A recent study has challenged this concept based on the observation that prothrombin fragment 2, which binds to exosite 2 on thrombin, does not influence the binding of hirudin (54–65) to exosite 1 (41). This discrepancy likely reflects differences in assay technique because our current findings indicate that exosite 2-directed ligands, including prothrombin fragment 2, attenuate the binding of $\alpha$-IIa to $\gamma_\alpha/\alpha_\lambda$-fibrin via exosite 1.

Both prothrombin fragment 2 and the $\gamma$-chain of fibrinogen possess clustered anionic residues that likely mediate its interaction with the electropositive exosite 2 domain on thrombin (22, 42, 43). Although no homology between the sequences that bind to exosite 1 or 2 can be discerned (22), the two exosites appear to have functionally distinct roles. In general, exosite 1 on thrombin is utilized by substrates, cofactors, and inhibitors to gain direct access to the active site of the enzyme. In contrast, exosite 2 binds heparin or prothrombin fragment 2, both of which are ligands that recruit tertiary reactants, antithrombin or factor V, respectively. This suggests that exosite 2 functions primarily as an anchoring site, whereas exosite 1 serves to align substrates or inhibitors. This is consistent with the observation that exosite 2 functions are retained when thrombin is complexed by antithrombin (35). The exosite 2-mediated anchoring of thrombin to $\gamma$-chain of fibrinogen enhances the capacity of fibrin to harbor catalytically active thrombin. Thrombin bound in this fashion would remain active and protected from inhibition by heparin-antithrombin because the heparin-binding site of the bound thrombin is ligated by the $\gamma$-chain. The observation of a correlation between plasma lev-
els of γδ/γ′-fibrinogen and the risk of coronary artery disease supports this contention (30).

From a structural perspective, our data suggest that the COOH-terminal portion of the γ'-chain must be in close proximity to the NH2 termini of the α- and β-chains of cross-linked γδ/γ′-fibrin to bind both thrombin exosites simultaneously. Supporting this concept are recent data revealing the proximity of the COOH terminus of the γ-chain to polymerization site “a” (44–46). The putative exosite 1-binding site that resides on the α-chain is located near the NH2-terminal “A” knob that binds to site “a” (19). The COOH terminus of the γ-chain is unthetered and flexible (44), and the γ'-chain is 16 residues longer than the γ-chain. Thus, the γ'-terminus could bind to exosite 2 of thrombin already bound to the α-chain via exosite 1. In this model, thrombin is likely bound first to the NH2 terminus of the α-chain via exosite 1 after cleavage of fibronectin A. This initiates polymerization and brings the γ'-chain into proximity of thrombin residing on the other strand of the protofibril.

In summary, the data suggest that the high affinity interaction of thrombin with γδ/γ′-fibrin requires simultaneous ligandation of both exosites on thrombin. Whereas exosite 2 binds to the γ'-chain, exosite 1 interacts with the NH2 terminus of the β-chain. Occupation of both exosites may induce conformational changes in the active site of thrombin. Such changes occur upon formation of the ternary thrombin-heparin-fibrin complex or when thrombin binds to thrombomodulin via exosite 1 and the chondroitin sulfate moiety of thrombomodulin binds to exosite 2. Conformational changes in the active site of thrombin or occupation of the heparin-binding site may limit its reactivity with serpins, thereby protecting thrombin from inhibition (5, 36).

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Evidence That Both Exosites on Thrombin Participate in Its High Affinity Interaction with Fibrin
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