Contribution of the Heavy and Light Chains of Factor Va to the Interaction with Factor Xa†

Michael Kalafatis, Jiachun Xue,‡ Casey M. Lawler,§ and Kenneth G. Mann*

Department of Biochemistry, Given Building, Health Science Complex, University of Vermont, College of Medicine, Burlington, Vermont 05405-0068

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ABSTRACT: The interactions of the isolated heavy and light chains of factor Va with factor Xa were evaluated using active-site-modified factor Xa [(carboxytetramethyl)rhodamine-Glu-Gly-Arg-factor Xa (ctr-EGR-Xa)]. The K_d for the factor Va heavy-chain interaction with ctr-EGR-Xa was 60 μM. A series of monoclonal antibodies directed against bovine factor Va were tested for their ability to inhibit thrombin formation in an assay using the fluorescent thrombin inhibitor dansylarginine *N,N*-(3-ethyl-1,5-pentanediyl)amide (DAPA). Monoclonal antibody αBFV-4, which recognizes the light chain of the cofactor, was found to inhibit the formation of thrombin. Similarly, monoclonal antibody αBFV-5, which is directed against the heavy chain of the cofactor, was found to inhibit thrombin formation. In contrast, monoclonal antibody αBFV-1, also directed against the heavy chain of the cofactor, did not inhibit thrombin generation by the *prothrombinase* complex. Monoclonal antibodies αBFV-4 and αBFV-5 inhibited the interaction of active-site-modified radiolabeled factor Xa (^{125}I-Xa-EGR) with factor Va bound to PC/PS-coated microtiter wells, whereas nonimmune mouse IgG did not have any effect on the ^{125}I-Xa-EGR·membrane-bound factor Va interaction. The antibodies effect upon the phospholipid-independent interaction between the cofactor and ctr-EGR-Xa was evaluated by analytical ultracentrifugation. Both αBFV-4 and αBFV-5 inhibited the phospholipid-independent interaction between factor Va and ctr-EGR-Xa. Immunoblotting experiments using factor Va, its subunits, and fragments resulting from cleavage of the cofactor with activated protein C (APC) demonstrated the presence of the αBFV-4 epitope on a fragment from the NH_2-terminal part of the light chain of factor Va (amino acids 1537–1752, A3 domain). The epitope for αBFV-5 was located to an M_r = 40 000 fragment containing the A1 domain of the cofactor (residues 1–306, A1 domain). Our data suggest that the factor Va–factor Xa interaction involves the A1 and A3 domains of the factor Va molecule.

The *prothrombinase* complex composed of factor Va, factor Xa, a phospholipid membrane, and Ca^{2+} plays a central role in the coagulation cascade (Mann et al., 1980, 1988, 1990). The *prothrombinase* complex has a catalytic efficiency 5 orders of magnitude higher than factor Xa acting alone (Nesheim et al., 1979a). Factor V circulates in plasma as a large single-chain procofactor with an M_r = 330 000 (Nesheim & Mann, 1979). The cDNA sequences for human and bovine factor V have been determined (Kane & Davie, 1986; Jenny et al., 1987; Guinto et al., 1992). The derived amino acid sequences correspond to a molecule with triplicated "A" domains, duplicated "C" domains, and a "B" region. Factor V is cleaved by thrombin and factor Xa to generate the active cofactor, factor Va (Nesheim & Mann, 1979; Foster et al., 1983b; Monkovic & Tracy, 1991). Bovine factor Va is composed of a heavy chain (A1–A2 domains, factor Va_{HC}, M_r = 94 000) derived from the NH_2-terminal portion of the factor V molecule and a light chain (A3–C1–C2, factor Va_{LC}, M_r = 74 000) derived from the COOH-terminal domain of factor V (Nesheim & Mann, 1979, Guinto et al., 1992). The two chains are noncovalently associated via divalent metal ions (Esmon, 1979; Krishaswamy et al., 1989).

Factor X circulates in plasma as a zymogen (M_r = 56 000) composed of a heavy chain factor (X_{HC}, M_r = 48 000) and a light chain (factor X_{LC}, M_r = 18 000) which are covalently associated by a disulfide bond (Fujikawa et al., 1972). Factor X is converted to its active form (factor Xa) either by the intrinsic *tenase* (factor IXa and its cofactor, factor VIIIa) or by the extrinsic *tenase* (factor VIIa in the presence of tissue factor) (Jackson & Nemerson, 1980; Lawson & Mann, 1991; Krishaswamy et al., 1992) by cleavage of factor X_{HC} between Arg_{193} and Ile_{194} (Titani et al., 1975; Jackson & Nemerson, 1980).

The binding of factor Xa to phospholipid vesicles is dependent upon the presence of divalent metal ions and is mediated through a process involving the γ-carboxyglutamic acid residues located at the NH_2-terminal part of factor Xa_{LC} and the acidic phospholipid (Skogen et al., 1984; Morita & Jackson, 1986; Krishaswamy et al., 1992). The binding of factor Va to phospholipid vesicles is independent of Ca^{2+} but requires acidic phospholipid and is promoted by two regions located on the light chain of the cofactor (Higgins & Mann, 1983; van de Waart, et al., 1983; Tracy & Mann, 1983; Krishnaswamy & Mann, 1988; Kalafatis et al., 1990, 1994; Ortel et al., 1992). In the absence of a membrane surface, the factor Va–factor Xa protein interaction is governed by a K_d of 0.8 μM and is dependent on the presence of Ca^{2+} (Pryzdial & Mann, 1991). In the presence of phospholipid vesicles and Ca^{2+}, the K_d of the factor Va–factor Xa association is decreased, to approximately 1 nM stabilized by protein–

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* To whom correspondence should be addressed.

‡ Present address: Howard Hughes Medical Institute, Research Laboratories, Washington University School of Medicine, Saint Louis, MO 63110.

§ Present address: Department of Medicine, Abbott Northwestern Hospital, Minneapolis, MN 55413.

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protein and protein–lipid interactions (Nesheim et al., 1979a; Lindhout et al., 1982; Skogen et al., 1984; Krishnaswamy, 1990).

The factor Xa binding site for factor Va is independent of the NH_2-terminal portion of Xa_{LC} (Skogen et al., 1984; Morita & Jackson, 1986; Persson et al., 1993) and has been suggested to involve residues 263–274 of factor Xa_{HC} (Chattopadhyay et al., 1992). The factor Va binding site for factor Xa involves Ca^{2+} and both chains of the cofactor (Tucker et al., 1983; Guinto & Esmon, 1984; Annamalai et al., 1987; Pryzdial & Mann, 1991). Each chain has been shown to independently interact with rx-EGR-Xa (Pryzdial & Mann, 1991). The present study was undertaken in order to estimate the contribution of the two chains of factor Va to the interaction with factor Xa and to identify the domains within the two chains of the cofactor involved in the factor Va–factor Xa association.

EXPERIMENTAL PROCEDURES

Materials and Reagents. Hepes, Q-Sepharose fast flow, Sepharose CL-4B, cyanogen bromide (CNBr), bovine serum albumin (BSA), 1-palmitoyl-2-oleoylphosphatidylserine (PS), and 1-palmitoyl-2-oleoylphosphatidylcholine (PC) were purchased from Sigma (St. Louis, MO). D-Phenylalanylprolylarginyl chloromethyl ketone (FPR-ck) and L-glutamylglycyl-L-arginine chloromethyl ketone (EGR-ck) were purchased from Calbiochem (San Diego, CA). Trifluoroacetic acid (TFA) was from Pierce (Rockford, Il). 5(6)-(Carboxytetramethyl)rhodamine succinimidyl ester was from Molecular Probes Inc. (Eugene, OR). The fluorescent thrombin inhibitor dansylarginine, *N*,*N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) was prepared as previously described (Nesheim et al., 1979b) and was a gift of Dr. Paul Haley (Haematologic Technologies Inc., Essex Junction, VT). Bovine prothrombin, thrombin, and factor Xa were purified as previously reported (Bajaj & Mann, 1973; Lundblad et al., 1976; Krishnaswamy et al., 1987). Bovine factor Va and factor Va subunits were purified as previously described (Nesheim et al., 1980; Katzmann et al., 1981; Kalafatis et al., 1993b). Phospholipid vesicles composed of 75% PC and 25% PS were prepared as previously described (Barenholz et al., 1977). The concentration of the phospholipid vesicles was determined by phosphorus assay as described earlier (Gomori, 1942). Monoclonal antibodies αBFV-1, αBFV-4, and αBFV-5 were provided as an ascites fluid by Dr. W. R. Church (University of Vermont) and were purified as described (Foster et al., 1982). The nomenclature given in the present paper is different from that used previously. For comparison, the antibodies are identical as follows: αBFV-1, previous designations IB6 and α_1D (Foster et al., 1982; Tucker et al., 1983); αBFV-4, previous designations IIC3 and α_1E (Foster et al., 1982, 1983a; Tucker et al., 1983); αBFV-5, previous designation IIIA5 (Foster et al., 1982). Purified αBFV-4 and αBFV-5 immunoglobins (IgGs) were immobilized on CNBr-activated Sepharose CL-4B as described (March et al., 1974). (Carboxytetramethyl)rhodamine-labeled chloromethyl ketone (ctr-EGRck) was prepared as previously reported (Williams et al., 1989), for the analogous synthesis of fluoresceinyl-EGRck. Incorporation of ctr-EGRck into factor Xa was performed as previously described (Pryzdial & Mann, 1991). The protein concentrations were determined spectrophotometrically by the absorbance at 280 nm and calculated using published molecular weights (M_r) and extinction coefficients ($\epsilon^{1\%}_{280nm}$) as follows: factor V_a, M_r = 168 000, 17.4; Va_{LC}, M_r = 74 000, 22.3; Va_{HC}, M_r = 94 000, 12.4 (Krishaswamy & Mann, 1988); factor Xa, M_r = 46 000, 11.6 (DiScipio et al., 1977). The concentration of ctr-EGRck was calculated using the molar extinction coefficient of the fluorophore at 560 nm (60 000 M^{-1} cm^{-1}) (Haugland, 1992).

Assay Measuring Thrombin Formation. Factor Va (33 nM) was incubated with monoclonal antibodies αBFV-1, αBFV-4, and αBFV-5 (333 nM) or with buffer [20 mM Hepes/0.15 M NaCl (HBS), 2 mM $CaCl_2$ (HBS+)] for 1 h on ice. Monoclonal antibodies αBFV-1, αBFV-4, and αBFV-5 are specific for bovine factor Va. Furthermore, antibodies αBFV-4 and αBFV-5 were shown to inhibit factor Va cofactor activity in clotting assays (Foster et al., 1982) whereas antibody αBFV-1 was shown to be specific for the heavy chain of the cofactor. Following incubation, factor Va cofactor activity was analyzed using the fluorescent thrombin inhibitor DAPA as described (Nesheim et al., 1979b; Kalafatis & Mann, 1993). The final concentration of factor Va was 1 nM.

Protein Iodination. Factor Va labeling was performed with $Na^{125}I$ (Amersham Corp., Arlington Heights, IL) using Iodo-Gen (Pierce Chemical Co.) as previously described by Fraker and Speck (1978). Free sodium iodide was removed by gel filtration using Excellulose F5 desalting columns (Pierce Chemical Co.).

Purified bovine factor Xa was incubated with a 10-fold molar excess of EGRck for 30 min, and the factor Xa mixture was labeled using Iodo-Gen (Pierce Chemical Co.) and $Na^{125}I$ (Amersham Corp.) as described above for factor Va. Free EGRck and free sodium iodide were removed by gel filtration using excellulose F5 desalting columns.

Phospholipid Plate Binding Assay. A solution containing 100% PS or a mixture composed of 75% PC and 25% PS was diluted in methanol to a concentration of 300 μg/mL, and the solution was kept under nitrogen at −20 °C. Prior to use, PC and PS were diluted to 3 μg/mL in methanol, and 100 μL of the solution was applied to a 96-well microtiter plate loaded with Removalwell strips (Immunlon 4; Dynatech Laboratories, Inc., Chantilly, VA). The wells were allowed to dry overnight at room temperature in a vacuum desiccator in order to prevent oxidation of the phospholipid. The wells were blocked with a solution of 20 mM Tris-HCl, 0.15 M NaCl, and 2 mM $CaCl_2$ (TBS, Ca^{2+}) containing 1% BSA (blocking buffer) for 2 h at room temperature. The wells were washed 2 times with TBS/0.1% Tween 20 (washing buffer), and 50-μL samples of iodinated factor Va at various concentrations were added to each well in duplicate. All dilutions of factor Va were performed in the blocking buffer. After 2-h incubation at room temperature, the wells were washed 5 times with the washing buffer and counted individually. In order to determine the PS coating efficiency on the microtiter wells, PS at 3 μg/mL (5 mL) was mixed with 50 μL of a solution of L-3-phosphatidyl-L-[3-^{14}C]serine (Amersham Corp.), with a specific activity of 55 mCi/mmol and a radioactive concentration of 10 μCi/mL. Following incubation at room temperature, an aliquot of the mixture (100 μL) was counted in a scintillation counter using scintillation fluid. Microtiter plates were loaded with the mixture (100 μL, ~19 500 cpm), and evaporation was allowed to proceed overnight as described above in a vacuum desiccator. The wells were incubated in the blocking buffer for 2 h and washed 2 times with the washing buffer. Increasing concentrations of unlabeled factor Va were added as described above and incubated for 2 additional h. The wells were washed 5 times and either counted directly in a scintillation fluid or incubated overnight in 10% SDS or 6 N HCl (200 μL), and then counted. In all cases, similar results were found (5900 cpm). Thus, the phospholipid-coating efficiency is approximately 30%. In order to correct for nonspecific binding of factor Va, identical experiments were

performed using wells incubated with methanol or 100% PC. The binding of factor Va to PC-coated plates was indistinguishable from the binding of factor Va to plates treated with methanol. Using the specific activity of iodinated factor Va, the cpm corresponding to the specific binding of factor Va were converted to moles of ^{125}I-factor Va bound, and the results were plotted as femtomoles of factor Va as a function of ^{125}I-factor Va added. A 96-well microtiter plate was loaded with a solution of 75% PC and 25% PS in methanol (3 μg/mL; 100 μL/well), and the plate was allowed to dry overnight at room temperature in a vacuum dessicator. The wells were incubated with the blocking buffer for 2 h at room temperature. The wells were washed with the washing buffer and loaded with 50 μL of various concentrations of bovine factor Va in duplicate (0–200 nM). Following 1-h incubation at room temperature, the wells were washed, and ^{125}I-Xa-EGR was added to the wells (100 nM). When the effect of the monoclonal antibodies (αBFV-4 and αBFV-5) on the factor Va–factor Xa interaction was tested, the radiolabeled EGR-factor Xa (100 nM) was preincubated with the antibodies (1 μM) for 1 h at room temperature and then added to the wells. Following incubation (1 h at room temperature), the wells were washed 5 times and counted in a γ-counter. The amount of ^{125}I-Xa-EGR bound to the plates (femtomoles) was calculated from the specific activity of the radiolabeled factor Xa.

Competition Assay. Microtiter plates were coated with 100% PS and blocked as described above. Iodinated factor Va (20 nM) in blocking buffer was incubated with various concentrations of unlabeled factor Va (0.5–1 μM), factor Va_{LC} (0.5–1 μM), factor Va_{HC} (0.5–1 μM), or monoclonal antibodies αBFV-4 and αBFV-5 (0.5–1 μM) for 30 min at room temperature. Samples of the mixture (50 μL) in duplicate were applied to the phospholipid-coated plates and incubated for 2 h at room temperature. The wells were washed 5 times with washing buffer and counted individually using a γ-counter. The value obtained using iodinated factor Va incubated with buffer under similar conditions was arbitrarily determined as 100% binding. The nonspecific binding was estimated using wells coated with methanol or with PC.

Analytical Ultracentrifugation. Sedimentation velocity studies were conducted using a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner. The sedimentation velocity data were acquired by scanning at 560 nm, and data acquisition and storage were performed as described (Pryzdial & Mann, 1991). In all experiments, samples were extensively dialyzed against 20 mM Hepes, 0.15 M NaCl, and 2 mM $CaCl_2$, pH 7.4 (unless otherwise noted), at 4 °C. Midpoint analysis was employed for scans for which resolved boundaries (i.e., free ctr-EGR-Xa and the complex ctr-EGR-Xa–factor Va) could be identified. Dissociation constants (K_d) were estimated using a formula for two interacting species and a stoichiometric interaction between factor Va and factor Xa (Pryzdial & Mann, 1991):

$$K_d = \frac{[\text{ctr-EGR-Xa}][\text{FVa}]}{[\text{ctr-EGR-Xa}\cdot\text{FVa}]}$$

The fractions of bound and free ctr-EGR-Xa were estimated from the absorbance of the sedimentation profile.

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analyses were performed using 5–15% and 8–18% gradient gels according to the method of Laemmli (1970). Proteins were visualized after staining with Coomassie Brilliant Blue in 50% methanol/10% acetic acid followed by destaining by diffusion using a solution of 50% methanol and 10% acetic acid.

Following SDS–PAGE, the proteins were transferred to nitrocellulose membranes (Trans-Blot nitrocellulose membranes; Bio-Rad, Hercules, CA) using a previously described method (Towbin et al., 1979). Nonspecific binding sites of the nitrocellulose membranes were blocked using 20 mM Tris, 0.15 M NaCl, pH 7.4, and 0.05% Tween 20 (TBS/Tween) for 1 h at room temperature followed by subsequent incubation with monoclonal antibody αBFV-4 or monoclonal antibody αBFV-5 (3 μg/mL), biotinylated goat anti-mouse secondary antibody (5 μg/mL; Vector Laboratories, Burlingame, CA), and ABC reagent (Vector Laboratories) in the presence of 0.5 M NaCl. The proteins were detected using H_2O_2 and 4-chloronaphthol in 20% methanol.

Preliminary results demonstrated that αBFV-5 showed limited interaction with the factor Va molecule or isolated heavy chain using conventional immunobloting techniques when SDS–PAGE was performed either under reducing or under nonreducing conditions. Thus, a more sensitive method was employed to detect factor Va_{HC} and its degradation products after cleavage by trypsin. The heavy chain was digested with trypsin, analyzed by SDS–PAGE under nonreducing conditions, and transferred to a nitrocellulose membrane. The nonspecific binding sites of the membranes were blocked by the addition of 5% (w/v) nonfat dry milk (Carnation Co., Los Angeles, CA) (w/v) in TBS/Tween for 1 h at room temperature followed by incubation with purified monoclonal antibody αBFV-5 at a final concentration of 5 μg/mL and conjugated horse anti-mouse–horseradish peroxidase IgG (Southern Biotechnology Associates, Inc.) at 1:5000 dilution, and finally the fragments were detected using the chemiluminescent substrate Luminol, essentially as described by the manufacturer (ECL Western blotting detection system, Amersham Corp.).

Factor Va Fragment Purification. Purified factor Va heavy chain was digested with TPCK treated-trypsin for 9 h at 37 °C at 1/500 (w/w ratio). The supernatant was applied to a 1 × 3 cm αBFV-5 immunoaffinity column (1.5 mL). The column was washed with 6 column volumes of HBS+, followed by 4 column volumes of 1 M NaCl, and finally 3 M sodium thiocyanate (NaSCN) was applied to elute the polypeptides. Fractions of 500 μL were collected manually, and the protein content was monitored by the absorbance at 280/320 nm. The peak containing the 3 M NaSCN eluate was extensively dialyzed against 0.05% TFA/H_2O (2 × 2 L), and further purification was performed using reverse-phase HPLC chromatography and an analytical Beckman Ultrapore RPSC-C3 column (Beckman). The purity of the purified polypeptides was assessed by SDS–PAGE (8–18% linear gradient).

Amino Acid Sequence Analysis. The NH_2-terminal sequence of purified peptides was determined using automatic Edman degradation on a Applied Biosystem 475A protein sequencing system as described (Kalafatis et al., 1990) in the Given Analytical Facility, Department of Biochemistry, University of Vermont.

RESULTS

Inhibition of the Generation of Thrombin. Among 13 monoclonal antibodies to bovine factor V, five were found to inhibit factor Va cofactor activity in a clotting assay (Foster et al., 1982). The effect of two of the described five inhibitory monoclonal antibodies was further tested in a purified system measuring thrombin formation. As shown in Figure 1 (*filled circles*), the rate of formation of thrombin under the experimental conditions utilized is approximately 500 nM IIa/min. In the presence of monoclonal antibody αBFV-1

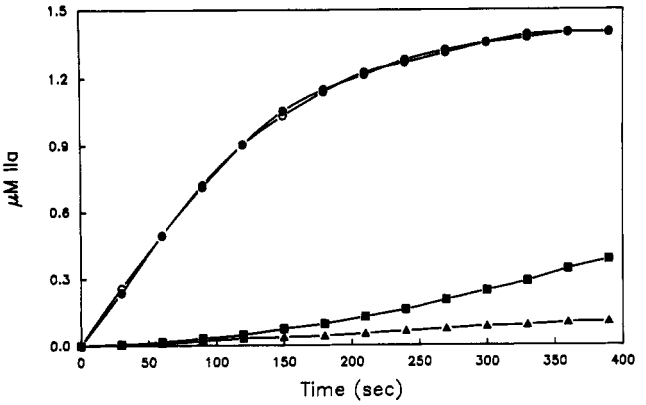

FIGURE 1: Thrombin formation in the presence of monoclonal antibodies. Thrombin generation was measured using the fluorescent thrombin inhibitor DAPA as described under Experimental Procedures. The plot shows thrombin formation as a function of time in the absence of monoclonal antibody (*filled circles*), in the presence of monoclonal antibody αBFV-1 (*open circles*), in the presence of monoclonal antibody αBFV-4 (*filled triangles*), and in the presence of monoclonal antibody αBFV-5 (*filled squares*).

specific for the heavy chain of bovine factor Va (Foster et al., 1983a; Tucker et al., 1983), identical results are obtained. Thus, the presence of a monoclonal antibody (i.e., M_r = 160 000) directed against factor Va *per se* in the reaction mixture does not influence the generation of thrombin by *prothrombinase* (Figure 1, *open circles*). In contrast, the generation of thrombin in the presence of monoclonal antibodies αBFV-4 and αBFV-5 (Figure 1, *filled triangles and filled squares*, respectively) is considerably slower (initial rate of ~7.2 nM IIa/min). These results indicate that these two anti-factor Va antibodies inhibit *prothrombinase* function by inhibiting one of factor Va's cofactor interactions. These data do not shed light upon the specific interaction within the *prothrombinase* complex inhibited by these anti-factor Va antibodies (i.e., factor Va–membrane interaction, factor Va–factor Xa interaction, or factor Va–prothrombin association). However, it has been previously established that αBFV-4 is directed against the light chain of the cofactor and inhibits the interaction between factor Va and membrane-bound factor Xa (Tucker et al., 1983).

Antibody Influence on the Binding of Factor Va to Phospholipid. Semiquantitative binding experiments using iodinated factor Va demonstrated specific and saturable binding of ^{125}I-factor Va to PS- and PC/PS-coated plates (Figure 2). The nonspecific binding of factor Va was measured using wells treated with methanol or PC-coated plates. In both cases, the binding was less than 5% of the total binding. Purified factor Va heavy and light chains, as well as monoclonal antibodies αBFV-4 and αBFV-5, were tested for their ability to competitively inhibit ^{125}I-factor Va binding to PS-coated plates. Total elimination of the binding was observed with unlabeled factor Va and factor Va_{LC} (Table 1). Half-maximum inhibition was reached with approximately 80 and 100 nM factor Va and factor Va_{LC}, respectively (IC_{50}, Table 1). Complete inhibition of the binding was obtained in the presence of 1 μM factor Va or factor Va_{LC}. In contrast, at concentrations as high as 1 μM, factor Va_{HC} failed to inhibit the binding of ^{125}I-factor Va to PS-coated plates. These data indicate, as already demonstrated, that the light chain of the cofactor alone can account for the total binding of ^{125}I-factor Va to phospholipid-coated plates. Under similar experimental conditions, the two monoclonal antibodies αBFV-4 and αBFV-5 failed to inhibit factor Va binding to PS-coated plates even at concentrations as high as 1 μM (Table 1). Thus, the two monoclonal antibodies which inhibit factor Va cofactor activity do not interfere with the binding of the cofactor to a phospholipid-coated surface.

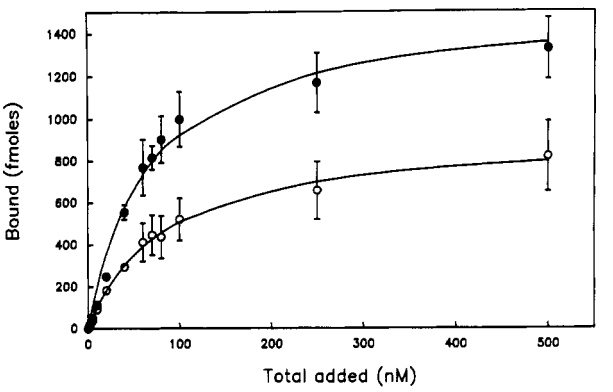

FIGURE 2: Binding of iodinated factor Va to phospholipid-coated plates. Binding of ^{125}I-factor Va to microtiter plates coated with 100% PS (*filled circles*) or 25% PS and 75% PC (*open circles*) was performed as described under Experimental Procedures. The nonspecific binding was achieved using methanol- and PC-coated plates. Both plots show the average specific binding found in three different experiments.

Table 1: Inhibition of ^{125}I-Factor Va Binding to PS-Coated Plates

unlabeled component	IC_{50}	unlabeled component	IC_{50}
factor Va	80 nM	αBFV-4	>1 μM
factor Va_{LC}	110 nM	αBFV-5	>1 μM
factor Va_{HC}	>1 μM		

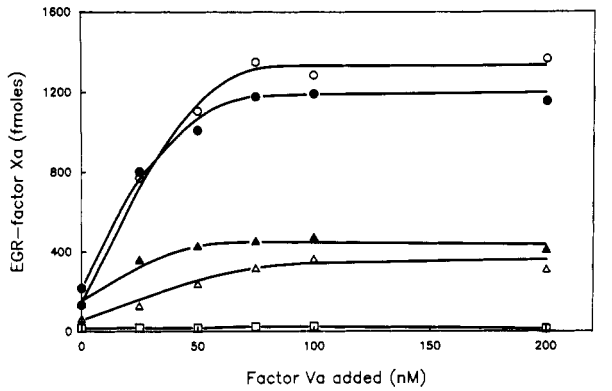

FIGURE 3: Binding of ^{125}I-Xa-EGR to phospholipid-coated plates in the presence of factor Va. Microtiter plates were coated with 100 μL of a solution of PC/PS (75% PC and 25% PS, 3 μg/mL), loaded with increasing concentrations of factor Va, and further incubated with ^{125}I-Xa-EGR as described under Experimental Procedures. When the effect of the monoclonal antibodies on the factor Va–factor Xa interaction was investigated, the monoclonal antibodies were pre-incubated with the radiolabeled factor Xa. The plot shows femtomoles of ^{125}I-Xa-EGR bound to PC/PS-coated wells in the presence of increasing concentrations of factor Va. *Open circles* show the binding of 100 nM ^{125}I-Xa-EGR; *closed circles* illustrate the binding of 100 nM ^{125}I-Xa-EGR preincubated with 1 μM nonimmune mouse IgG; *closed triangles* and *open triangles* illustrate the binding of 100 nM ^{125}I-Xa-EGR preincubated with 1 μM monoclonal antibody αBFV-5 and αBFV-4, respectively. No significant binding of ^{125}I-Xa-EGR is observed in the presence of 25 mM EDTA (*open squares*).

Antibody Inhibition of the Interaction between Factor Xa and Membrane-Bound Factor Va. Figure 3 illustrates the binding of ^{125}I-Xa-EGR to unlabeled factor Va immobilized on PC/PS-coated microtiter plates. In the presence of increasing concentrations of factor Va, there is a 10-fold increase in the binding of ^{125}I-Xa-EGR to the PC/PS-coated plates (Figure 3, *open circles*). In the presence of a 10-fold excess of monoclonal antibodies αBFV-4 and αBFV-5 (Figure 3, *open triangles* and *filled triangles*, respectively), the binding of ^{125}I-Xa-EGR to PC/PS-coated wells is decreased and

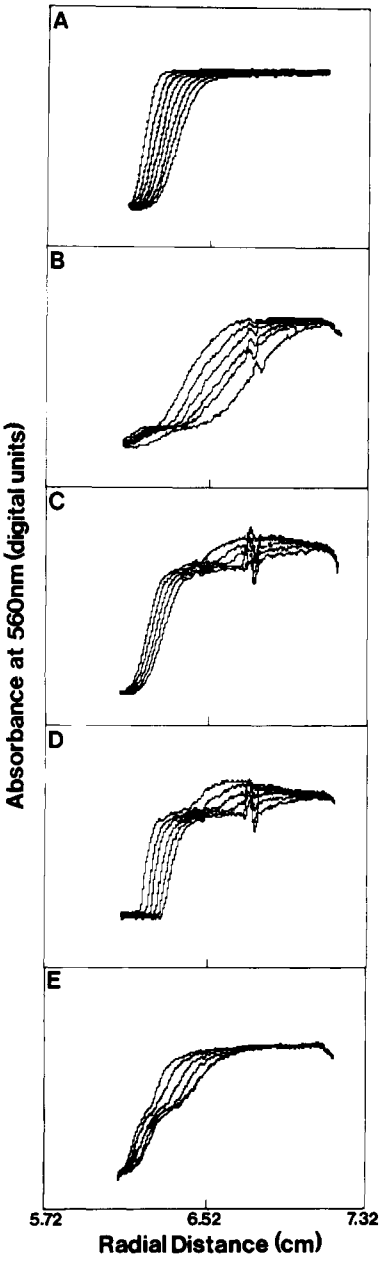

FIGURE 4: Analysis of the phospholipid-independent interaction between ctr-EGR–Xa and factor Va by sedimentation velocity. Sedimentation velocity analysis of ctr-EGR-Xa was performed at 60 000 rpm in 20 mM Hepes, 0.15 M NaCl, and 2 mM $CaCl_2$, pH 7.4 at 20.8 °C. Eight scans taken at 4-min intervals are illustrated in panel A; six scans taken at 6-min intervals are shown in panels B, D, and E whereas five scans at 6-min intervals are depicted in panel C. Panel A, 9.8 μM ctr-EGR-Xa; panel B, 5 μM ctr-EGR-Xa, 15 μM factor Va; panel C, sample as in panel B in the presence of 30 μM monoclonal antibody αBFV-4; panel D, sample as in panel B in the presence of 30 μM monoclonal antibody αBFV-5; panel E, 3.6 μM factor Va, 3.73 μM ctr-EGR-Xa, and 5.5 μM nonimmune mouse IgG.

reaches approximately the same level of binding as the binding of ^{125}I-Xa-EGR to PC/PS-coated wells in the absence of factor Va (see Figure 3, at 0 nM factor Va added). No effect on the factor Xa–factor Va binding interaction was observed when a 1 μM sample of a nonimmune mouse IgG was included in the mixture (Figure 3, *filled circles*). In all cases (i.e., in the presence and absence of the monoclonal antibodies), the binding of ^{125}I-Xa-EGR to PC/PS-coated plates in the absence of factor Va was between 6 and 10% of the total applied material. The *open squares* depict the results obtained in the presence of 25 mM EDTA. These data demonstrate that the

Table 2: Inhibition of ctr-EGR-Xa Binding to Factor Va (FVa) by Monoclonal Antibodies αBFV-4 and αBFV-5

components	% bound	% free
9.8 μM ctr-EGR-Xa		100
5 μM ctr-EGR-Xa + 15 μM FVa	85	15
5 μM ctr-EGR-Xa + 15 μM FVa + 30 μM αBFV-4	18	82
5 μM ctr-EGR-Xa + 15 μM FVa + 30 μM αBFV-5	23	78
3.73 μM ctr-EGR-Xa + 3.64 μM FVa + 5.5 μM nonimmune mouse IgG	49	51

two monoclonal antibodies inhibit the interaction between factor Xa and membrane-bound factor Va.

Antibody Inhibition of the Interaction between Factor Xa and Factor Va in the Absence of Phospholipid Vesicles. The sedimentation scans for ctr-EGR-Xa are shown in Figure 4A. The sedimentation coefficient ($s_{20,w}$) observed for ctr-EGR-Xa was 3.79 S. The sedimentation coefficients observed for the interaction of the purified factor Va subunits with ctr-EGR-Xa were 4.82 S for factor Va_{HC}–ctr-EGR-Xa and 4.56 S for factor Va_{LC}–ctr-EGR-Xa. The increase in the sedimentation coefficient of ctr-EGR-Xa observed when combined with factor Va_{HC} or factor Va_{LC} demonstrates interaction between ctr-EGR-Xa and each of the isolated factor Va subunits. A K_d of 60 μM for the factor Va_{HC}–ctr-EGR-Xa interaction was estimated directly from the absorbance profile, using the equation described under Experimental Procedures, in which a partial boundary between bound and free species is observed. No distinct boundary between free and bound ctr-EGR-Xa was detected when the latter was incubated with factor Va_{LC}.

The phospholipid-independent interactions between factor Va and factor Xa were studied using sedimentation velocity experiments. Active-site-labeled factor Xa has been previously used to study the factor Va–factor Xa interaction and demonstrated that the incorporation of the chromophore inhibitor into the active site of factor Xa has little effect on the sedimentation properties of the enzyme or upon its capacity to interact with factor Va (Pryzdial & Mann, 1991). Sedimentation velocity experiments performed with factor Va and ctr-EGR-Xa in the presence and absence of antibodies are depicted in Figure 4. When 5 μM ctr-EGR-Xa is incubated with 15 μM factor Va, the rhodamine signal is divided into a slow and fast migrating species (Figure 4B). The slower boundary corresponds to free ctr-EGR-Xa and represents ~15% of the signal (Table 2). The faster moving boundary represents the complex between ctr-EGR-Xa and factor Va and corresponds to ~85% of the absorbance at 560 nm (Table 2). Under similar conditions in the presence of either monoclonal antibody αBFV-4 (Figure 4C) or monoclonal antibody αBFV-5 (Figure 4D), most of the rhodamine signal (~80% of the total signal, Table 2) sediments as free ctr-EGR-Xa. Control experiments using final concentrations of 3.7 μM ctr-EGR-Xa and 3.6 μM factor Va, performed in the presence of a nonimmune mouse IgG (5.5 μM), resulted in 49% complex formation. These results are in agreement with previously published data (Pryzdial & Mann, 1991) which showed approximately 50% complex formation when equimolar concentrations of factor Va and rx-EGR-factor Xa were mixed (in the micromolar range, when studying the factor Va–factor Xa interaction in the absence of a membrane surface) and demonstrated no effect of the immunoglobulin *per se* on the factor Va–ctr-EGR-Xa interaction (Figure 4E and Table 2). These data demonstrate that the presence of an excess protein within the mixture does not have a major effect on the sedimentation of ctr-EGR-Xa or on the sedimentation of the factor Va–ctr-EGR-Xa complex. Collectively these data (Figures 1–4) indicate that monoclonal

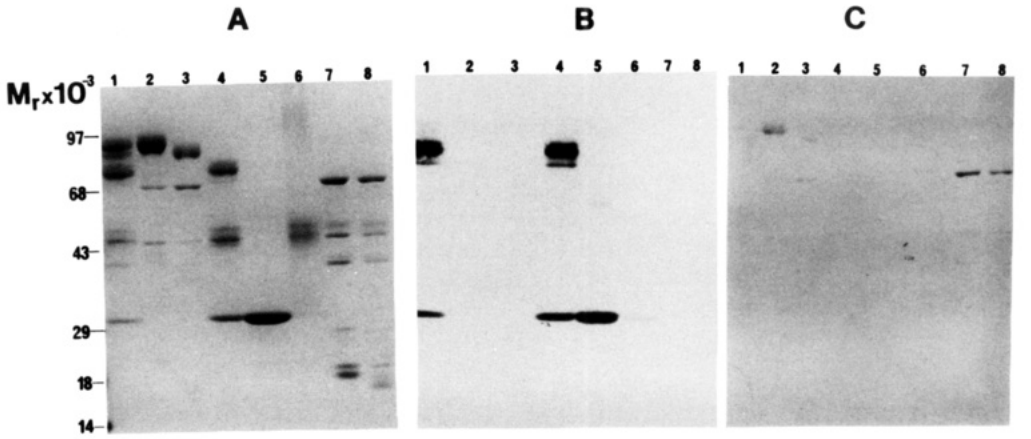

FIGURE 5: Localization of the epitopes of the inhibitory monoclonal antibodies. After SDS–PAGE anlaysis, factor Va or factor Va subunits or their degradation products following APC digestion were stained with Coomassie Blue (Panel A) or transferred onto a nitrocellulose membrane and displayed by using monoclonal antibody αBFV-4 (panel B) or monoclonal antibody αBFV-5 (panel C) as described under Experimental Procedures using H_2O_2 and 4-chloronaphthol. Lane 1, heterogeneous factor Va; lane 2, factor Va_{HC} (H94); lane 3, factor Va heavy-chain degradation product which lacks the acidic COOH-terminal portion (factor Va_{H90}; Kalafatis et al., 1993a,b); lane 4, factor Va_{LC}; lane 5, NH_2-terminal portion of factor Va_{LC} (residues 1537–1752); lane 6, COOH-terminal part of factor Va_{LC} (residues 1753–2183); lane 7, proteolytic fragments deriving from APC digestion of factor Va_{HC}; lane 8, proteolytic fragments deriving from APC digestion of factor Va_{H90}. The position of the molecular weight markers is indicated on the left of panel A.

antibodies αBFV-4 and αBFV-5 inhibit factor Va cofactor function through the specific inhibition of factor Xa interactions with the light and the heavy chain of the cofactor, respectively.

Antibody Specificity. The epitopes of the two monoclonal antibodies (i.e., αBFV-4 and αBFV-5) were further identified using purified factor Va components and their degradation products after APC digestion of the cofactor in the absence of phospholipid (Odegaard & Mann, 1987; Kalafatis & Mann, 1993). Immunoblotting experiments revealed that αBFV-4 recognizes an epitope located on the light chain of the cofactor at the NH_2-terminal part of the light chain (residues 1537–1752, Figure 5, panel B, lanes 1, 4, and 5). Similar experiments showed that αBFV-5 recognizes an epitope located on the NH_2-terminal portion of the factor V molecule (residues 1–505, Figure 5, panel C, lanes 2, 7, and 8). These data suggest that the NH_2-termini of the two chains of the cofactor contain motif(s) which is (are) recognized by each of the antibodies which inhibit the factor Va–factor Xa association.

In order to delineate a smaller region within the bovine factor V molecule containing the epitope of αBFV-5, the heavy-chain H90 fragment (Kalafatis et al., 1993a,b) was digested with trypsin, and the derived fragments were tested for their ability to interact with αBFV-5 under nonreducing conditions in the presence of SDS. Initial experiments showed that trypsin generates an M_r = 40 000 fragment which was recognized by αBFV-5. Control experiments demonstrated that upon reduction of the M_r = 40 000 fragment with β-mercaptoethanol, αBFV-5 no longer recognizes its epitope on H90. Thus, binding of αBFV-5 to its specific epitope on factor Va_{HC} depends on the integrity of the disulfide bridges. Following trypsin digestion of H90, the mixture was applied to an αBFV-5 immunoaffinity column, and elution was performed using 3 M NaSCN (Figure 6, peak 3 and inset, lane 3). Further purification of the polypeptide contained in peak 3 was achieved using reverse-phase HPLC (Figure 7). A major peak was obtained (indicated by the arrow in Figure 7). The polypeptide contained within this peak (M_r = 40 000) was analyzed for NH_2-terminal sequence (Table 3). Edman degradation followed by phenylthiohydantoin analysis of the isolated M_r = 40 000 fragment demonstrated an NH_2-terminal sequence which matches the NH_2-terminal portion of bovine factor Va (residues 1–17 of the bovine cofactor, Table 3).

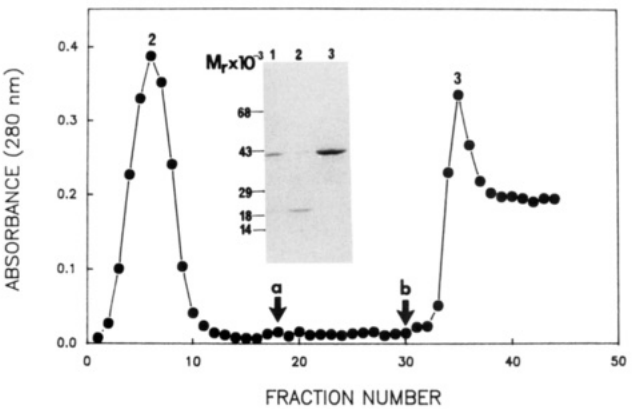

FIGURE 6: Immunoaffinity purification of an M_r = 40 000 fragment containing the epitope for monoclonal antibody αBFV-5. Factor Va_{H90} was digested with trypsin at a 1:500 (w/w) enzyme:substrate ratio for 9 h at 37 °C. The mixture was directly applied to a 3-mL column containing monoclonal antibody αBFV-5 coupled to Sepharose CL-4B. Once the flow-through eluted, the nonspecific peptides were eluted with a solution of 1 M NaCl (arrow a), and the immunoadsorbed fragments were eluted with a solution of 3 M NaSCN (arrow b). The inset shows a 5–15% gradient SDS–PAGE gel stained with Coomassie Blue. Lane 1 corresponds to the total digest applied to the column; lane 2, fragments contained in peak 2 (flow-through); lane 3, fragments contained in peak 3. The position of the molecular weight standards is indicated on the left.

Recent data identified all fragments derived from membrane-bound bovine factor Va after cleavage by APC (Kalafatis & Mann, 1993). Thus, APC digestion of membrane-bound factor Va was further employed to identify the carboxy-terminal part of the M_r = 40 000 fragment. Following digestion of factor Va by APC in the presence of PC/PS vesicles, SDS–PAGE analysis under nonreducing conditions, electroblotting and immunostaining with αBFV-5, the M_r = 70 000 fragment (amino acids 1–505) and the M_r = 40 000 fragment containing amino acids 1–306 (Kalafatis & Mann, 1993) were recognized by αBFV-5. Thus, the epitope for αBFV-5 is contained within residues 1–306 of bovine factor V.

DISCUSSION

Using antibodies directed against the bovine factor Va molecule which inhibit factor Va cofactor activity, sedimentation velocity experiments, a plate binding assay, and assays measuring thrombin formation, we demonstrate that the NH_2-

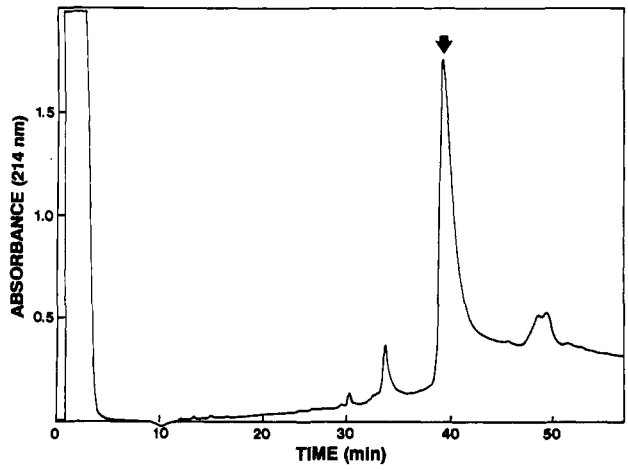

FIGURE 7: Reverse-phase HPLC purification of the M_r = 40 000 fragment containing the epitope for monoclonal antibody αBFV-5. The fragment contained within peak 3 in Figure 6 was collected and extensively dialyzed against 0.05% TFA/H_2O. The sample was injected onto a 4.6 mm × 7.5 cm Ultrapore RPSC C-3 reverse-phase HPLC column operated at a flow rate of 1 mL/min at 22 °C. A gradient of 0% buffer B to 60% buffer B over 40 min was initiated simultaneously with sample injection. Elution was monitored by the absorbance at 214 nm as shown on the left of the chromatogram. The vertical arrow represents the major peak which is eluted at approximately 40 min and was analyzed for NH_2-terminal sequence in Table 3.

Table 3: Comparison of the NH_2-Terminal Sequences of the Tryptic Peptide with the NH_2-Terminal Sequence of the Heavy Chain of Human and Bovine Factor Va

cycle no.	tryptic fragment	human NH_2-terminal[d]	bovine NH_2-terminal[e]
1	ND[a]	A	A
2	K (49.5)[b]	Q	K
3	L (47.5)	L	L
4	R (3.36)	R	R
5	Q (42.6)	Q	Q
6	F (35.0)	F	F
7	Y (25.7)	Y	Y
8	V (28.7)	V	V
9	A (27.3)	A	A
10	A (29.9)	A	A
11	Q (29.2)	Q	Q
12	ND	G	S
13	I (11.3)	I	I
14	R NQ[c]	S	R
15	ND	W	W
16	ND	S	N
17	YNQ	Y	Y

[a] Not determined. [b] The number in parentheses indicates picomoles of amino acid at the given cycle. [c] Not quantitated. [d] From Jenny et al. (1987). [e] From Guinto et al. (1992).

termini of both heavy and light chains of factor Va (i.e., the A1 and A3 domains of the cofactor) are involved in the interactions of the cofactor with factor Xa.

Factor Va and factor Xa interact stoichiometrically, and it has been shown that in the absence of phospholipid both the heavy and light chains of the cofactor are involved in the interaction with factor Xa (Annamalai et al., 1987; Pryzdial & Mann, 1991). This becomes apparent when comparing the sedimentation coefficients of ctr-EGR-Xa complexed with the individual chains and intact factor Va. The sedimentation coefficient of ctr-EGR-Xa increases when mixed with both purified factor Va_{HC} and purified factor Va_{LC}, providing direct physical evidence for that interaction. Previous studies established that the factor Va–factor Xa interaction is governed by a dissociation constant of 0.8 μM (Pryzdial & Mann, 1991). The K_d for the interaction of factor Va_{HC} with ctr-EGR-Xa is approximately 60 μM. Under these conditions, the Gibbs free energy contributed by factor Va_{HC}–factor Xa complex formation represents 70% of the energy liberated when factor Xa interacts with factor Va. Using monoclonal antibodies to bovine factor Va, we found that inhibition of the interaction of either one of the factor Va subunits with factor Xa completely abolishes factor Va cofactor activity (Figure 1). Thus, both chains of the cofactor are required for its interaction with factor Xa and the expression of cofactor activity. These data also suggest that both factor Va subunits are necessary not only for the direct binding of factor Va to factor Xa but also for the stabilization of the bimolecular interaction. Direct analyses of phospholipid-dependent complex formation illustrate that these antibodies inhibit the factor Va–factor Xa interaction in the presence as well as in the absence of phospholipid.

Earlier data using light fluorescence polarization measurements and gel exclusion chromatography showed that monoclonal antibody αBFV-4 which recognizes factor Va_{LC} inhibits factor Va interaction with factor Xa (Tucker et al., 1983). The present data extend those findings and localize the epitope of monoclonal αBFV-4 to the NH_2-terminal portion of factor Va_{LC} (amino acids 1537–1752). This portion of the light chain also contains a region which is involved in phospholipid binding of factor Va (amino acids 1654–1752; Kalafatis et al., 1990, 1994). Our findings also demonstrate that monoclonal antibody αBFV-5, which is directed against the heavy chain of factor Va and inhibits the factor Va–factor Xa interaction in the presence and absence of phospholipid vesicles, recognizes an epitope located on the NH_2-terminal portion of the heavy chain (residues 1–306). These data demonstrate that the NH_2-termini of both the heavy and light chains of the cofactor (A1 and A3 domains) are involved in the binding of factor Va to factor Xa. However, it is noteworthy that our study does not define as to whether the monoclonal antibodies which inhibit the factor Va–factor Xa interaction recognize the specific site involved in the factor Va–factor Xa interaction (i.e., competitive inhibitor) or inhibit the interaction between the two proteins by steric hindrance, a noncompetitive inhibition.

The interaction of factor Xa with factor Va on a phospholipid surface produces an enzymatic complex extremely efficient in activating prothrombin. The NH_2-terminal part of factor Xa_{LC} does not appear to participate in the factor Va–factor Xa interaction. In the absence of phospholipid, the addition of saturating concentrations of factor Va to Gla-domainless factor Xa increased the rate of thrombin formation approximately 550-fold when compared to the Gla-domainless factor Xa alone acting on prothrombin (Skogen et al., 1984). The role of factor Va in the *prothrombinase* complex is to localize factor Xa on the phospholipid membrane, and since the binding of factor Va to factor Xa does not affect its catalytic efficiency toward small synthetic *p*-nitroanilide substrate (Nesheim et al., 1981), it has been proposed that the increase in the catalytic efficiency of the complex is due to the stabilization of the substrate binding site (i.e., prothrombin) rather than because of alterations of the catalytic site of the enzyme (Walker & Krishnaswamy, 1993). Using synthetic peptides from selected regions of the factor Xa molecule (Furie et al., 1982; Chattopadhyay et al., 1992), it has been shown that the serine protease domain contains motifs which inhibit thrombin formation by *prothrombinase*. Thus, the high-affinity binding site(s) for factor Va appear(s) to involve the serine protease domain of the factor Xa molecule. Additionally, cross-linking experiments suggest that a peptide containing residues 263–274 of factor Xa_{HC} binds to the light chain of factor Va. However, peptide 254–269 of factor Xa_{HC} strongly inhibits thrombin formation by the *prothrombinase*

complex, and peptides 211–222 and 263–274 of factor Xa_{HC} demonstrated mutual exclusivity. Altogether, these data suggest that more than one region of factor Xa is involved in its interaction with factor Va. This conclusion is also suggested by our data since two binding sites for factor Va imply that there should be at least two binding sites for factor Xa.

In summary, using monoclonal antibodies to factor Va that inhibit factor Va cofactor activity, we have demonstrated that the factor Va–factor Xa interaction is promoted by the NH_2-termini of the two chains of the cofactor.

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