Identification of Distinct Sequences in Human Blood Coagulation Factor Xa and Prothrombin Essential for Substrate and Cofactor Recognition in the Prothrombinase Complex*  

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To identify amino acid sequences in factor Xa (fXa) and prothrombin (fII) that may be involved in prothrombinase complex (fXa-factor Va-fII-phospholipid) assembly, synthetic peptides based on fXa and fII sequences were prepared and screened for their ability to inhibit fXa-induced clotting of normal plasma. One fII peptide (PT557–571 homologous to chymotrypsin (CHT) residues 225–239) and two fXa peptides (X404–418, CHT231–244, and X415–429, CHT241–252) potently inhibited plasma clotting and prothrombinase activity with 50% inhibition between 41 and 115 μM peptide. Inhibition of prothrombinase by PT557–571 and X415–429 was fVa-independent, whereas the inhibition by X404–418 was fVa-dependent. X404–418 inhibited the binding of fVa to fluorescein-labeled, inhibited fXa, in the presence of phosphatidylcholine/phosphatidylerine vesicles, whereas X415–429 inhibited binding of fII to phospholipid-bound fluorescein-labeled, inhibited fXa. PT557–571 altered the fluorescence emission of fluorescein-labeled fXa, showing that PT557–571 binds to fXa. These data suggest that residues 404–418 in fXa provide fVa binding sites, whereas residues 557–571 in fII and 415–429 in fXa mediate interactions between fXa and fII in the prothrombinase complex.

Prothrombin (fII)1 activation is the final step in a cascade of zymogen activations that are precisely regulated by a variety of molecular interactions (1). Since thrombin can effect a variety of procoagulant, anticoagulant, antifibrinolytic, or cellular responses depending on the local environment, fII activation has been extensively studied. fII is converted to α-thrombin by proteolytic cleavages at Arg-271–Thr-272 and at Arg-320–Ile-321 (2, 3). This is optimally accomplished by the assembly of the so-called prothrombinase complex that consists of the serine protease coagulation factor Xa (fXa) with its non-enzymatic cofactor, factor Va (fVa), on a negatively charged phospholipid surface in the presence of Ca2+ (4–8). Assembly of the prothrombinase complex results in a 278,000-fold enhancement in the rate of fII activation as compared with fXa alone (5). fVa functions both as a K-type and a V-type effector in the reaction, decreasing the Km and increasing the kcat, respectively. Additionally, in the presence of fVas and phospholipids, fII activation proceeds almost exclusively via a meizothrombin intermediate (initial cleavage at Arg-320), whereas in the absence of these two components, the activation proceeds via a prethrombin 2 intermediate (initial cleavage at Arg-271) (2, 3, 9). Therefore, the substrate specificity of fXa is altered in the presence of fVas and phospholipids.

fXa bears extensive structural and sequential homology to other vitamin K-dependent serine proteases (10). The mature glycoprotein circulates in plasma as two chains connected by a single disulfide linkage. The N-terminal region of the light chain is rich in post-translationally modified γ-carboxyglutamic acid (Gla) residues and hence is termed the Gla domain. The Gla domain is followed by two domains that bear structural similarity to epidermal growth factor (epidermal growth factor-like repeats). The heavy chain of fXa contains the serine protease domain. The heavy chain of fXa is highly homologous to other serine proteases such as thrombin, chymotrypsin, and activated protein C, and six variable regions and seven conserved regions have been identified (11). fII also belongs to the vitamin K-dependent protein family and hence has a Gla domain (residues 1–40) that is homologous to fXa Gla domain (10). However, instead of the two epidermal growth factor-like repeats in fXa, fII has two kringle domains (residues 41–156 and 156–271, respectively) followed by the serine protease domain (residues 272–579).

Identifying functionally important amino acid sequences that mediate protein-protein interaction is a central problem in biochemistry. For the prothrombinase complex, some progress has been made although the picture is still very incomplete. For example, of the 139 amino acids in the light chain of fXa, a few of the amino acids seem to be critical in the fXa-fVa interaction. Gla domainless-fXa shows impaired binding to fVas and phospholipid-bound fVas (12–14). Additionally, the second epidermal growth factor repeat in fXa may also bind fVas, albeit weakly (14, 15). In the heavy chain of fXa, amino acids 211–222 (CHT32–44) and 254–274 (CHT74–94) in the variable region 1 and 2, respectively, of the catalytic domain of fXa may be...
involved in FVa binding (16, 17), whereas amino acids representing the regions 275–287 (CHT95–107) and 415–425 (CHT241–252C) were suggested to play a key role in substrate recognition (16). In another study using an exosite-directed antibody, overlapping regions, namely amino acids 262–271 (CHTS2–91) and 282–296 (CHT102–116), were suggested to be critical for substrate recognition (18). Furthermore, Arg-347 (CHTArg165) and the autolysis loop might contribute to the interaction of FXa with FVa (17, 19, 20). As in FXa, several FVa binding loops vulnerable to exist on FII. Both FVII tringle domains have been reported to interact with FVa (21, 22). Additionally, the proexosite I and the Gla domain of FII have also been reported to participate in FVa binding (23, 24).

Mesters et al. (25–27) identified sequences in human activated protein C essential for interactions with its substrate FVa by the use of synthetic peptides. Based on the homology of protein C with FXa and FII (28–30), we used synthetic peptides comprising related sequences in FXa and FII to test their ability to inhibit assembly of the prothrombinase complex. Here, we identified distinct sequences in FXa and FII that are essential for interactions with each other and with their FVa cofactor.

**EXPERIMENTAL PROCEDURES**

**Materials—**Human FII was isolated as reported previously (31). Protein concentrations were determined using a molecular weight of 72,000 daltons and an extinction coefficient (ε280 nm) of 1.44 (32). For some experiments, purified human FVa was obtained as a gift from Drs. Guido Tan and Jan Roosing (University of Maastricht, Maastricht, The Netherlands) (33). For other experiments, FVa was purchased from Hematologic Technologies (Essex Junction, VT). Human thrombin and human FXa were obtained from Enzyme Research Laboratories (South Bend, IN), and the molar concentrations were determined by active site titration as described (34). All proteins appeared to be >95% purity judged by SDS-PAGE. The chromogenic substrates SFM and S3286 were purchased from Kabi-Vitrum (Franklin, OH), and CBS 34-47 was purchased from Diagnostica Stago Inc. (Passypanny, NJ). Rabbit brain cephalin was obtained from Sigma. Bovine serum albumin, human fibrinogen, and n-Phenylalanyl-L-prolyl-L-arginyl (FPF) chloromethylketone were purchased from Calbiochem, and normal human citrate-anticoagulated plasma (NHP) was obtained from George King Bio-Medical, Inc. (Overland Park, KS). Fluorescein-labeled FXa (Fl-FXa) was prepared as described (35). Small unilamellar vesicles were made as described (36) using synthetic dioleoylphosphatidylcholine (PC) and dioleoylphosphatidylinerine (PS) purchased from Avanti Polar Lipids (Alabaster, AL). t-3-Phosphatidylcholine-1, 2-di (13C)oleyl PC was obtained from Bio Synthesis Inc. (Lewisville, TX). Mass spectrometric analyses of the purified peptides using the previously reported method yielded, in each case, the exact expected molecular weight for each individual peptide. Solutions of each peptide were prepared, and the concentrations were determined as described (25).

**Peptide Synthesis and Characterization—**Fifteen-amino-acid-olig synthetic peptides were prepared, purified to homogeneity using reverse-phase high performance liquid chromatography, and characterized as described earlier (25). For the control experiments, scrambled peptides were obtained from Bio Synthesis Inc. (Lewisville, TX). Mass spectrometric analyses of the purified peptides using the previously reported method yielded, in each case, the exact expected molecular weight for each individual peptide. Solutions of each peptide were prepared, and the concentrations were determined as described (25).

**Fibrinogen Clotting—**Human thrombin (0.5 units/ml) was preincubated with the peptide of interest (0–1 nm) at five different concentrations in 200 μl of buffer containing TBS-BSA plus 2.5 mM CaCl2 for 10 min at 37 °C in polystyrene cuvettes. Clotting was initiated by addition of 200 μl of human fibrinogen (5 mg/ml) in TBS-BSA plus 2.5 mM CaCl2, and time in seconds from addition of fibrinogen to clot formation was subsequently measured in the second step using a chromogenic substrate S-2238 or CBS 34-47. Amounts of FXa, phospholipids, and FII present in the assay were such that the rate of thrombin generation was linearly proportional to the amount of FXa present in the reaction mixture. In a typical experiment, the peptide of interest was preincubated with FVa (0.4 nm final) in TBS-BSA plus 2 mM CaCl2 for 20 min at 22 °C. Before preincubation, an aliquot of this solution was added to a solution containing FII (1.2 μM final) and PC/PS vesicles (50 μM final) in TBS-BSA plus 2 mM CaCl2. Thrombin generation was initiated by the addition of FXa (1 nm). To measure the amount of thrombin generated, a 20-μl aliquot of the activation mixture was withdrawn every minute and quenched into 80 μl of buffer containing TBS-BSA plus 10 mM EDTA. Finally, the amount of thrombin present was quantified by adding 50 μl of 2 mM S-2238, and rate of substrate hydrolysis was measured at 405 nm for 2.5 min. The change in A405 was directly proportional to the thrombin generation. For some kinetic studies, a modified prothrombinase assay was used to conserve reagents. Briefly, different concentrations of FII (ranging from 0 to 6.5 μM) in 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM CaCl2, 0.1 mM MnCl2, 0.5% BSA, and 0.02% NaN3 were incubated with PC/PS (20 μM) in the presence or absence of different concentrations of the peptide of interest at room temperature for 10 min in a total volume of 35 μl. FXa activation was initiated with 10 μl of FXa (320 pm) in the same buffer. After 2.5 min, the reaction was quenched with 50 μl of buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 10 mM EDTA. Thrombin generated was estimated by using CBS 34-47 (50 μl, 1.3 μM), and the rate of substrate hydrolysis was measured at 405 nm for 5 min. The change in A405 was directly proportional to the thrombin generation.

For FII activation without FVa, different concentrations of FII (ranging from 0 to 1.2 μM) in 150 μl of buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM CaCl2, 0.1 mM MnCl2, and 0.5% BSA were incubated with PC/PS (20 μM) in the presence of the peptide of interest at room temperature. FII activation was then initiated with FXa (7.5 nm). A aliquots (15 μl) were withdrawn at 10, 20, 40, 60, and 80 min, and the reaction was quenched in 50 μl of buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, and 10 mM EDTA. Finally, the amount of thrombin generated was quantified using 50 μl of CBS 34-47 (1.5 mM) as described above.

**Spectral Measurements—**Steady state fluorescence intensity and anisotropy measurements were made using a SLM AB2 Luminescence spectrometer (SLM Aminco, Rochester, NY) equipped with a 150-watt xenon arc lamp, two holographic gratings in the excitation light path, and an IBM computer for data analysis. Fluorescein fluorescence was measured using its excitation and emission maxima wavelengths at 490 and 525 nm, respectively, using a 345-nm cut-off filter (Schott Glass Technology, Duryea, PA) in the excitation beam path. Shutters were closed except during measurements to minimize photodegradation of samples. A circulating water bath was used to maintain the sample compartment at 25 °C. All fluorescence experiments were performed in 5 × 5 mm quartz cuvettes. Samples were excited using a 480-nm 2-mm magnetic spinbar as described (37, 38). Absorption of protein to the cuvette walls was minimized by coating the cuvettes with 100% PC vesicles as described (39).

For a typical fluorescence experiment, two samples were prepared in parallel. The sample cuvette received 200 nm Fl-FXa in 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM CaCl2, and 0.02% NaN3, while the control cuvette received an equal concentration of unlabelled active site inhibited FFR-Xa in the same buffer. The initial net fluorescence intensity of the sample, designated F0, was obtained by subtraction of a dye-free blank cuvette from the sample cuvette signal. For the peptide titrations,
equimolar concentrations of peptides were added to each cuvette. The signal of the blank cuvette was never greater than 0.2% of the sample cuvette. After the signal observed in the absence of the dye (sample B) was subtracted from that in the presence of the dye (sample A), the net volume-corrected signal was designated F at that point in the titration. Phospholipid and protein titrations in the presence or absence of peptides were performed analogously.

Steady state anisotropy was measured using Glan-Thompson prism polarizers on both the excitation and emission beams. The emission intensity measured when the sample was excited by vertically (V) plane-polarized light and the emission was detected through a horizontal (H) polarizer is termed $I_{HV}$, $I_{HH}$, $I_{VV}$, and $I_{VH}$ are defined analogously. The component intensities of a dye-free blank were subtracted from the component sample intensities to give the net emission intensities. Anisotropy ($r$) was then calculated from the net intensities using $r = (I_{VV} - G I_{HV})/ (I_{VV} + 2 G I_{HH})$, where the grating factor, G, equals $I_{HV}/I_{HH}$.

**Curve Fitting and Data Analysis**—The curves obtained from the fluorescence assays were fit using GraphPad Prism Version 3.03 using procedures similar to those described earlier (40). The maximum theoretical change in fluorescence parameter obtained for each titration (F$_{max}$) was then used to normalize all curves to 100% maximum change.

**RESULTS**

**Inhibition of FXa-induced Clotting Activity by fII-derived and fXa-derived Peptides**—Table I illustrates homology between functionally important sequences in protein C comprising residues 390–404 (25) and residues 142–155 (27) and sequences in fXa (41) and fII (42). Synthetic peptides of fII and fXa derived from these regions of homology were screened for their ability to inhibit fXa clotting activity in normal human plasma. Table II shows the amino acid sequences that were assayed and the residual fXa activity observed in an fXa-1 stage clotting assay in the presence of 500 μM of each peptide. Three peptides, namely PT557–571, X404–418, and X415–429, inhibited more than 80% of fXa clotting activity at 500 μM peptide, whereas peptide PT294–306, which is homologous to amino acid stretch 142–155 of protein C, inhibited only about 25% of fXa activity.

The three peptides that potently inhibited fXa activity were characterized further using the same clotting assay, and a scrambled sequence of each peptide was also tested for their ability to inhibit fXa-induced clotting. Two of the three scrambled sequences did not show any significant inhibition in the fXa-1 stage assay, whereas the X415–429SCR, a scrambled sequence of amino acids 415–429 in fXa, showed ~46% inhibition as compared with 93% inhibition by the native sequence (Table II). Thus, none of the scrambled sequences was as effective as PT557–571, X404–418, and X415–429 at inhibiting fXa-induced clotting, thus suggesting sequence specificity of these peptide in inhibiting fXa activity. In dose-response studies, PT557–571, X404–418, and X415–429 inhibited fXa clotting activity with 50% inhibition between 41 and 115 μM (Fig. 1). Inhibition of clotting by X415–429SCR was due to an artificial inhibition because this peptide was found to inhibit clotting of fibrinogen by thrombin. None of the other peptides inhibited clotting of fibrinogen by thrombin (data not shown).

**fVa-dependent Inhibition of Prothrombinase by Peptide X404–418**—To characterize the inhibitory mechanisms of the three most anticoagulant peptides in a prothrombinase assay both in the presence and absence of fVa, studies were performed using purified proteins. Each peptide was tested for its ability to inhibit the generation of thrombin by fXa. Fig. 2 shows that peptide X404–418 dose-dependently inhibited prothrombinase activity in the presence of fVa with 50% inhibition at 83 μM. However, X404–418 did not significantly affect prothrombinase activity in the absence of fVa for the concentrations of peptide tested (Fig. 2). Thus, X404–418 absolutely required the presence of fVa to exert its inhibitory effect on prothrombinase activity. The control scrambled peptide, X404–418SCR, did not inhibit prothrombinase in either the presence or the absence of fVa (Fig. 2). Thus, the fVa-dependent inhibition of prothrombinase by X404–418 was sequence-specific. To further elucidate the mechanism of inhibition by X404–418, prothrombinase assays were performed at varying fII and X404–418 concentrations (Fig. 2 Inset). Plots of 1/II against 1/velocity of reaction generated (Fig. 2, inset, Lineweaver-Burk plot) straight lines when X404–418 was not present or present in increasing concentrations. The inhibition pattern can be described as non-competitive inhibition. The plot of X404–418 concentration against the slope gave an estimated inhibition constant, K$_i$, value of 22 μM.

**fVa-independent Inhibition of Prothrombinase by Peptides PT557–571 and X415–429**—In contrast to the fVa-dependent

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**Table I**

| Protein       | Residues | Sequence$^a$ |
|---------------|----------|--------------|
| Protein C     | 390–404  | GYTFRKVSYLDWHP | $^a$ Bold and underlined letters indicate amino acids that are identical among Protein C, fX, and fII. |
| Factor X      | 409–423  | GYTFRVATLPKWDH |
| Prothrombin   | 557–571  | GYTRVVLRLKKIMQ |
| Chymotrypsin  | 225–239  | RGGTPRKKPPRTGGTTRV |
| Protein C     | 142–155  | RPRTKVVRKRSKSL |
| Prothrombin   | 294–304  | RPRTF. . . . . .BK. . . |

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**Table II**

| Peptide | Amino acid sequence | Residual fXa activity (%) |
|---------|---------------------|--------------------------|
| PT294–306 | GLRPPEKKSLED     | 75                      |
| PT557–571 | YGFTYHVRKLKWKIQ | 8                       |
| PT557–571SCR | KVQKRTLFLFYWGY | 103                     |
| X404–418     | ARRKGTYITVKTFAP  | 6                       |
| X404–418SCR | TVYVF2AKAGKGI    | 100                     |
| X415–429     | VTAFLKWDMSKTR    | 7                       |
| X415–429SCR | SATRFMRKIVKTDL   | 54                      |

**Fig. 1.** fXa clotting activity in the presence of various peptides. fXa (0.15 nM) clotting activity was measured in the presence of peptides: PT557–571 (solid square), X404–418 (solid triangle), X415–429 (solid circle), PT557–571SCR (open square), X404–418SCR (open triangle), X415–429SCR (open circle). The fII-derived peptides are shown as solid lines, whereas the fX-derived peptides as dashed lines.
FIG. 2. Prothrombinase activity in the presence of peptide X404–418. The thrombin generation assay was performed using 1 nM fXa, 0.4 nM fVa, 1.2 μM fII, or 2.5 nM fVa and 1.2 μM fII in the absence of fVa in TBS-BSA, 2.5 mM CaCl$_2$, pH 7.4, and 50 μM PC/PS vesicles. Solid lines represent prothrombinase activity in the presence of fVa, and dashed lines represent prothrombinase activity in the absence of fVa. X404–418 in the presence and absence of fVa is represented by solid and open triangles, respectively, and X404–418SCR in the presence and absence of fVa is represented by open solid and open inverse triangles, respectively. In the inset, prothrombinase activity was examined at various peptide and fII concentrations as described under “Experimental Procedures.” X404–418 concentrations were 0 (open circles), 45 (closed diamonds), 91 (open triangles), and 182 μM (closed inverted triangles), respectively. The control X404–418SCR concentration was 100 μM (open diamonds). A K$_\text{d}$ of 22 μM was obtained.

Effects of X404–418, PT557–571 and X415–429 exhibited similar dose-dependent inhibition of prothrombinase activity in the presence and in the absence of fVa (Fig. 3) with half-maximal effects between 25 and 50 μM peptide. Thus, the inhibition of fXa by PT557–571 and X415–429 was fVaindependent. Scrambled peptides PT557–571SCR and X415–429SCR did not significantly inhibit prothrombinase (Fig. 3), indicating that the inhibition of prothrombinase by PT557–571 and X415–429 was sequence-specific.

When prothrombinase assays were performed at different fII and peptide concentrations and in the absence of fVa, PT557–571 showed an inhibition pattern similar to that of a classical non-competitive inhibitor (Fig. 3, inset) with the all lines arising from different peptide concentrations having the same X intercept but different Y intercepts. An inhibition constant (K) of 38 μM was obtained from the plot of PT557–571 concentration versus apparent 1/V$_\text{max}$ (not shown). When similar experiments were performed for X415–429, the peptide showed a pattern typical of a classical competitive inhibitor (Fig. 3, inset) with all the lines having the same Y intercept but different X intercepts, and a plot of X415–429 concentration versus slope gave an estimated K value of 178 μM (not shown).

Effects of X404–418, X415–429, and PT557–571 on the Active Sites of fXa or Thrombin—To test whether these three peptides that inhibited fXa clotting activity as well as prothrombinase activity directly inhibited the active site of fXa or thrombin, studies were performed using small chromogenic oligopeptide substrates. fXa or thrombin was incubated with the peptide of interest before the addition of the respective chromogenic substrate. None of the three peptides significantly inhibited the activities of fXa and thrombin toward their respective oligopeptide substrates, S-2222 and CBS 34–47 (or S-2238), over a wide range of substrate and peptide concentrations (data not shown). Additionally, none of the three peptides had any significant effect on the clotting of purified fibrinogen by thrombin when tested at five different peptide concentrations (0–500 μM final concentration). Thus, these experiments indicate that X404–418, X415–429, and PT557–571 do not inhibit the active sites of fXa and thrombin.

Effects of Inhibitory Peptides on Protein-Protein Interactions—To test the hypothesis that X404–418 inhibited prothrombinase activity by blocking the binding of fXa to fVa, we used fluorescence to monitor protein-protein interactions. The fluorescein in Fl-fXa is sensitive to the interactions of fXa with each of the different components of the prothrombinase complex, namely, phospholipids, fVa, and fII (35). Thus, the fluorescein probe can monitor spectroscopically the complete assembly of the prothrombinase complex.

To determine the effects of X404–418 on fXa-fVa interactions, these interactions were monitored in the absence and presence of X404–418. Samples of PC/PS-bound Fl-fXa were prepared with excess vesicles sufficient to bind both fXa and an excess of fVa. The anisotropy of the fluorescein dye in these samples varied between 0.233 and 0.237. Then these samples were incubated with either buffer containing X404–418 (48 μM) or buffer alone for 10 min at room temperature. Finally, both sets of samples were titrated with fVa, and the binding of fVa to PC/PS-bound Fl-fXa was measured by monitoring fluorescein anisotropy (Fig. 4). Upon addition of fVa to the buffer-alone sample, the fluorescein anisotropy increased to a value of 0.306 and reached a plateau at a concentration of about 350 nM fVa. However, for the sample containing the peptide X404–418, the titration was significantly shifted to higher [fVa] and
reached the same plateau only upon addition of about 1.0 μM fVa (Fig. 4). Thus, peptide X404–418 inhibited the binding of fXa to fVa.

In other experiments, PC/PS-bound Fl-fXai was incubated with peptide X415–429, and the fVa titration was performed as detailed above. As in the case of buffer-only fVa titration, the fluorescein anisotropy in the presence of X415–429 also increased sharply and reached a plateau at ~420 nM fVa, suggesting that in contrast to X404–418, peptide X415–429 did not inhibit fXa binding to fVa.

The hypothesis that peptide X415–429 inhibited the binding of fXa with its substrate fII was tested. fII binds to Fl-fXai (Fig. 5) suggesting that in contrast to X404–418, not inhibit fXa binding to fVa. 

FIG. 4. Effect of fX-derived peptides on fXa-fVa interactions. Fl-fXai (initially 200 nM) in 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM CaCl2 was mixed with increasing amounts of PC/PS (4:1) vesicles (data not shown) until the anisotropy of the free protein increased from a value of 0.203 and reached a plateau of 0.257 at ~25 μM phospholipid. Then either 48 μM peptide or an equal volume of buffer was added. After incubation for 10 min at room temperature, fVa was added into each cuvette, and the binding of phospholipid-bound Fl-fXai to fVa was monitored as a change in anisotropy. The fVa titrations in the presence of peptide X404–418 (solid triangles), X415–429 (solid circles), or buffer (open circles) are shown. rwas the anisotropy of the sample before the addition of fVai, and r was the anisotropy observed at each fVa concentration. Each curve was fit to a one-phase exponential association using GraphPad Prism version 3.03, and then the curves were normalized to the maximum change value.

FIG. 5. Effect of fX-derived peptides on fXa-fII interactions. Fl-fXai (initially 200 nM) in 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM CaCl2 was mixed with increasing amounts of PC/PS (4:1) vesicles (data not shown) as described in the legend for Fig. 4. Then fX-derived peptides (90 μM) or buffer was added to the samples. After 10 min incubation, fII was titrated into the sample, and complex formation was monitored by changes in anisotropy in the presence of peptide X404–418 (solid triangles), X415–429 (solid circles), or buffer (open circles). r was the anisotropy before addition of fII, and r was the anisotropy observed at each fII concentration. Curves were fit and normalized as described in the legend for Fig. 4.

FIG. 6. Effect of fII-derived peptides on Fl-fXai emission. Fl-fXai (initially 200 nM) in 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM CaCl2 was titrated with either PT557–571 (solid squares) or PT557–571SCR (open squares), and the fluorescein fluorescence intensity was monitored. F was the fluorescence at each peptide concentration, whereas F0 was the fluorescence when no peptide was present.

Peptide PT557–571 SCR that was titrated with either PT557–571 (solid squares) or PT557–571SCR (open squares), and the fluorescein fluorescence intensity was monitored. F was the fluorescence at each peptide concentration, whereas F0 was the fluorescence when no peptide was present.

of fXa in clotting and prothrombinase assays but not in amidolytic assays. Thus, to test the hypothesis that the sequence of PT557–571 SCR may provide a binding site on fII for fXa and that the PT557–571 SCR sequence directly binds to fXa, Fl-fXai was titrated with PT557–571, and the fluorescein emission intensity was monitored. When PT557–571 was titrated into a solution containing Fl-fXai, fluorescein emission remarkably decreased by almost 70% of the initial value and reached a plateau at concentrations above 16 μM peptide (Fig. 6). When a similar titration was performed with PT557–571 SCR that was
not inhibitory, no remarkable changes in Fl-fXai, were detected. These experiments strongly suggest that PT557–571 binds to Fl-fXa.

Binding of Fl-fXa, to PC/PS vesicles decreases fluorescein emission intensity by ~40% (35). In control studies, we found that none of the inhibitory peptides (X404–418, X415–429, and PT557–571) altered the affinity of Fl-fXai for phospholipid vesicles (data not shown).

**DISCUSSION**

FX and FII are two vitamin K-dependent proteins that bear extensive structural and sequential homology to other vitamin K-dependent proteins in blood coagulation such as protein C. Earlier, Mesters et al. (25–27) identified, using synthetic peptides, three specific stretches in protein C that are essential for its interaction with its substrate FVa. To test the hypothesis that peptides derived from these homologous regions in factor X and FII contribute to functionally important exosites that mediate protein-protein interactions in the prothrombinase complex, four different peptides were synthesized, purified, and screened for activity using an fXa-induced clotting assay. Three of the peptides comprising sequences from either factor X or FII, namely PT557–571, X404–418, and X415–429, were potent inhibitors of fXa clotting activity at 500 μM peptide in NHP. This inhibition was not due to the interference of the peptides with thrombin-fibrinogen interaction because the three peptides had no effect on the ability of thrombin to clot fibrinogen or hydrolyze chromogenic substrates. The peptides did not also alter the activity of fXa on the S-2222 chromogenic substrate, showing that these peptides did not alter the structural integrity of the fXa active site. Scrambled sequences of these peptides were not as effective as the respective peptides at inhibiting fXa induced clotting, indicating sequence specificity of inhibition. All three peptides dose-dependently inhibited thrombin generation by the prothrombinase complex without affecting the interaction of Fl-fXai with phospholipid vesicles, suggesting that their effects were directly on protein-protein interactions in the prothrombinase complex.

Of the three potent inhibitory peptides, two of them, X415–429 and PT557–571, inhibited the conversion of fII to thrombin by blocking the assembly of the fXa cofactor) was strongly supported by the fluorescence experiments using active site-labeled fXa, in which X404–418 inhibited the binding of fVa to Fl-fXa. Interestingly, peptide X415–429, which is an overlapping peptide downstream of X404–418, had little effect on the fXa-fVa interaction, showing the specificity of X404–418 in inhibiting fXa-fVa interaction. Recently, Rudolph et al. (20) reported that when Lys-414 was
mutated to Ala in fXa this mutant was defective in binding fVa. They also identified two other amino acids, namely Arg-347 and Arg-351, in fXa that are critical for fVa binding. Thus, our peptide studies are in complete agreement with these Ala scanning studies, and together, they strongly suggest that the region represented by X404–415 could be part of an extended fVa binding site on fXa. It is also noteworthy that X404–415 is located on the same three-dimensional face of the fXa molecule (Fig. 7A) that is reported to contain other fVa binding sites (16, 17, 20).

Peptides X415–429 and PT557–571 show similar dose-dependent inhibition of prothrombinase activity in the presence or absence of fVa, indicating that these regions might be important for the mutual recognition of fXa and fII. The observation that the inhibitory effect of X415–429 on prothrombinase activity reaches a plateau at 50% inhibition may reflect the existence of multiple fII binding exosites on fXa (16, 18). X415–429 appears as a C-terminal α-helix in the structure of fXa, and it is more cationic in fXa than in other serine proteases (8, 11). Because of the fVa independence of its inhibitory effects, we hypothesized that this peptide blocked the binding of fII to fXa. This was shown to be the case using fluorescence binding assays, where the fII bound very weakly to Fl-fXai, whereas the scrambled sequence PT557–571 showed similar dose-dependent inhibition. Mutated to Ala in fXa this mutant was defective in binding fVa. Moreover, caution should be exercised because it is possible that a synthetic peptide might disrupt the native structure of a protein by partially inserting itself into the protein it is meant to mimic, although examples of this artifact are rare.

In summary, peptide inhibition studies combined with the use of fluorescent reporters of protein-protein interactions in the prothrombinase complex support the novel suggestions that the threedimensional structures of fXa contains contiguous sequences (residues 404–418 and 415–429) that directly bind fVa and fII, respectively. The data also suggest that the C-terminal helix of fII containing residues 557–571 binds directly to fXa.

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