Factor Va Residues 311–325 Represent an Activated Protein C Binding Region*

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Activated protein C (APC) inactivates factor Va (fVa) by proteolytically cleaving fVa heavy chain at Arg506, Arg306, and Arg679. Factor Xa (fXa) protects fVa from inactivation by APC. To test the hypothesis that fXa and APC share overlapping fVa binding sites, 15 amino acid-overlapping peptides representing the heavy chain (residues 1–709) of fVa were screened for inhibition of fVa inactivation by APC. As reported, VP311–325, a peptide comprising residues 311–325 in fVa, dose-dependently and potently inhibited fVa-dependent prothrombin activation by fXa in the absence of APC. This peptide also inhibited the inactivation of fVa by APC, suggesting that this region of fVa interacts with APC. The peptide inhibited the APC-dependent cleavage of both Arg506 and Arg306 because inhibition was observed with plasma-derived fVa and recombinant R506Q and RR306/679QQ fVa. VP311–325 altered the fluorescence emission of dansyl-active site-labeled APCi, but not a dansyl-active site-labeled thrombin control, showing that the peptide binds to APCi. This peptide also inhibited the resonance energy transfer between membrane-bound fluorescein-labeled fVa (donor) and rhodamine-active site-labeled S360C-APC (acceptor). These data suggest that peptide VP311–325 represents both an APC and fXa binding region in fVa.

Factor Va (fVa)² serves as a cofactor for the enzyme factor Xa (fXa) in the activation of prothrombin (II) to thrombin (IIa). The conversion of II to IIa by fXa is enhanced ~13,000-fold in the presence of fVa (1, 2), an effect that is primarily on the kcat of the reaction. fVa is a two-chain protein with a heavy chain (1–709 residues) consisting of the A1 (residues 1–303) and A2 (residues 317–656) domains noncovalently bound via divalent metal ions to the light chain (residues 1546–2196, A3-C1-C2 domains) (3). Both the light chain and the heavy chain of fVa have been shown to interact with fXa (4).

Activated protein C (APC) down-regulates blood coagulation by proteolytic inactivation of the cofactors fVa and fVIIIa.

APC cleaves fVa rapidly at Arg506 followed by slower cleavages at Arg306 and Arg679 in the heavy chain of fVa (5, 6). The importance of specific cleavages has been clarified using point mutants of fVa where Arg506 and Arg306 have been replaced with Gln, and the A2 domain has been covalently cross-linked to the A3 domain (7–10). A partial loss of activity occurs when APC cleaves fVa at Arg506 (~30% loss in fXa cofactor activity), whereas complete inactivation of fVa requires the cleavage at Arg306. The cleavage at Arg679 is believed to be of minor importance for the inactivation of fVa. Inactivation of fVa results in the loss of ability of fVa to bind fXa and also in the dissociation of the A2 domain of fVa from the rest of the cleaved fVa molecule (10).

Kojima et al. (11) reported that a peptide comprising residues 311–325 in fVa inhibits the prothrombinase complex containing fVa (11). The region of residues 311–325 is in close proximity to the Arg306 cleavage site for APC (12) and also near the region of residues 493–506, which likely interacts with fXa (13). Other fXa binding sites on fVa have also been reported. Residues 323–331 (14) and amino acids 467, 511, and 652 (15) are important for fXa binding. Additionally, residue 1683 in the light chain of fVa is critical for fXa binding (15). Currently, no data are available on APC binding sites on fVa.

fVa is protected from APC-mediated inactivation by the presence of fXa (16–18), suggesting that APC binds to fVa on or near at least one fXa binding site(s). The hypothesis that fXa and APC share overlapping binding sites on fVa was tested using peptide inhibition studies. Fifteen amino acid-overlapping peptides derived from the heavy chain of fVa were screened for inhibiting the activation of II by the fXa/fVa complex and the inactivation of fVa by APC. Fluorescence spectroscopy of labeled proteins was used to verify inhibition of protein-protein interactions by peptides. The results of these studies are presented here.

EXPERIMENTAL PROCEDURES

Reagents—Human fVa, II, fXa, and APC were obtained from Hematologic Technologies Inc. (Essex Junction, VT) for most experiments. Prothrombin was also purchased from Enzyme Research Laboratories (South Bend, IN). Enzyme concentrations were determined by active site titrations using p-nitrophenyl p′-guanidinobenzoate as described previously (19).

Synthetic 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine and 1,2-dioleoyl-sn-glycero-3-phosphatidylserine were obtained from Avanti Polar Lipids (Alabaster, AL). 1,2-Di[1-14C]oleoyl-L-3-phosphatidylcholine was purchased from Amersham Biosciences. Dansyl-Glu-Arg chloromethylketone (DEGR-ck) was obtained from Calbiochem. Chromogenic substrates Pefachrome TH and PCa were purchased from Pentapharm.
APC Binding Region on fVa Heavy Chain

L tidy were obtained from Biosynthesis Inc. (Lewisville, TX).

Peptides—For the fVa heavy chain screens, 15 amino acid-overlapping synthetic peptides were prepared, purified, to homogeneity using reverse phase high performance liquid chromatography, and characterized as described previously (20). Peptides were prepared N-acetylated and as C-terminal carboxamides to avoid zwitterionic end-effects. Some peptides were prepared with a serine-to-cysteine mutation to avoid intermolecular disulfide formation at high peptide concentrations. For detailed studies subsequent to screening assays, peptides were obtained from Biosynthesis Inc. (Lewisville, TX).

Preparation of fVa and APC Variants—R506Q fVa was prepared from plasma as described (21). Recombinant fV and its variants (R506Q and RR306/679QQ) were constructed on a B-domain-deleted S2183A platform as described (10).

The active site serine mutant S360A protein C was expressed activated and purified as described earlier (22). To facilitate active site-specific labeling, the active site serine-to-cysteine protein C variant, S360C protein C, was prepared similarly.

Labeling of Proteins with Fluorescent Probes—Human plasma-derived APC was active site specifically labeled using DEGR-c-k and purified from the excess reagents as described (23). IIa was labeled with DEGR-c-k and purified as described previously (24). Human fVa was labeled with light-with-a-bite, a multicomponent, thiol-reactive reagent, as described (25, 26). The fluorescence component of light-with-a-bite is a fluorochrome (FL) moiety, and thus the labeled molecule was designated FL-fVa.

Recombinant S360C APC was labeled with tetramethylrhodamine-5-iodoacetamide (5-TMRMIA, Molecular Probes, Eugene, OR) to provide an acceptor for the RET experiments with FL-fVa. Briefly, S360C APC (5.4 μM) in 50 mM Hepes (pH 7.4), 150 mM NaCl, and 2 mM EDTA was incubated with a 28-fold molar excess of TMRMIA in Me2SO at room temperature in the dark for 3.5 h. Rh-S360C APC was separated from the free label using size exclusion chromatography on a Nap-10 G-25 column (GE Healthcare) equilibrated with 50 mM Hepes (pH 7.4), 150 mM NaCl, and 1 mM EDTA, and then appropriately pooled and dialyzed overnight against the same buffer (3 × 1000 ml). Rh-S360C APC was further purified on an anion exchange column (MonoQ 5/5, GE Healthcare) equilibrated in the same buffer and resolved with a gradient of 0.1–0.6 mM NaCl in 20 ml. Tubes containing Rh-S360C APC were pooled together, concentrated, and changed to 50 mM Hepes (pH 7.4), 150 mM NaCl buffer using a centrifri-microfilter. Protein concentrations were estimated from a value at 280 nm of 1.45 for APC. Dye-to-protein ratios were determined using a value for ε280 of 87,000 M–1 cm–1 for TMRMIA. Samples were stored as conveniently sized aliquots at −80 °C after flash-freezing in liquid nitrogen.

Lipid Vesicles—Small unilamellar vesicles of phosphatidylcholine/phosphatidylserine (PC/PS, 4:1 w/w) were prepared by sonication under a flow of nitrogen using a microtip sonicator followed by extensive centrifugation to separate multimellar vesicles as described previously (27). Lipid concentrations were estimated post-centrifugation using a 14C-PC tracer.

Screening of fVa Peptides—fVa-derived peptides were screened for inhibition of inactivation of fVa by APC and for inhibition of II activation by fXa/fVa using the following conditions. fVa (20 pm final) was incubated with PC/PS (4:1, 25 μM) and peptide (100 μM) in 40 μl of buffer containing 50 mM Tris (pH 7.4), 100 mM NaCl, 5 mM CaCl2, 0.1 mM MnCl2, 31 μM PC/PS, and 0.02% NaN3. After a 30-s incubation of this mixture, APC (51 pm final) or buffer was added, and the mixture was incubated for 30 min at room temperature. Subsequently, 50 μl of fXa (1.25 nM) and 10 μl of II (0.3 μl) were added to activate II. Aliquots (17 μl) of the reaction mixture were withdrawn at time (t) = 0, 0.5, 1, 1.5, 2, and 2.5 min, and IIa generation was quenched with 50 μl of 50 mM Tris (pH 7.4), 100 mM NaCl, 10 mM EDTA, 0.5% BSA, and 0.02% NaN3 buffer. Finally, IIa production was quantified by the addition of 35 μl of 4-carboxybenzenesulfonyl fluoride (0.6 mM), and color generation was monitored at 405 nm using a Vmax plate reader ( Molecular Devices, Sunnyvale, CA).

Some peptides were not soluble in 50 mM Hepes (pH 7.4), 150 mM NaCl (HBS) buffer. This was presumably because of either high hydrophobicity or high acidity of some of the peptides. Solubility in HBS improved after neutralization of pH to 7.0 with NaOH in some cases. In other cases, insoluble peptides were precipitated by centrifugation, and the supernatant was assayed for inhibition. Peptide concentrations were then estimated using the extinction coefficient of tryptophans and tyrosines, if present, or using the estimated extinction coefficient of the peptide bond of the unfolded peptide.

APC Inactivation of fVa—Subsequent to identification of potential inhibitory fVa-derived peptides in screening assays, select peptides were evaluated in detailed fVa inactivation assays using APC. A three-step reaction was used to evaluate the APC-mediated inactivation of fVa. The first step involved the inactivation of fVa by APC in the presence of phospholipid vesicles with or without peptide. The second step involved the generation of IIa by the fXa/fVa-II-PL complex. The final step involved the quantification of IIa generated by the II-ase complex with the aid of a chromogenic substrate. One notes that both meizo-thrombin and alpha-thrombin exhibited similar amidolytic activity, and herein we describe pro-thrombin activation without distinguishing meizo-thrombin from alpha-thrombin.
tion mixture into 55 \mu l of 50 mM Tris (pH 7.4), 100 mM NaCl, 10 mM EDTA, 0.5% BSA, and 0.02% NaN₃ buffer. The IIa produced was quantified by the addition of 35 \mu l of Pefachrome TH (0.6 mM) to the mixture, and color generation was monitored at 405 nm on an OPTImax tunable plate reader (Molecular Devices).

Inactivation assays of plasma-derived R506Q fVa and recombinant-R506QfVa were performed essentially as described above except that the concentration of APC was increased to 6 and 14 nm, respectively, because APC cleavage at Arg³⁰⁶ is much slower than at Arg⁵⁰⁶.

Activated Partial Thromboplastin Time (aPTT) Assays—The activity of Rh-labeled S360C APC was compared with that of plasma-derived APC using the following protocol. Rh-S360C APC (30 nm), S360C APC, or an equimolar concentration of plasma-derived APC in 50 \mu l was mixed with 50 \mu l of chromogenic substrate Pefachrome PCa and p-nitroaniline generation monitored at 405 nm as described.

Amidolytic Assays—The effect of VP311–325 on the amidolytic activity of APC was tested. APC (20 nm) was incubated with five different concentrations of VP311–325 peptide at varying concentrations of Pefachrome PCa and p-nitroaniline generation monitored at 405 nm as described.

Amidolytic activity of Rh-labeled S360C APC was compared with that of plasma-derived APC using an aPTT assay (28). Briefly, normal human plasma (50 \mu l) was incubated with the aPTT reagent Platelin LS (50 \mu l) at 37 °C for 3 min. Subsequently, different concentrations of APC (2 \mu M) were added to this mixture, and clotting was initiated by the addition of 50 \mu l of buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 25 mM CaCl₂, and 0.5% BSA. Clotting times were recorded using a microtiter plate reader at 405 nm as described above.

Activated Partial Thromboplastin Time (aPTT) Assays—The activity of Rh-labeled S360C-APC was compared with that of plasma-derived APC using an aPTT assay (28). Briefly, normal human plasma (50 \mu l) was incubated with the aPTT reagent Platelin LS (50 \mu l) at 37 °C for 3 min. Subsequently, different concentrations of APC (2 \mu M) were added to this mixture, and clotting was initiated by the addition of 50 \mu l of buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 25 mM CaCl₂, and 0.5% BSA. Clotting times were recorded using a microtiter plate reader at 405 nm as described above.

Gel Electrophoresis—The inhibition of APC-dependent inactivation of fVa by peptide VP311–325 was followed by SDS-PAGE. fVa (0.75, 1, or 1.2 \mu M) in buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM CaCl₂, and 150 mM PC/PS vesicles was incubated with APC (100 \mu M) for 90 min at 37 °C in the presence (0.5 or 1 mM) or absence of VP311. Subsequently, fVa inactivation was stopped by the addition of 5 mM EDTA followed by addition of hot SDS with reducing agent, and samples were analyzed by gel electrophoresis using 4–12% BisTris gels (Invitrogen). bands were visualized using SimplyBlue (Invitrogen) stain according to manufacturer’s protocols.

Spectral Measurements—Steady state fluorescence intensity 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 in Fl-fVa was detected at its emission and excitation maximal wavelengths of 490 and 521 nm, respectively, and using a 345-nm cut-off filter (Schott Glass Tech., Duryea, PA) in the excitation beam path. Dansyl fluorescence in DEGR-APCi, was detected at an excitation and emission maximum of 340 and 540 nm, respectively. A circulating water bath was used to maintain the sample compartment at 25 °C. All fluorescence experiments were performed in 5 x 5 mm quartz cuvettes. Samples were mixed using a small Teflon-coated 2 x 2 mm magnetic spinbar as described (29). Adsorption of pro-tein to the cuvette walls was minimized by coating the cuvettes with 100% PC vesicles as described (30).

For the binding of peptide VP311–325 to DEGR-APCi, two samples were prepared in parallel. The sample cuvette received DEGR-APCi (100 nm) in 50 mM Hepes (pH 7.4), 150 mM NaCl, and 2 mM CaCl₂, whereas the blank cuvette received an equal concentration of unlabeled active site inhibited FFR-APCi in the same buffer. The initial net fluorescence intensity of the sample, designated F₀, was obtained by the subtraction of a dye-free blank cuvette from the sample cuvette signal. Subsequent, equimolar concentrations of VP311–325 peptide were added to each 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. Dissociation constants were obtained by plotting (F₀ – F)/F₀ versus peptide concentrations and fitting the curves to a quadratic equation as described (23, 31). The titration of VP311 with DEGR-IIa, was performed analogously.

RET Experiments—The interaction of PC/PS membrane-bound fVa with APC was monitored using singlet-to-singlet energy transfer. The fluorescein dye on Fl-fVa served as a donor, whereas the Rh dye in the active site of Rh-labeled S360C-APC served as the acceptor. Four samples were prepared in parallel for each energy transfer experiment: cuvette D (containing donor) and cuvette DA (containing donor and acceptor) received 25 nm Fl-fVa (donor), whereas cuvettes A (containing acceptor) and B received 25 nm unlabeled fVa. The net initial emission intensity (F₀) was obtained by the subtraction of the signal of B from D, DA, and A. Prior to the APC titration, all cuvettes received 25 \mu M (final) of PC/PS. Samples D and B were titrated with S360A APC, whereas samples DA and A were titrated Rh-S360C APC. The net intensity of D, DA, or A (Fᵣ, Fᵣᵦ, and Fᵣᵢ, respectively) was obtained by subtracting the signal from the B cuvette and correcting for dilution. To correct for any signal in the DA sample because of direct excitation of the acceptor, the net dilution-corrected emission intensity from A was subtracted from the DA sample signal. Making the reasonable assumption that the absorption of Fl is unaffected by the presence of Rh-labeled APC, the ratio of donor quantum yields in D and DA samples is given by

\[
\frac{Q_D}{Q_A} = \frac{[F_D/(F_D)₀]}{[(F_DA - F_A)/(F_DA - F_A)₀]} \quad (Eq. 1)
\]

where F is the net dilution-corrected emission intensity of a sample at some point in the titration and the subscript o is used to denote the initial intensity of the sample.

RESULTS

APC-dependent Inhibition of II-ase—fXa has been shown to inhibit the inactivation of fVa by APC (16–18, 32). However, the mechanism(s) by which this occurs is yet unclear. The converse, that APC inhibits fXa activity in an II-ase complex, was tested. Varying concentrations of S360A APC, a variant of APC with no amidolytic activity, were used in II-ase assays. Upon increasing S360A APC concentrations, inhibition of IIa generation was observed in the II-ase assays. This inhibition was abolished upon increasing fXa concentrations in II-ase...
APC Binding Region on fVa Heavy Chain

![Graph showing S360A APC inhibition of IIa generation by the II-ase complex.](image)

FIGURE 1. S360A APC inhibition of IIa generation by the II-ase complex. S360A APC (0–3 nM) was incubated with PC/PS-bound fVa (0.55 nM initial) before the cofactor activity of fVa was tested in a II-ase assay using varying concentrations of fXa (0.6–5 nM) as described under “Experimental Procedures.”

complex. A plot of 1/[fXa] versus 1/IIa, generated as velocity in mOD/min, is shown in Fig. 1. This plot shows that S360A-APC is a classic competitive inhibitor of fXa in the II-ase complex. Similarly, S360A APC also inhibited IIa generation by Gla-domainless fXa (not shown), suggesting that the inhibition by S360A APC was because of abrogation of fXa-fVa protein-interactions. These data suggest that fXa and APC could share overlapping binding sites on fVa.

Screening of fVa Peptides—Fifteen amino acid-long peptides representing the entire sequence of the fVa heavy chain and overlapping adjacent peptides by 5 amino acids were tested for inhibiting APC-dependent fVa inactivation. In parallel experiments, screens were also performed in the absence of APC to assess the direct effect of the peptides in the II-ase assay. Fig. 2 shows the results of such screening assays. The peptide comprising amino acids 1–15 was termed VP1 and peptide comprising the amino acids 11–25 was termed VP11. All other peptides were designated analogously by the first residue number.

The left side of Fig. 2 shows that only a few peptides inhibited IIa assay. Under our assay conditions, fVa typically retained ~25–29% of its activity after incubation with PC/PS vesicles and APC for 30 min. The right side of Fig. 2 shows that a few peptides inhibited fVa inactivation by APC. The vast majority of the peptides tested did not have significant effects in either of the assays employed. However, a small selection of peptides had a significant effect in one or both of the assays. Four types of patterns were observed from these screens: (a) peptides that inhibited only the IIa assay; (b) peptides that inhibited the APC-dependent inactivation of fVa but had no effect on IIa assay; (c) peptides that inhibited both IIa assay and fVa inactivation by APC; and (d) peptides that inhibited IIa assays but enhanced fVa inactivation by APC. Peptides that conformed to the first pattern included VP21, VP61, and VP401. Peptide VP351 inhibited APC inactivation of fVa but not the IIa assay. Peptides VP251, VP311, VP321, and VP511 inhibited both APC-dependent inactivation of fVa and IIa assay, and inhibited both assays. Finally, peptide VP491 inhibited IIa assay but enhanced APC-dependent inactivation of fVa.

Effect of Peptide VP311 on Inactivation of fVa by APC—Of the four peptides that inhibit both fVa-dependent activity in IIa assay and APC-dependent inactivation of fVa, peptide VP311 was the most potent inhibitor of APC-dependent inactivation of fVa. To further characterize the inhibitory effects of peptide VP311, the inactivation of fVa by APC was monitored over 30 min (Fig. 3). Four different reactions were performed in parallel: (a) reaction containing both APC and VP311 (+, +); (b) reaction containing APC but no VP311 (+, −); (c) reaction containing no APC but containing peptide (−, +); and (d) reaction containing no APC or peptide (−, −). APC inactivated fVa over the course of 30 min in a biphasic reaction. In the absence of APC and peptide (−, −) fVa activity was stable for 30 min. Peptide VP311 by itself inhibited fVa activity as reported (11), but this effect was slight (~10%) and constant over the 30-min time period. VP311 significantly inhibited APC-dependent inactivation of fVa (Fig. 3). These data show that peptide VP311 is a good inhibitor of the APC-dependent inactivation of fVa. Fig. 4A shows that VP311 inhibited APC activity in a dose-dependent fashion with a 50% inhibition of ~70 μM.

To verify that the VP311 peptide did not inhibit APC-dependent fVa inactivation by inhibiting the amidolytic activity of APC, the amidolytic activity of APC was measured. Fig. 4B shows that peptide VP311 did not have any significant effect on the ability of APC to cleave the chromogenic substrate Pefachrome PCa. Additionally, the peptide did not significantly alter the ability of IIa to cleave Pefachrome TH (not shown). Thus, peptide VP311 specifically inhibited APC-dependent inactivation of fVa.

Effect of Peptide VP311 Inactivation of R506Q fVa and RR306/679QQ fVa by APC—To test the hypothesis that VP311 inhibits the Arg306 bond cleavage in fVa, APC was incubated with R506Q fVa in the presence or absence of peptide VP311 before quantifying the residual activity of R506Q fVa in a II-ase assay. Because APC cleaves after Arg residues, R506Q fVa is resistant to cleavage by APC at position 506 and lacks the typical biphasic inactivation profiles observed for normal fVa upon APC inactivation. Fig. 5A and B shows that VP311 inhibited the APC-dependent inactivation of both plasma-derived and recombinant R506Q fVa, respectively.

To test whether peptide VP311 inhibited the Arg306 bond cleavage in fVa by APC, a recombinant double mutant RR306/679QQ fVa was prepared and was incubated in the presence or absence of VP311 prior to the determination of residual fVa activity by IIa assay. Fig. 5C shows that VP311 also inhibited cleavage at Arg306.

Effect of Peptide VP311 on fVa Inactivation by APC/Protein S—Protein S is the nonenzymatic protein cofactor of APC in the inactivation of fVa. Protein S acts as a cofactor to APC by preferentially enhancing the Arg306 cleavage in fVa by APC (33). Recent studies indicate that fXa protects fVa from APC cleavage at Arg306 but enhances the Arg306 cleavage by APC (32). Although protein S ablates the protective effect of fXa in the APC cleavage of fVa at Arg306 it enhances the fXa-dependent increase at Arg306.

To test the effect of peptide VP311 on fVa inactivation by APC/protein S, fVa was incubated with APC/protein S in the presence or absence of peptide VP311 prior to estimation of fVa activity by IIa assay. Fig. 6 shows that protein S enhanced the activity of APC in the inactivation of fVa compared with APC alone (Fig. 3). Further, in the presence of peptide VP311, mix-
Subsequently, S360C APC was labeled with a molar excess of TMRIA acceptor dye and the labeled protein purified from the excess reactants as described under “Experimental Procedures.” The dye-to-protein ratio was determined to be 1:1 using a molar extinction coefficient of 87,000 M⁻¹ cm⁻¹ for Rh. When a mock labeling was performed with plasma-derived APC, <5% of the APC was labeled with 5-TMRIA, suggesting that the labeling was primarily at the mutation site (C360) in Rh-S360C APC. Rh-S360C APC was compared with wild-type recombinant APC in SDS-PAGE analysis and in APC functional assays. Fig. 8A shows that Rh-S360C APC and wild-type APC (wt-APC) controls had very similar mobility on SDS-PAGE. This implies that the introduction of a serine-to-cysteine mutation in the active site of APC did not introduce dimerization artifacts on APC. Fig. 8B shows that the Rh-S360C APC did not significantly prolong the aPTT compared with wt-APC, suggesting that Rh-S360C APC is a poor anticoagulant compared with wt-APC.

To show that Rh-S360C APC is a poor anticoagulant because it does not hydrolyze the amide bond in the substrate, Rh-S360C APC and wt-APC were compared for their ability to hydrolyze a small chromogenic peptide substrate. Fig. 8C shows that Rh-S360C APC did not hydrolyze a small chromogenic substrate, unlike wt-APC controls. These results were expected as the cysteine variant should not form the tetrahedral intermediate with the carbonyl carbon on the P₁ site of the substrate.
because this variant lacks the lone pair of electrons from the hydroxyl group from serine 360. Consequently, there is no formation of the acyl enzyme intermediate and thus a lack of substrate hydrolysis. This Rh-S360C APC is a convenient tool to study APC binding to fVa because it can be easily labeled in the active site and retains all fVa binding exosites but lacks fVa cleaving activity, which would otherwise complicate biophysical assays.

Singlet-to-Singlet Energy Transfer from Fl-fVa to Rh-S360C APC—To monitor the interaction of fVa with APC by RET, fVa was labeled with a fluorescein donor according to procedures described previously (26), and membrane-bound Fl-fVa was titrated with Rh-S360C APC (acceptor). When Fl-fVa was titrated with PC/PS (4:1 mole ratio) vesicles, the fluorescein intensity increased (−5%) and reached a plateau at −8–10 μM PC/PS vesicles, suggesting that Fl-fVa was membrane-bound (not shown). These results are in accordance with previously reported RET experiments with Fl-fVa and Rh-labeled PC/PS vesicles. To ensure that all of the Fl-fVa molecules were membrane-bound, an excess of PC/PS vesicles (25 μM) was added sufficient to bind fVa saturably. When PC/PS-bound Fl-fVa (donor) was titrated with S360A-APC control, a small decrease (−7%) in the Fl-fVa fluorescence was observed in the D cuvette. However, when PC/PS-bound Fl-fVa was titrated with Rh-S360C APC, a significant decrease in Fl-fVa signal (−20%) was observed in the DA cuvette reaching a plateau value −125 nm of Rh-S360C APC (Fig. 9). To facilitate analysis, the data have been normalized and expressed as a ratio of donor quantum yields in the presence and absence of acceptor (QD/QA) and as a function of Rh-S360C APC concentration in Fig. 9. This Rh-S360C APC-dependent decrease in Fl-fVa intensity (Fig. 9) indicates that the donor dyes on Fl-fVa are close enough to the acceptor dyes on APC for RET to occur. To investigate the effect of peptide VP311 on the Fl-fVa to Rh-S360C APC RET, samples of membrane-bound Fl-fVa were prepared. Then a bolus of VP311 (100 μM) was added to each cuvette before Rh-S360C APC was titrated into these samples. The peptide itself caused a small change (−5%) in Fl-fVa fluorescence at the high concentrations used. Upon titration of Rh-S360C APC into these samples, the ratio of donor quantum yields in the presence and absence of acceptor remained constant suggesting that the Fl-fVa molecules are not close enough to the acceptor dyes on APC for RET to occur. This is most likely because of VP311 peptide competing with Fl-fVa for Rh-S360C APC binding.

**DISCUSSION**

The present study reveals that peptide VP311, a peptide corresponding to the amino acid residues 311–325 and situated in a surface-exposed loop between the A1 and A2 domains of fVa, represents an APC binding site on fVa. This conclusion is based on the following observations: (a) VP311 is a potent inhibitor of APC-dependent inactivation of fVa; (b) the peptide directly binds to and alters the conformation of DEGR-APCi; and (c) the peptide interferes with RET that reflects the binding of Fl-fVa to Rh-labeled APC.

S360A APC, a variant of APC with no amidolytic activity, inhibits IIa generation by the II-ase complex. This inhibition is not solely because of competition for phospholipids because S360A APC inhibited IIa generation by the Glα-domainless fxα+fvα complex as well, suggesting that the inhibition was due to disruption of protein-protein interactions. S360A APC can inhibit IIa generation by binding to fvα and sterically hindering the assembly of the II-ase complex, or by binding to and altering the structure of fvα such that fxα and II binding sites on fvα are no longer available for interaction with these proteins. The for-
mer possibility is supported by the fact that fXa inhibits APC-dependent inactivation of fVa. In other words, fXa and APC mutually inhibit their respective interactions with fVa. Given the fact that S360A APC-dependent inhibition of IIa is competitive with respect to fXa (Fig. 1), it is most likely that fXa and APC could share binding sites on fVa.

The hypothesis that fXa and APC share binding sites on fVa was tested using inhibitory peptides derived from the fVa heavy chain. Seventy-one 15-mer peptides encompassing the entire fVa heavy chain were screened in two different assays, one for inhibiting APC-dependent fVa inactivation and one for inhibiting IIa-assay in the presence of fVa. Only a few peptides had any significant effects in these assays, consistent with the idea that only certain limited regions of fVa are involved in interactions with APC and fXa. Peptides VP251, VP311, VP321, and VP511 inhibited both assays, seemingly having opposite effects on IIa-generation in the presence and absence of APC. Notably, regions representative of peptides VP311, VP321, and VP511 have been shown to be fXa binding sites using either peptide inhibition techniques and/or fV mutagenesis approaches (11, 14, 15). Thus, our peptide screens are not only consistent with previously published data but suggest that these regions could also represent APC binding sites on fVa. The present study characterizes in detail the effects of peptide VP311 on the IIa-ase complex in the presence and absence of APC.

Peptide VP311 was earlier reported to inhibit, in a fVa-dependent fashion, IIa generation in a IIa-assay (11). In this new study, the peptide inhibited both APC inactivation of fVa and fXa-dependent effects in IIa-assays. Furthermore, the inhibitory effects of the peptide were dose-dependent (Fig. 4). The peptide did not significantly inhibit the amidolytic activity of APC. These data suggest that the region represented by the VP311 peptide on fVa could provide binding sites on fVa for both fXa and APC.

The inactivation of fVa by APC is a complex process that involves proteolysis at Arg^{506}, Arg^{306}, and Arg^{679}. Although the
hydrolysis at Arg506 is kinetically favored, it leads to only a partial inactivation of fVa, whereas the hydrolysis of Arg306 leads to the complete inactivation of fVa. The differences in kinetics between the Arg506 and Arg306 cleavages are presumably because of differences in specific interactions between exosites on APC and fVa surfaces surrounding the cleavage sites. Approximately 34 Å separates Arg506 and Arg306 in the structures of fVa (12), suggesting it is likely that at least two modes of binding are involved in APC binding fVa to cleave the two sites on fVa. In support of this hypothesis, it was earlier demonstrated that three loops of APC, namely the autolysis loop (28, 34), the 37-loop, and the calcium binding loop (22), define an extended fVa binding site on APC and that mutations in these loops of APC can discriminate and account for much of the difference in cleavage rates for the Arg506 and Arg306. Therefore, the effect of peptide VP311 on individual bond cleavages in fVa by APC was investigated using various fVa mutants. Results showed that peptide VP311 inhibits the APC cleavage at both Arg506 and Arg306 in fVa.

Protein S enhances the ability of to inactivate fVa. Recent evidence indicates that the protein enhances cleavages at both Arg506 and Arg306 of fVa although the rate of cleavage at the former site is enhanced to a greater extent than at the latter site. Peptide VP311 inhibited the inactivation of fVa by the APC-protein S complex.

To see whether peptide VP311 specifically binds to APC, we used fluorescence spectroscopy. Upon titration with peptide VP311, the steady state emission of dansyl in DEGR-APCi was altered, whereas no change in the fluorescence of the dansyl dye was observed with similarly labeled DEGR-IIai. These data suggest that peptide VP311 binds specifically to APC. Kojima et al. (11) earlier reported that peptide VP311 binds to and alters the steady state fluorescence of DEGR-IXai. In conjunction with the present data, this suggests that the region represented by peptide VP311 in fVa is a binding site for both APC and fXa. In a recently published model of fXa docked to fVa, Autin et al. (12) showed extensive interactions between fXa and fVa and involving residues 311–325 on fVa. In the Autin model, residue Lys320 of fVa seems to make key contacts with Thr177, Ile175, Gln178, Thr177, and Asn179 of fXa (all chymotrypsin numbering).

The inhibition of APC-dependent fVa inactivation by VP311 is most likely because of the peptide binding to an APC exosite and interfering with fVa-APC interactions. This hypothesis was tested using the singlet-to-singlet RET approach. The observed RET between PC/PS vesicle-bound Fl-fVa (donor) and Rh-S360C APC (acceptor) suggests that the two molecules bind in the absence of peptide VP311, whereas the absence of RET between the two molecules in the presence of excess peptide VP311 suggests that the peptide inhibits the interaction of fVa and APC. Notably in these assays, RET was used only qualitatively to monitor binding between fVa and APC, and no quantitative data such as distances were extracted. This is because the exact location of the label on fVa is as yet unknown. In an earlier study, we reported that fVa treated with the cysteine-

FIGURE 9. Effect of peptide VP311 on Fl-fVa to Rh-S360C APC singlet-to-singlet RET. PC/PS-bound Fl-fVa (22.5 nM initial) was titrated with Rh-S360C APC in the absence (solid circles) or presence (open squares) of 100 µM peptide VP311 as described under “Experimental Procedures.” Ratios of quantum yield of the donor in the absence and in the presence of the acceptor ($Q_D/Q_A$) were determined accordingly.
specific light-with-a-bite dye labeled predominantly in the heavy chain of fVa, but some adventitious labeling was also observed in the light chain of the molecule (26). Because of the presence of multiple donors on fVa, conclusions about precise distances between donors on fVa and acceptors on APC are avoided here.

In conclusion, various interactions between fVa, fXa, and APC were investigated here. The present findings suggest that APC and fXa compete for binding to residues 311–325 in fVa.

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REFERENCES

1. Nesheim, M. E., Taswell, J. B., and Mann, K. G. (1979) J. Biol. Chem. 254, 10952–10962
2. Rosing, J., Tans, G., Govers-Riemslag, J. W., Zwaal, R. F., and Hemker, H. C. (1980) J. Biol. Chem. 255, 274–283
3. Nicolaes, G. A. F., and Dahlback, B. (2002) Arterioscler. Thromb. Vasc. Biol. 22, 530–538
4. Kalafatis, M., Xue, J., Lawler, C. M., and Mann, K. G. (1994) Biochemistry 33, 6538–6545
5. Kalafatis, M., Rand, M. D., and Mann, K. G. (1994) J. Biol. Chem. 269, 31869–31880
6. Nicolaes, G. A., Tans, G., Thomassen, M. C., Hemker, H. C., Pabinger, I., Varadi, K., Schwarz, H. P., and Rosing, J. (1995) J. Biol. Chem. 270, 21158–21166
7. Heeb, M. J., Rehemtulla, A., Moussalli, M., Kojima, Y., and Kaufman, R. J. (1999) Eur. J. Biochem. 260, 64–75
8. Egan, J. O., Kalafatis, M., and Mann, K. G. (1997) Protein Sci. 6, 2016–2027
9. Norstrom, E. A., Steen, M., Tran, S., and Dahlback, B. (2003) J. Biol. Chem. 278, 24904–24911
10. Gale, A. J., Xu, X., Pellequer, J. L., Getzoff, E. D., and Griffin, J. H. (2002) Protein Sci. 11, 2091–2101
11. Kojima, Y., Heeb, M. J., Gale, A. J., Hackeng, T. M., and Griffin, J. H. (1998) J. Biol. Chem. 273, 14900–14905
12. Axtin, L., Steen, M., Dahlback, B., and Villoutreix, B. O. (2006) Proteins 63, 440–450
13. Heeb, M. J., Kojima, Y., Hackeng, T. M., and Griffin, J. H. (1996) Protein Sci. 5, 1883–1889
14. Kalafatis, M., and Beck, D. O. (2002) Biochemistry 41, 12715–12728
15. Steen, M., Villoutreix, B. O., Norstrom, E. A., Yamazaki, T., and Dahlback, B. (2002) J. Biol. Chem. 277, 50022–50029
16. Walker, F. I., Sexton, P. W., and Esmon, C. T. (1979) Biochim. Biophys. Acta 571, 333–342
17. Nesheim, M. E., Canfield, W. M., Kisiel, W., and Mann, K. G. (1982) J. Biol. Chem. 257, 1443–1447
18. Suzuki, K., Stenflo, J., Dahlback, B., and Teodorsson, B. (1983) J. Biol. Chem. 258, 1914–1920
19. Chase, T., and Shaw, E. (1969) Biochemistry 8, 2212–2224
20. Mesters, R. M., Houghten, R. A., and Griffin, J. H. (1991) J. Biol. Chem. 266, 24514–24519
21. Heeb, M. J., Kojima, Y., Greengard, J. S., and Griffin, J. H. (1995) Blood 85, 3405–3411
22. Gale, A. J., Tsavalier, A., and Griffin, J. H. (2002) J. Biol. Chem. 277, 28836–28840
23. Krishnaswamy, S., Williams, E. B., and Mann, K. G. (1986) J. Biol. Chem. 261, 9684–9693
24. Lu, R., Esmon, N. L., Esmon, C. T., and Johnson, A. E. (1989) J. Biol. Chem. 264, 12956–12962
25. Yegneswaran, S., Fernandez, J. A., Griffin, J. H., and Dawson, P. E. (2002) Chem. Biol. 9, 485–494
26. Yegneswaran, S., Mesters, R. M., Fernandez, J. A., and Griffin, J. H. (2004) J. Biol. Chem. 279, 49019–49025
27. Husten, E. J., Esmon, C. T., and Johnson, A. E. (1987) J. Biol. Chem. 262, 12953–12961
28. Gale, A. J., Heeb, M. J., and Griffin, J. H. (2000) Blood 96, 585–593
29. Dell, V. A., Miller, D. L., and Johnson, A. E. (1990) Biochemistry 29, 1757–1763
30. Ye, J., Esmon, N. L., Esmon, C. T., and Johnson, A. E. (1991) J. Biol. Chem. 266, 23016–23021
31. Koppaka, V., and Lentz, B. R. (1996) Biophys. J. 70, 2930–2937
32. Norstrom, E. A., Tran, S., Steen, M., and Dahlback, B. (2006) J. Biol. Chem. 281, 31486–31494
33. Rosing, J., Hoekema, L., Nicolaes, G. A., Thomassen, M. C., Hemker, H. C., Varadi, K., Schwarz, H. P., and Tans, G. (1995) J. Biol. Chem. 270, 27852–27858
34. Friedrich, U., Blom, A. M., Dahlback, B., and Villoutreix, B. O. (2001) J. Biol. Chem. 276, 24122–24128