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Amino Acids Glu^{323}, Tyr^{324}, Glu^{330}, and Val^{331} of Factor Va Heavy Chain Are Essential for Expression of Cofactor Activity*

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We have recently demonstrated that amino acid region 323–331 of factor Va heavy chain (9 amino acids, AP4′) contains a binding site for factor Xa (Kalafatis, M., and Beck, D. O. (2002) *Biochemistry* 41, 12715–12728). To ascertain which amino acids within this region are important for the effector and receptor properties of the cofactor with respect to factor Xa, we have synthesized three overlapping peptides (5 amino acids each) spanning the amino acid region 323–331 and tested them for their effect on prothrombinase complex assembly and function. Peptide containing amino acids ^{323}EYFIA^{327} alone was found to increase the catalytic efficiency of factor Xa but had no effect on the fluorescent anisotropy of active site-labeled factor Xa (human factor Xa labeled in the active site with Oregon Green 488; [OG$_{488}$]-EGR-hXa). In contrast, peptide containing the sequence ^{327}AAEEV^{331} was found to interact with [OG$_{488}$]-EGR-hXa with half-maximal saturation reached at ~150 μM, but it was unable to produce a cofactor effect on factor Xa. Peptide ^{325}FIAAE^{329} inhibited prothrombinase activity and was able to partially decrease the fluorescent anisotropy of [OG$_{488}$]-EGR-hXa but could not increase the catalytic efficiency of factor Xa with respect to prothrombin. A control peptide with the sequence FFFIA did not increase the catalytic efficiency of factor Xa, whereas a peptide with the sequence AAEMI was impaired in its capability to interact with [OG$_{488}$]-EGR-hXa. Two mutant recombinant factor Va molecules (Glu^{323} → Phe/Tyr^{324} → Phe, factor Va^{FF}; Glu^{330} → Met/Val^{331} → Ile, factor Va^{MI}) showed impaired cofactor activity when used at limiting cofactor concentration, whereas the quadruple mutant (Glu^{323} → Phe/Tyr^{324} → Phe and Glu^{330} → Met/Val^{331} → Ile, factor Va$^{FF/MI}$) had no cofactor activity under similar experimental conditions. Our data demonstrate that amino acid residues Glu^{323}, Tyr^{324}, Glu^{330}, and Val^{331} of factor Va heavy chain are critical for expression of factor Va cofactor activity.

The maintenance of hemostasis is a complex event requiring the controlled interaction of proteases, zymogens, cofactors, and inhibitors on surfaces provided by platelets and endothelial cells, culminating in timely α-thrombin formation at the site of a vascular injury. The enzymatic complex prothrombinase, which activates prothrombin, is required for normal blood clotting and is composed of the enzyme, factor Xa, and the protein cofactor, factor Va, associated on a cell surface in the presence of divalent metal ions (1, 2). Whereas factor Xa possesses the enzymatic capability to activate prothrombin, the incorporation of factor Va into prothrombinase and its interaction with factor Xa increase the catalytic efficiency of the enzyme by 5 orders of magnitude as compared with factor Xa alone (3). The increase in the catalytic efficiency of prothrombinase as compared with factor Xa alone is believed to arise from a 100-fold decrease in the K_m and a 3000-fold increase in the k_{cat} of the enzyme (4–7). The decrease in the K_m of the reaction appears to be the result of the interaction of the complex with the cell surface, resulting in higher local concentration of substrate. The increase in the k_{cat} of prothrombinase is attributed to the binding of factor Va to factor Xa. The bulk of the data generated suggest that incorporation of factor Va into prothrombinase and its interaction with factor Xa and prothrombin do not have any significant effect on the catalytic site of the enzyme (8–10). The accumulated data suggest that binding of factor Va to factor Xa induces a conformational transition in the structure of the enzyme exposing a prothrombin-binding exosite on the enzyme, resulting in more efficient catalysis. However, the cofactor itself may also represent a portion of the exosite for prothrombin. Evidence for a direct interaction of factor Va with prothrombin has been repetitively provided (11–14). The amino acids responsible for this dual effect of factor Va on factor Xa remain to be identified.

Human factor V circulates in plasma as a single chain glycoprotein of M_r 330,000 consisting of multiple domains A1-A2-B-A3-C1-C2, at a concentration of ~20 nM (15–18). The factor V gene is 80 kb in length and contains 24 introns, whereas the mRNA is 6.9 kb long (19). Single chain factor V does not appear to interact with factor Xa and to have cofactor activity. Factor V is cleaved sequentially by α-thrombin at Arg^{709}, Arg^{1018}, and Arg^{1545} to produce the active cofactor, factor Va, that consists of a heavy chain (M_r 105,000) and a light chain (M_r 74,000). The heavy chain derives from the NH_2-terminal part of factor V (A1-A2 domains, residues 1–709), whereas the light chain represents the COOH-terminal end of the procofactor (A3-C1-C2 domains, residues 1546–2196) (17). The heavy and light chains are noncovalently associated via divalent metal ions (20). Activation of the procofactor by α-thrombin is required for expression of its cofactor activity (*i.e.* interaction with factor Xa and prothrombin). Both chains of the cofactor are required for the high affinity interaction with factor Xa (21–23). The data accumulated thus far indicate that the binding of factor Va and/or factor Xa to the lipid bilayer most likely results in conformational changes in one or both proteins that contribute to aspects of the factor Va-factor Xa interaction. Factor Va and factor Xa interact stoichiometrically in the presence and absence of phos-

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pholipids. In the absence of phospholipids, the K_d for the interaction is 0.8 μM and is dependent upon the presence of Ca^{2+} ions (24). Upon binding of both proteins to lipids, the K_d of the interaction decreases by ~1000-fold to 1 nM (4, 5), suggesting that multiple points of contact between the two proteins are exposed, resulting in the tighter interaction. Because of the physiological concentration of the two proteins in plasma, the only physiologically relevant K_d for the interaction between the two proteins is the K_d observed in the presence of a membrane surface. Factor Va is inactivated by activated protein C (APC),[1] following cleavage at Arg^{506}, Arg^{306}, and Arg^{679} only in the presence of a membrane surface (25, 26). Cleavage of factor Va by APC at Arg^{506} results in a 10-fold decrease in the affinity of the molecule for factor Xa and is required for efficient cleavage at Arg^{306} (25–27). Subsequent cleavage at Arg^{306}, which is lipid-dependent and is the inactivating cleavage site, completely abolishes the ability of the cofactor to interact with factor Xa because of the dissociation of the heavy chain fragments from the light chain, hence eliminating the factor Va-factor Xa interaction (27). Thus, whereas activation of factor V by α-thrombin allows for proper interaction of factor Va with factor Xa, APC inhibits this interaction by eliminating the binding site(s) of the cofactor for the enzyme. These combined data suggest that the regulation of normal hemostasis and control of thrombosis is exerted following the activation and inactivation processes of the cofactor, which ultimately and explicitly control its interaction with factor Xa. As a consequence, a complete understanding of prothrombinase assembly and function, which is a prototype for most membrane-bound complexes contributing to the blood clotting processes, necessitates the identification of the distinct amino acids from each protein responsible for their interaction as well as a complete understanding of the macromolecular interactions that occur between them.

We have shown that a synthetic peptide containing a sequence of amino acids from the middle portion of factor Va heavy chain (amino acid residues 307–348; N42R) (every peptide is identified by the first and last amino acids, with the number of residues composing the peptide in the middle) inhibits factor Va cofactor activity (28). We have recently demonstrated, using overlapping peptides, that a nonapeptide from N42R containing amino acid sequence 323–331 of factor Va (AP4′), is the active inhibitory portion of N42R and represents a direct binding site for factor Xa (29). Both peptides, N42R and AP4′, also produced a "cofactor" effect on factor Xa, increasing the catalytic efficiency of the enzyme by 21- and 4-fold, respectively, as compared with factor Xa alone (29). The present study was undertaken to identify the amino acid residues within AP4′ that are responsible for its biological activities.

EXPERIMENTAL PROCEDURES

Materials, Reagents, and Proteins—Hepes, Trizma (Tris base), Q-Sepharose fast flow, Sepharose CL-4B, bovine serum albumin, Coomassie Brilliant Blue R-250, diisopropyl fluorophosphate (DFP), *o*-phenylenediamine dihydrochloride (OPD), and factor V-deficient plasma were purchased from Sigma. Heparin-Sepharose and the chemiluminescent reagent ECL^+ were from Amersham Biosciences. Anti-mouse and anti-sheep IgG coupled to peroxidase were from Southern Biotechnology Associates Inc. (Birmingham, AL). L-α-Phosphatidylserine (PS) and L-α-phosphatidylcholine (PC) where from Avanti Polar Lipids (Alabaster, AL). Normal reference plasma, the chromogenic substrate for thrombin, Spectrozyme-TH, and hirudin were from American Diagnostica Inc. (Greenwich, CT). The thromboplastin reagent used in the clotting assays was purchased from Organon Teknika Corp. (Durham, NC). Polyethylene glycol (M_r 8000) was from J. T. Baker Inc. The fluorescent thrombin inhibitor dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amide (DAPA), human factor Xa, human α-thrombin, human prothrombin, the monoclonal antibody αHFV 1 coupled to Sepharose, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, EGR-hXa (glutamylglycinylarginyl chloromethyl ketone active site-blocked human factor Xa), and human factor Xa labeled in the active site with Oregon Green 488 ([OG_{488}]-EGR-hXa), as previously described (30), were from Hematologic Technologies Inc. (Essex Junction, VT). The cDNA for factor V was purchased from American Type Tissue Collection (ATCC number 40515 pMT2-V; American Type Culture Collection; Manassas, VA). The sequence of this cDNA molecule is identical to the cDNA published by Jenny *et al.* (17). All restriction enzymes were from New England Biolabs (Beverly, MA), and all other molecular biology and tissue culture reagents and media were from Invitrogen or as indicated. The two monoclonal antibodies to human factor V (against the heavy and light chains of the cofactor, *i.e.* αHFV_{HC} 17 and αHFV_{LC} 9) were provided by Dr. Kenneth G. Mann (Department of Biochemistry, College of Medicine, University of Vermont, Burlington, VT). These antibodies have been thoroughly characterized (31–34). Overlapping peptides from the region 323–331 of factor V were synthesized in the analytical facility of Dr. Satya Yadav at the Cleveland Clinic Foundation (Cleveland, OH), purified by high performance liquid chromatography, and characterized by mass spectrometry as described (29). Human factor V was purified as previously described using the monoclonal antibody αHFV 1 coupled to Sepharose (35, 36). Factor Va was obtained as recently described using heparin-Sepharose (29). Prior to use, factor Va was concentrated using Centricon (M_r 30,000 cut-off YM-30; Millipore Corp., Bedford, MA) and stored on ice at 4 °C. Because of the instability of the active cofactor following prolonged storage on ice at 4 °C, factor Va preparations were made as needed and in small quantities. The quality of each preparation was assessed prior to use by measuring the activity of the factor Va molecule systematically by a clotting assay using factor V-deficient plasma and standardized to the percentage of control (35). Phospholipid vesicles composed of 75% PC and 25% PS (referred to as PCPS vesicles throughout) were prepared as previously described (37). The concentration of the phospholipid vesicles was determined by phosphorous assay as described (38) and is given as the concentration of inorganic phosphate.

Assay Measuring Thrombin Formation—The formation of thrombin was analyzed using the fluorescent thrombin inhibitor DAPA as described (25, 26, 29, 39) using a PerkinElmer LS-50B Luminescence Spectrometer with λ_{ex} = 280 nm, λ_{em} = 550 nm, and a 500-nm-long pass filter in the emission beam (Schott KV-500). The buffer used in all cases was composed of 20 mM Hepes, 0.15 M NaCl, 5 mM $CaCl_2$, pH 7.4 (HBS(Ca^{2+}), "assay buffer"). The final concentration of factor Va in the mixture was 4 nM, with factor Xa at 10 nM. Under these conditions, the rate of thrombin formation is linearly related to the amount of active cofactor (factor Va). The initial rate of thrombin formation (nM IIa/min) was calculated as described (25, 26, 29). The concentration of peptide given in each figure is the final concentration of the peptide in the assay mixture. All data were initially analyzed and stored using the software FL WinLab (PerkinElmer Life Sciences) provided by the manufacturer and further analyzed and plotted with the software Prizm (GraphPad, San Diego, CA).

Fluorescence Anisotropy Measurements—Fluorescence anisotropy of [OG_{488}]-EGR-hXa was measured using a PerkinElmer LS-50B Luminescence Spectrometer in L format as recently described (29). Anisotropy measurements were performed in a quartz cuvette under constant stirring (low) with λ_{ex} = 490 nm and λ_{em} = 520 nm with a long pass filter (Schott KV-520) in the emission beam. At each addition, anisotropy was measured for 20 s and averaging eight successive readings. In all cases, the total addition of peptide did not exceed 10% of the volume of the reaction. The concentration of peptide given in each graph is the final concentration of the peptide in the assay mixture. All data were initially analyzed and stored using the software FL WinLab (PerkinElmer Life Sciences) and further analyzed and plotted with the software Prizm (GraphPad). Some of the data were also plotted using DeltaGraph (DeltaPoint, Monterey, CA).

[1] The abbreviations used are: APC, activated protein C; DFP, diisopropyl fluorophosphate; OPD, *o*-phenylenediamine dihydrochloride; PS, L-α-phosphatidylserine; PC, L-α-phosphatidylcholine; PCPS, small unilamellar phospholipids vesicles composed of 75% PC and 25% PS (w/w); DAPA, dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amide; EGR-hXa, glutamylglycinylarginyl chloromethyl ketone active site-blocked human factor Xa; [OG_{488}]-EGR-hXa, human factor Xa labeled in the active site with Oregon Green 488; PVDF, polyvinylidene difluoride; ELISA, enzyme-linked immunosorbent assay(s); factor Va^{FF}, recombinant human factor Va with the mutations $Glu^{323} \rightarrow Phe/Tyr^{324} \rightarrow Phe$; factor Va^{MI}, recombinant human factor Va with the mutations $Glu^{330} \rightarrow Met/Val^{331} \rightarrow Ile$; factor $Va^{FF/MI}$, quadruple mutant, recombinant human factor Va with the mutations $Glu^{323} \rightarrow Phe/Tyr^{324} \rightarrow Phe$ and $Glu^{330} \rightarrow Met/Val^{331} \rightarrow I$; mOD, 10^{-3} OD.

Mutagenesis and Transient Expression of Recombinant Factor V Molecules—The complete 6.909-kb-long cDNA sequence for factor V containing a leader sequence (from 91 to 174 bp) that encodes the signal peptide was inserted into the mammalian expression vector pMT2 at *Sal*I sites. Mutant molecules were designed by keeping in mind that in order to positively identify the amino acids responsible for the effector and receptor properties of factor Va, we have to express recombinant factor V molecules with these specific amino acids mutated in such a manner that the entire conformation of the recombinant factor V molecules possesses a tertiary conformation as close as possible to the wild type factor Va molecule. For these reasons, we have made three conservative and one nonconservative mutation. As conservative substitutions, phenylalanine was substituted for a tyrosine in order to test the function of the hydroxyl present on the later amino acid. Methionine has a similar core structure as glutamic acid; however, the carboxyl group of the latter is replaced by a thioether group in the former. Although valine and isoleucine are both hydrophobic in nature, the structure of the side chain of the latter is significantly different from the structure of the side chain of the former. Finally, we have previously observed that the presence of a glutamic acid at position 323 was a prerequisite for optimum inhibitory potential of AP4′ (29). We have thus performed a nonconservative mutation by replacing the carboxyl group of glutamic acid with a phenyl group found in phenylalanine to eliminate any potential contribution of this hydrophilic amino acid to the interaction with factor Xa. PCR-based site-directed mutagenesis was used for constructing the factor V mutants (*i.e.* pMT2-FV ($Glu^{323} \rightarrow Phe/Tyr^{324} \rightarrow Phe$, factor V^{FF}), pMT2-FV ($Glu^{330} \rightarrow Met/Val^{331} \rightarrow Ile$, factor V^{MI}), and pMT2-FV ($Glu^{323} \rightarrow Phe$, $Tyr^{324} \rightarrow Phe$, $Glu^{330} \rightarrow Met$, $Val^{331} \rightarrow Ile$, factor $Val^{FF/MI}$)). The mutagenic primers for the mutant $Glu^{323} \rightarrow Phe/Tyr^{324} \rightarrow Phe$ were 5′-GAAGAGGTGGTTCTTCTTCATTGCTGC-3′ (sense) and 5′-GCAGCAATGAAGAAGGAACCACCTCTTC-3′ (antisense), whereas for the mutant $Glu^{330} \rightarrow Met/Val^{331} \rightarrow Ile$ the primers were 5′-CATTGCTGCAGAGATGATCATTTGGGACTATGC-3′ (sense) and 5′-GCATAGTCCCAAATGATCATCTCTGCAGCAATG-3′ (antisense) (underlined letters indicate the mismatch). The outer PCR primers were 5′-ACATCCACTACCGCAATATGAC-3′ (sense) and 5′-CCTCAGGCAGGAACAACACCATGA-3′ (antisense), corresponding to nucleotide positions 964–979 (upstream of a *Xcm*I restriction site) and 1481–1504 (downstream of a *Bsu*361 site), respectively (according to Jenny *et al.* (17); GenBank™ accession number M16967). Each site-directed mutagenesis was carried out by three-step PCRs. The first two PCRs were performed using the sense outer primer and antisense mutagenic primers or antisense outer primer and sense mutagenic primers. The complete DNA fragment removed from the pTM2-FV wild type sequence by digesting with *Sal*I was used as a template for the PCR. The third reaction was performed using outer sense and antisense primers and the purified PCR products of the first two reactions as templates. All PCRs were performed with high fidelity *Taq* polymerase. PCRs were programmed as follows: denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 68 °C for 1 min/kb in a GeneAmp PCR system 9700 DNA thermal cycler (PerkinElmer Life Sciences). The amplicons of each third reaction were ligated into the cloning plasmid vector pGEM-T, and the integrity of the constructs was verified following sequencing using an ABI Prism automatic DNA sequencer and the universal primers T7 and SP6 as well as other factor V sequence-specific primers as needed at the sequencing facility at the Cleveland Clinic Foundation. The quadruple mutant pMT2-FV (E323F/Y324F/E330M/V331I) was made using the pGEM-T plasmid vector containing the DNA insert of factor V^{FF} as template and the mutagenic primers used for factor V^{MI}. The inserts (nucleotides 964–1504) were removed from the pGEMT-FV plasmid constructs (mutant fragments) following digestion with *Xcm*I and *Bsu*361 restriction enzymes. Following purification of the inserts from the agarose gel, the factor V inserts that possess the mutations (nucleotide positions 964–1504) were religated into pMT2-FV, in which the DNA fragment between the *Xcm*I and *Bsu*361 restriction sites was removed. The ligated plasmids were transformed into JM109 bacterial competent cells. Positive ampicillin-resistant clones for pMT2-FV mutants were selected. The correct sequences and orientations of the inserts were established by DNA sequence analysis with factor V-specific primers. The wild-type pMT2-FV and mutant pMT2-FV plasmids were isolated from the bacterial culture by the QIAfilter plasmid Midi kit (Qiagen Inc., Valencia, CA).

Recombinant Factor V-Wild Type and Factor V Mutant Transfection into COS-7 Cells—COS-7 cells (ATTC) or COS-7L, (Invitrogen) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 μg/ml streptomycin and 100 IU/ml penicillin) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The purified plasmids pMT2-FV wild type, pMT2-FV(E323F/Y324F), pMT2-FV(E330M/V331I), and pMT2-FV(E323F/Y324F/E330M/V331I) were used to transfect into COS-7/COS-7L cells as follows. Each plasmid (1–2 μg) was transfected into 50–80% confluent COS-7/COS-7L cells in a 100-mm culture plate using lipid-based transfection reagent FUGENE 6 (Roche Diagnostics) according to the manufacturer's instructions. Following 48 h of transfection, the medium was removed. Cells were washed twice with serum-free medium, and 6–10 ml of conditioned medium VP-SFM supplemented with 4 mM of L-glutamine was added. After growing the cells in the conditioned medium for 24 h, medium was harvested, and 6–10 ml of fresh condition medium was added. After growing the cells for another 24 h, the media were harvested again. The harvested medium containing recombinant factor V was briefly centrifuged at 4,500 rpm at 4 °C to remove insoluble particles. All control media and solutions containing the recombinant factor V molecules were concentrated using centrifugal ultrafiltration (Centricon YM 30,000; Millipore Corp.), washed with 20 mM Tris, 0.15 M NaCl, 5 mM $CaCl_2$, pH 7.4, following repeated concentration steps, and stored on ice at 4 °C. The activity and the integrity of the molecules were verified before and after thrombin activation by clotting assays using factor V-deficient plasma and by Western blotting using the appropriate monoclonal and polyclonal antibodies. The recombinant molecules were stable under these conditions for at least 1 week. The concentrations of the recombinant molecules were determined by an ELISA developed in our laboratory and described below. The values found in our ELISA were similar to the concentration of recombinant factor V determined using the commercially available factor V ELISA kit (Affinity Biologicals, Hamilton, Ontario, Canada). It is noteworthy that in all experiments performed herein, before concentration of the recombinant molecules, the double mutant (factor $V^{FF/MI}$) was found to be consistently secreted in lower concentrations than the wild type procofactor molecule or the single mutants (*i.e.* the mutants with only two amino acids mutated at the time, factor V^{FF} or factor V^{MI}). However, its concentration was normalized and adjusted in all prothrombinase assays. Thus, all recombinant molecules were assayed under similar experimental conditions.

Determination of the Concentration of the Recombinant Factor V Molecules—The concentration of factor V was determined with an ELISA. 20 μl of polyclonal anti-human factor V (10 mg/ml) was diluted into 20 ml of coating buffer (0.077 M $NaHCO_3$, 0.007 M Na_2CO_3, pH 9.5). A 96-well microtiter plate (Costar, Corning Glass) was loaded with 200 μl/well of the polyclonal antibody mixture and incubated overnight at 4 °C. The final concentration of the antibody in each well was 10 μg/ml. The plate was washed three times with 25 mM Tris, 0.15 M NaCl, pH 7.4, 0.05% Tween 20 (TBS-Tween, washing buffer), following by incubation with 100 μl/well of 5% nonfat dry milk in TBS-Tween at 37 °C for 1 h. Following extensive washing, the plate was loaded (100 μl/well) with either serial dilutions of purified plasma factor V in VP-SFM medium with 4 mM L-glutamine within the range of 20 μg to 5 ng of factor V in triplicate or with various volumes of recombinant factor V solutions in triplicate. Normal plasma factor V was diluted in the medium in order to rule out the possibility that the cell culture media would interfere with the ELISA assay. Following incubation at 37 °C for 1 h, the plate was washed three times followed by incubation with αHFV_{HC} 17 at 5 μg/ml final concentration (per well) for 1 h at 37 °C. Following extensive washing, a 1:2000 dilution of goat anti-mouse antibody coupled to peroxidase was loaded on the wells (100 μl/well), and incubation was allowed to proceed at 37 °C for an additional 1 h. The plates were developed with OPD tablets (5 mg), which were dissolved in 0.1 M citric acid, 0.1 M Na_2HPO_4 (12 ml). Immediately prior to use, 12 μl of 30% H_2O_2 was added to the OPD solution (developing solution), and 100 μl was applied per well. Following incubation at room temperature, the reaction was stopped with 150 μl of 1.5 M H_2SO_4. The absorbance at 490 nm was monitored using an Amersham Biosciences THERMOMAX microplate reader. Because of slight differences in time of incubation with the substrate, in every experiment a plasma factor V standard (serial dilutions of purified plasma factor V) was run, and all values obtained with the recombinant molecules were compared with the plasma factor V standard values within the same 96-well plate. No comparison in concentration was made between recombinant molecules from one plate and the other. The determination of the concentration of the recombinant molecules was performed by averaging the value found for each sample run in triplicate.

Measurement of Rates of α-Thrombin Formation in a Prothrombinase Assay—Since even after concentration, the recombinant molecules resulting from the transient expression were obtained in limited amounts, cofactor activity measurements using DAPA were not possible. The activity of all recombinant factor V molecules was thus measured in a discontinuous assay as follows. All factor V species were

activated with 10 nM thrombin for 20 min followed by the addition of DFP (5 mM). The factor Va solution was then incubated for an additional 30 min on ice. Control experiments demonstrated that under these conditions, no interference of the DFP with the assay could be observed, since DFP is readily hydrolyzed in aqueous solution. Assay mixtures contained PCPS vesicles (20 μM), DAPA (3 μM), various recombinant factor Va species (0.5 nM), and prothrombin (1.4 μM) in 20 mM HEPES, 0.15 M NaCl, 5 mM $CaCl_2$, pH 7.4. The mixture was allowed to incubate at ambient temperature for 5 min, and the reaction was initiated by the addition of factor Xa (5 nM). Aliquots of the reaction mixture were removed at various time intervals, as indicated in the figures, and diluted 3-fold in 20 mM HEPES, 0.15 M NaCl, 50 mM EDTA, 0.1% polyethylene glycol 8000, pH 7.4, in a 96-well microtiter plate (Costar, Corning Glass) to quench the reaction. The formation of α-thrombin in each sample was monitored using the chromogenic substrate Spectrozyme TH (0.4 mM, final concentration). The change in the absorbance at 405 nm was monitored using the Amersham Biosciences THERMOMAX microplate reader. The initial rate of α-thrombin generation under these conditions is linear, and in all experiments no more than 10% of prothrombin was consumed during the initial course of the assay. An excess of the specific α-thrombin inhibitor DAPA was included in all experiments to prevent potential feedback reactions catalyzed by α-thrombin that is generated during the assay. All data were analyzed with the software Prizm (GraphPad). Some of the data were also plotted using DeltaGraph (DeltaPoint).

Gel Electrophoresis and Western Blotting—SDS-PAGE analyses were performed using 5–15% gradient gels according to the method of Laemmli (40). When necessary, proteins were visualized after staining with Coomassie Brilliant Blue. In several experiments, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp.) according to the method described by Towbin *et al.* (41). After transfer to nitrocellulose, factor V heavy and light chain(s) were detected using the appropriate monoclonal and polyclonal antibodies (31–34). Immunoreactive fragments were visualized with chemiluminescence.

Analysis of the Inactivation of Membrane-bound Factor Va (with or without EGR-hXa)—The inactivation of factor Va by APC was studied in the presence or absence of synthetic peptides. EGR-hXa was preincubated with peptides and added to purified membrane-bound factor Va. The final concentrations of all reagents were as follows: factor Va, 20 nM; EGR-hXa, 50 nM; PCPS vesicles, 5 μM; pentapeptides, 200 μM; AP4′, 8 μM; A9A, 8 μM. The reaction was initiated by the addition of APC (5 nM). Samples of the mixture were withdrawn at selected time intervals (indicated in the legends to the figures), mixed with 2% SDS, and heated for 5 min at 90 °C prior to analysis by gel electrophoresis. Following transfer to PVDF membranes, factor Va-immunoreactive fragments were visualized with monoclonal antibody αHVa_{HC} 17 and chemiluminescence.

RESULTS

Inhibition of Prothrombinase Assembly and Function by Synthetic Peptides from the Region 323–331 of Factor Va Heavy Chain—We have recently shown that a 9-amino acid peptide containing the region 323–331 of factor Va heavy (AP4′) chain inhibits factor Va cofactor activity in a noncompetitive manner with respect to substrate (prothrombin) with a K_i of 5.7 μM (29). We have also shown that binding of factor Va and AP4′ to factor Xa are mutually exclusive (29). In order to differentiate between the amino acids of the nonapeptide that are important for its functions, we synthesized three overlapping peptides, of five amino acids each, spanning the 323–331 region (Fig. 1*A*). Each pentapeptide, except the first and the last, has 3 amino acids in common with the preceding and the following peptide of the series (Fig. 1*A*). Thus, the first peptide (E5A) has its first 2 amino acids that are unique to its sequence, whereas the third peptide (A5V) has its last 2 amino acids unique to its sequence. We have also synthesized and tested two control pentapeptides that are mutated at the extremities of AP4′ (Fig. 1*B*). Control peptides were designed by keeping in mind that in order to positively identify the amino acids responsible for the effector and receptor properties of factor Va, we will ultimately have to express recombinant factor V molecules with these specific amino acids mutated in such a manner that the entire conformation of the recombinant factor V molecules possesses a tertiary conformation as close as possible to the wild type factor Va molecule (see below).

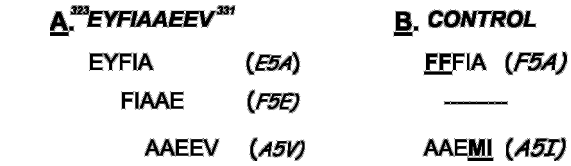

FIG. 1. **Peptides from amino acid region 323–331 of factor V.** *A*, overlapping peptides (5 amino acids each) from the region 323–331 of factor V. Every peptide is identified by the first and last amino acids, with the number of residues composing the peptide in the *middle*. For ease of reading of this report and the explicit identification of every amino acid identified in our work, Glu^{323} and Val^{331} are identified as the beginning and the end of the sequence of interest. *B*, control peptides used in the study. The changed amino acids are in *boldface type* and *underlined*. F5A is the control peptide for E5A, whereas A5I is the control peptide for A5V.

These peptides were first assayed for inhibition of prothrombinase function. Under the conditions employed, all three peptides showed various degrees of inhibition, with E5A being more potent at low concentrations, whereas A5V was more potent at high concentrations of peptide (Fig. 2, *dark filled bars*). Thus, as previously suggested, the pentapeptides contain amino acids that all appear to contribute to the inhibitory potential of AP4′ (29). Experiments using the control peptides demonstrated that replacing Glu^{323} and Tyr^{324} by 2 phenylalanines resulted in diminished inhibitory potential of F5A (Fig. 2, *hatched bars*, 100 and 300 μM), whereas replacing Glu^{330} and Val^{331} by methionine and isoleucine, respectively, resulted in a diminished inhibitory potential of A5I (Fig. 2, *hatched bar*, 500 μM). However, both control peptides had an inhibitory effect on prothrombinase, albeit less potent than their parent peptides. The *open bars* in Fig. 2 depict the positive and negative control peptides AP4′ and A9A previously characterized (29).

We have shown that AP4′ interacts with $[OG_{488}]$-EGR-hXa with half-maximal saturation of ~65 μM (29). We have thus tested the three pentapeptides for their ability to interfere with the fluorescence anisotropy of membrane-bound $[OG_{488}]$-EGR-hXa in the absence of factor Va (Fig. 3). The data show that increasing concentrations of A5V produce a significant decrease (quench) in the fluorescent anisotropy of $[OG_{488}]$-EGR-hXa (Fig. 3, *filled inverted triangles*), demonstrating direct interaction between the peptide and $[OG_{488}]$-EGR-hXa. Half-maximal saturation was reached at ~150 μM peptide. A control pentadecapeptide (P15H) previously shown to have no effect on prothrombinase assembly and function (29) produced a ~20% decrease in the anisotropy of $[OG_{488}]$-EGR-hXa when used at 300 μM (not shown). Peptide E5A had a similar effect on the anisotropy of $[OG_{488}]$-EGR-hXa (~22% decrease in the anisotropy, assuming that the decrease observed in the presence of A5V is the maximum possible effect) (Fig. 3, *filled diamonds*). Under similar experimental conditions, and in the presence of 400 μM peptide F5E, there was a 42% decrease in the anisotropy of $[OG_{488}]$-EGR-hXa (Fig. 3, *filled triangles*). This effect of F5E remained constant even at concentrations as high as 700 μM peptide. The data indicate that A5V alone appears to contain most of the amino acids that are responsible for the direct interaction between $[OG_{488}]$-EGR-hXa and AP4′. However, an additional contribution of 3 amino acids common to F5E and A5V is also apparent. No significant effect of the control peptide A5I was observed on the fluorescent anisotropy of $[OG_{488}]$-EGR-hXa (the decrease in the anisotropy observed was similar to that of a control peptide inducing ~20% change in the fluorescent anisotropy) (Fig. 3, *filled squares*). Overall, these data point to two amino acids, Glu^{330} and Val^{331}, within the sequence of AP4′ that appear to be largely responsible for the

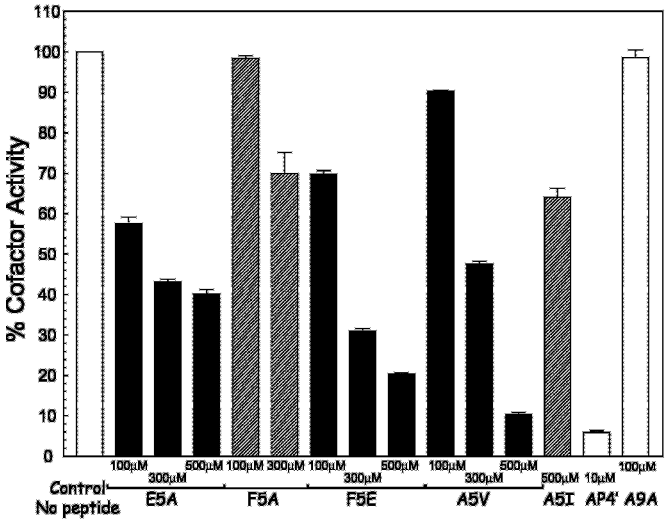

FIG. 2. **Inhibition of prothrombinase function by synthetic overlapping peptides from AP4′.** Peptides were incubated with factor Xa as detailed under "Experimental Procedures" at a fixed concentration (shown at the *bottom* of each *histogram*). The final concentration of factor Xa in the mixture was 10 nM (with human factor Va at 4 nM). The percentage of factor Va cofactor activity was calculated by comparing the activity of prothrombinase in the presence of a given peptide (the rate of thrombin formation) with the activity of prothrombinase determined in a control reaction in the absence of peptide and in the presence of factor Xa. The amino acid sequence and identification of each peptide are given in Fig. 1. The data represent the average of the results found in three independent measurements. The identity of the peptide used in each group of measurements is given at the *bottom*. *Filled bars*, pentapeptides from AP4′; *hatched bars*, control peptides; *open bars*, controls for the entire experiment.

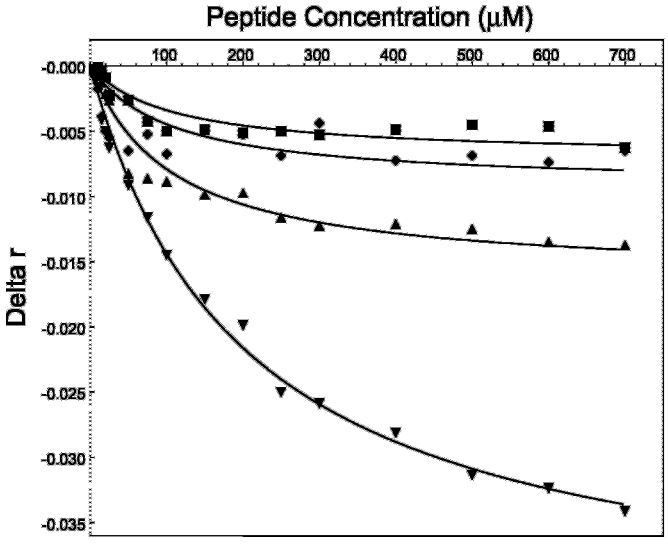

FIG. 3. **Direct interaction with of [OG_{488}]-EGR-hXa.** [OG_{488}]-EGR-hXa (10 nM) in the presence of PCPS vesicles (10 μM) but in the absence of factor Va was titrated with increasing concentrations of E5A (*filled diamonds*), F5E (*filled triangles*), A5V (*filled inverted triangles*), and A5I (*filled squares*) as previously described (29) and briefly detailed under "Experimental Procedures." Fluorescence anisotropy was measured at 25 °C, and the Δr was calculated as previously described (29, 30) and detailed under "Experimental Procedures."

decrease of the anisotropy of [OG_{488}]-EGR-hXa when incubated with the peptide. It is noteworthy that since all three peptides inhibit prothrombinase, they most likely interact with factor Xa; however, only A5V appears to contain amino acids capable of producing a significant decrease of the fluorescent anisotropy of [OG_{488}]-EGR-hXa.

High concentrations of AP4′ and N42R were shown to have a cofactor effect on factor Xa (29). To understand which amino

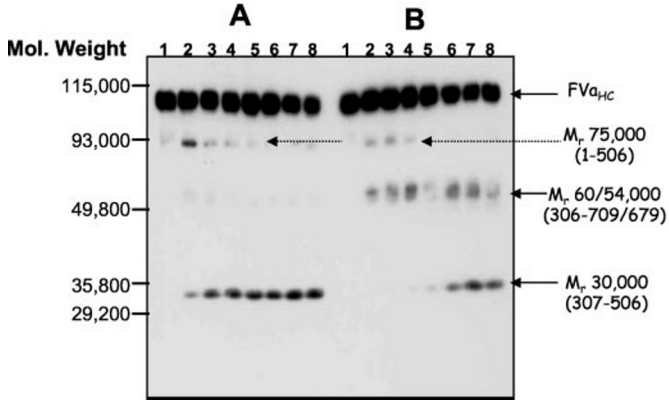

FIG. 4. **Cleavage of factor Va by APC in the presence of active site blocked factor Xa.** Factor Va (40 nM) was cleaved by APC (5 nM) in the absence (*A*) or presence (*B*) of EGR-hXa (80 nM) and PCPS. Samples were analyzed on 5–15% linear gradient SDS-PAGE gels. Following transfer to PVDF membranes, fragments were detected using monoclonal antibody αHFVa_{HC} 17. *Lane 1*, membrane-bound factor Va; *lanes 2–8*, factor Va at 1, 3, 5, 8, 10, 15, and 30 min following the addition of APC. The positions of molecular weight markers are indicated at the *left*, whereas the positions of the factor Va fragments recognized by the monoclonal antibody are indicated at *right*. In *parentheses*, *under* the identification of each fragment, the amino acid sequence of each fragment is also given.

acids from AP4′ are responsible for this effect, we assayed the pentapeptides for their ability to increase the catalytic efficiency of factor Xa during activation of prothrombin in the absence of factor Va. Incubation of E5A (100 μM) with factor Xa resulted in a modest but significant and reproducible increase of the activity of factor Xa with respect to prothrombin activation (~150%) when compared with the activity of factor Xa alone (not shown). No increase in the activity of factor Xa was observed in the presence of any of the other peptides shown in Fig. 1. Thus, amino acids Glu^{323} and Tyr^{324} may be responsible for the cofactor effect of AP4′ on factor Xa (29). Overall, these data demonstrate that Glu^{323}, Tyr^{324}, Glu^{330}, and Val^{331}, which represent amino acids located at the extremities of AP4′, are crucial for the inhibitory potential of the peptide on both prothrombinase assembly and function. These amino acids may be thus required for expression of factor Va cofactor function.

Elimination of the Protective Effect of Factor Xa during Membrane-dependent Inactivation of Factor Va by APC—It has been established that factor Xa protects factor Va from inactivation by APC (42–44). APC inactivation of factor Va following cleavage of the membrane-bound cofactor at Arg^{506}/Arg^{306} and generation of the M_r 30,000 fragment (containing amino acid residues 307–506) is fast and occurs within 1 min (Fig. 4*A*) (26). Following preincubation of the cofactor with EGR-hXa, the appearance of the M_r 30,000 fragment of factor Va is considerably delayed (Fig. 5*B*, *lanes 2–8*), and an increase in the concentration of an M_r 60,000/54,000 doublet is observed (Fig. 4*B*, *lanes 2–6*). This doublet represents initial cleavage of the cofactor at Arg^{306} (26, 31). Our data are in agreement with a previous report showing that upon incubation of the membrane-bound cofactor with EGR-hXa, the rate of cleavage at Arg^{506} is considerably impaired (44). Altogether, these data suggest that upon interaction with the membrane-bound cofactor, the EGR-hXa molecule impairs cleavage by APC at Arg^{506} by hindering access of APC to specific amino acids that are required for its interaction with factor Va and subsequent catalysis. Thus, by preincubating the cofactor with EGR-hXa, we are creating an artificial and transient factor V^{LEIDEN} molecule (31). However, since the factor Va molecule still possesses an arginine at position 506, inactivation of the molecule will

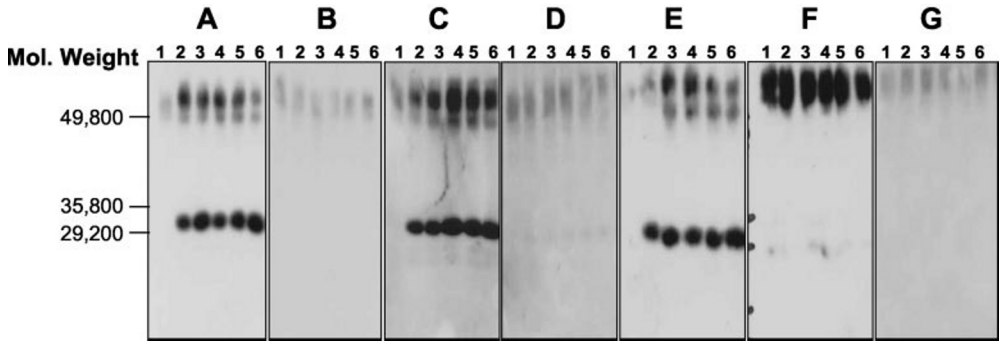

FIG. 5. **Competition for factor Va binding.** Factor Va was incubated with PCPS vesicles. In a different reaction tube, EGR-hXa was incubated with various synthetic peptides. The final concentration of EGR-hXa and the concentration of each peptide are given under "Experimental Procedures." The EGR-hXa molecule, which was preincubated with the peptides, was added to the membrane-bound factor Va solution. APC was then added, and aliquots were withdrawn at selected time intervals and analyzed on 5–15% linear gradient SDS-PAGE gels. Following transfer to PVDF membranes, fragments were visualized using monoclonal antibody $\alpha HFVa_{HC}$ 17. *Lane 1*, membrane-bound factor Va, no APC; *lanes 2–6*, factor Va at 1, 3, 5, 8, and 10 min following the addition of APC. *A*, EGR-hXa preincubated with AP4′; *B*, EGR-hXa preincubated with A9A; *C*, EGR-hXa preincubated with E5A; *D*, EGR-hXa preincubated with F5E; *E*, EGR-hXa preincubated with A5V; *F*, EGR-hXa preincubated with A5I; *G*, EGR-hXa preincubated with F5A. Molecular weight markers are indicated at the *left* of *A*.

still proceed but with the order of cleavages reversed; the first cleavage at Arg^{306} will be followed by a slow cleavage at Arg^{506} and generation of the M_r 30,000 fragment. As a consequence, the appearance of the M_r 30,000 will be delayed, whereas the M_r 60/54,000 doublet will be more pronounced compared with the cleavage of factor Va in the absence of EGR-hXa (Fig. 4*B*, *lanes 2–8*). These data verify the crucial importance of prior cleavage at Arg^{506} for cleavage at Arg^{306} and rapid inactivation of the cofactor as previously suggested (26, 31, 32). These data also demonstrate that we can distinguish between the binding of EGR-hXa to the membrane-bound cofactor or not by the absence or presence, respectively, of the M_r 30,000 fragment (at the beginning of the reaction). Our previous (29) and present data show that a factor Xa binding site is contained within amino acid residues 323–331 of factor Va heavy chain. Thus, we can formulate the hypothesis that upon preincubation of EGR-hXa with a peptide from factor Va that contains a binding site for factor Xa, binding to the cofactor will be prevented, resulting in the elimination of the protective effect of EGR-hXa from APC inactivation. As a consequence, membrane-bound factor Va will be rapidly cleaved by APC with the appearance of the M_r 30,000 fragment. The data resulting from such an experiment are illustrated in Fig. 5. To simplify the figure and for the easy analysis of the data, we have focused on the portion of the gels around the region showing the M_r 30,000 fragment resulting from cleavage of factor Va heavy chain at Arg^{506}/Arg^{306}. The data only show the progress of the reaction through the first 10 min.

Under the conditions employed, upon preincubation of EGR-hXa with AP4′, inactivation of membrane-bound factor Va by APC occurred normally with rapid cleavages at Arg^{506}/Arg^{306} and appearance of the M_r 30,000 fragment (Fig. 5*A*). The appearance of the M_r 30,000 fragment was not observed when EGR-hXa was preincubated with A9A, a peptide containing a scrambled version of AP4′ (29) (Fig. 5*B*), demonstrating that 1) AP4′ inhibits the interaction of EGR-hXa with membrane-bound factor Va, and thus, allows APC cleavage of the cofactor, and 2) A9A, which does not interact with factor Xa, allows protection of the membrane-bound cofactor by EGR-hXa from cleavage by APC. Preincubation of EGR-hXa with either E5A or A5V results in the appearance of the M_r 30,000 fragment following incubation of the membrane-bound cofactor with APC (Fig. 5, *C* and *E*). These data strongly suggest that the two pentapeptides contain amino acids capable of interfering with the binding of EGR-hXa to membrane-bound factor Va, thus inhibiting the protective effect of the cofactor by EGR-hXa and allowing APC cleavage of the heavy chain at Arg^{506}/Arg^{306}, resulting in formation of the M_r 30,000 fragment. Consistent with the findings shown in Figs. 2 and 3, the control peptides, A5I (Fig. 5*F*) and F5A (Fig. 5*G*), did not interfere with the ability of EGR-hXa to protect membrane-bound factor Va from inactivation by APC. Interestingly, pentapeptide F5E, which inhibited prothrombinase function and induced a modest decrease in the fluorescent anisotropy of $[OG_{488}]$-EGR-hXa, was unable to inhibit EGR-hXa protection of factor Va under the conditions employed (Fig. 5*D*). Overall, these data confirm our previous findings and demonstrate that peptides E5A and A5V contain specific amino acids that are involved in their direct interaction with factor Xa. It is noteworthy that in control experiments, a delay in factor Va inactivation (a decrease in the rate of appearance of the M_r 30,000 fragment) was observed when APC was preincubated with high concentrations of AP4′ prior to the addition to the purified membrane-bound factor Va molecule in the absence of EGR-hXa. The bulk of data thus far accumulated demonstrate that amino acids Glu^{323}, Tyr^{324}, Glu^{330}, and Val^{331} of factor Va heavy chain, which are located at the extremities of AP4′, are crucial for the interaction of the cofactor with factor Xa.

Expression and Activation of Recombinant Human Factor Va—In view of the data shown above, we used recombinant technology to assess the effect of these 4 amino acids residues on factor Va cofactor function. Three recombinant factor V molecules were made: factor V^{FF} and factor V with the mutations $Glu^{323} \rightarrow Phe/Tyr^{324} \rightarrow Phe$, factor V^{MI} and factor V with the mutations $Glu^{330} \rightarrow Met/Val^{331} \rightarrow Ile$, and factor $V^{FF/MI}$ and factor V with four substitutions $Glu^{323} \rightarrow Phe$, $Tyr^{324} \rightarrow Phe$, $Glu^{330} \rightarrow Met$, and $Val^{331} \rightarrow Ile$. All recombinant molecules were expressed in COS-7L cells. The concentration of each molecule prior to all assays was calculated using an ELISA recently developed in our laboratory. The capture antibody was a polyclonal antibody (sheep anti-factor V), whereas the detecting antibody was the monoclonal antibody αHFV_{HC} 17 (31–34). For comparison, we have also used a commercially available kit containing two polyclonal antibodies, previously employed by other investigators, to assess the concentration of recombinant factor V in conditioned media (45–48). A linear correlation between the absorbance at 490 nm and increasing concentrations of purified plasma factor V was observed between 10 ng and 10 μg of plasma factor V in both our assay and the commercial ELISA kit (not shown). Further, all plasma and recombinant molecules gave similar results in the two ELISA (*i.e.* similar absorbance values at 490 nm were observed for a given volume containing a recombinant molecule); thus, all molecules were recognized with similar affinities by the detect-

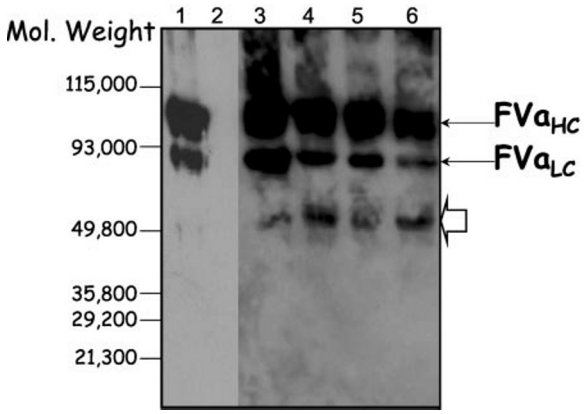

FIG. 6. **Electrophoretic analyses of recombinant factor V molecules.** Recombinant factor V molecules were activated by thrombin as described under "Experimental Procedures" and analyzed on 5–15% linear gradient SDS-PAGE. Following transfer to PVDF membranes, the heavy chain and light chain of the recombinant molecules were detected using a mixture of monoclonal antibodies αHFVa$_{HC}$ 17 (recognizing the heavy chain) and αHFVa$_{LC}$ 9 (recognizing the light chain). *Lane 1*, plasma factor Va; *lane 2*, media harvested from cells without the factor V construct (mock-transfected cells); *lane 3*, wild type recombinant factor Va; *lane 4*, factor Va^{FF}; *lane 5*, factor Va^{MI}; *lane 6*, factor Va$^{FF/MI}$. The positions of the molecular weight markers are indicated at the *left*. On the *right*, the positions of the heavy and light chains of factor Va are shown. The *open arrowhead* identifies a degradation product from factor Va that is recognized by the monoclonal antibody directed to the light chain of the cofactor.

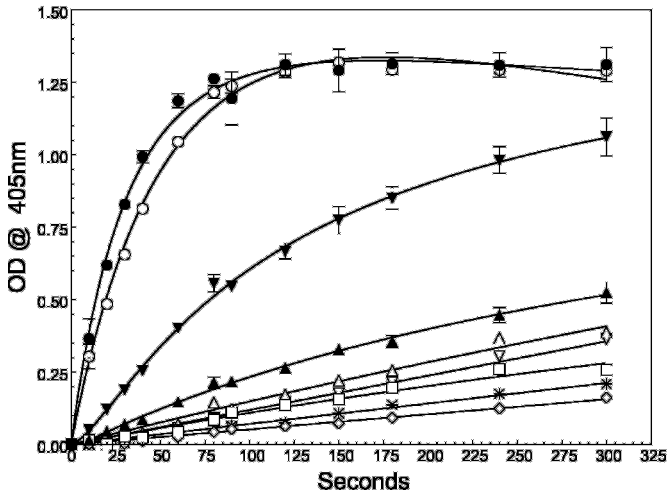

FIG. 7. **Comparison of the cofactor activities of the recombinant factor Va molecules.** The cofactor activities of the various factor Va molecules were measured following activation by thrombin in an assay system using purified reagents and employing spectrozyme-TH to probe for thrombin generation as described under "Experimental Procedures." Initial rates of prothrombin activation were determined at ambient temperature using 500 pM factor Va and 5 nM factor Xa. The various factor Va species are depicted as follows. *Filled circles*, wild type recombinant factor Va; *open circles*, plasma-derived factor Va; *filled inverse triangles*, factor Va^{FF}; *filled triangles*, factor Va^{MI}; *open squares*, factor Va$^{FF/MI}$ at 1 nM; *open triangles*, factor Va$^{FF/MI}$ at 2 nM; *open inverted triangles*, factor Va$^{FF/MI}$ at 5 nM; *open diamonds*, factor Xa alone, no factor Va; *stars*, factor Xa in the presence of media harvested from control cells without the factor Va construct. The data represent the average of the results found in three independent experiments.

ing antibodies. The concentrations of the recombinant molecules in the media before concentration, using centrifugal ultrafiltration, varied from 300 ng/ml to 3.2 μg/ml. Fig. 6 shows an SDS-PAGE of the secreted molecules used in our experiments following concentration and incubation with α-thrombin. Fig. 6, *lane 1*, is a control sample representing the heavy and light chains of plasma factor Va and demonstrates that all heavy and light chains of the recombinant factor Va molecules (wild type or mutated) (Fig. 6, *lanes 3–6*) have similar molecular weights, which do not appear to be significantly different from the molecular weight of the heavy and light chain(s) of the plasma-derived factor Va molecule (Fig. 6, *lane 1*). Thus, introduction of the mutations in factor Va heavy chain does not alter the ability of the recombinant molecules to be recognized by the monoclonal antibodies; nor does it alter their mobility on an SDS-PAGE. In Fig. 6, *lane 2*, a control sample is shown where all steps were carried out with cells that do not contain the cDNA for factor V (mock transfection). The *open arrow* identifies a M_r 50,000 fragment that is recognized by the monoclonal antibody to the light chain of the cofactor. This band is present in the plasma-derived cofactor molecule (visible upon prolonged exposure of the autoradiogram) and is also recognized by a polyclonal antibody to human factor V. Overall, the data demonstrate that the quality of the recombinant molecules is similar to the quality of plasma-derived factor Va.

Functional Characterization of the Recombinant Molecules—The recombinant molecules were first screened for clotting activity in a one- and two-stage clotting assay (35). Whereas factor V^{FF} and factor V^{MI} had ~10-fold less clotting activity than the wild type recombinant factor Va molecule, the double mutant (factor Va$^{FF/MI}$) was unable to promote clotting under the conditions employed in a one- or two-stage clotting assay using factor V-deficient plasma (*i.e.* the clotting activity of the double mutant was similar to the clotting activity observed when using the concentrated media obtained from mock-transfected COS-7L cells; not shown). Thus, whereas mutation of 2 amino acids at the time results in a cofactor with impaired clotting activity, substitution of all 4 amino acid residues resulted in the elimination of factor Va clotting activity. These data suggest that these 4 amino acids are of crucial importance for the expression of factor Va clotting activity.

We next investigated the ability of the thrombin-activated recombinant molecules (at 0.5 nM) to interact with factor Xa (5 nM) and be assembled into prothrombinase using an assay employing purified reagents and a chromogenic substrate. Since the assay is conducted with limiting cofactor concentrations, any dearth in cofactor activity may be explained by the inability of the recombinant molecules to act as a cofactor for factor Xa. The data demonstrated that under the conditions employed wild type recombinant factor Va (Fig. 7, *filled circles*) displayed similar cofactor activity as plasma-derived factor Va (Fig. 7, *open circles*), 2550 and 2100 mOD/min, respectively. Under similar experimental conditions, factor Va^{FF} (Fig. 7, *filled inverted triangles*) and factor Va^{MI} (Fig. 7, *filled triangles*) showed decreased cofactor activities, of 450 and 150 mOD/min, respectively. Thus, the activity of the two mutants is 5.7- and 17-fold less compared with wild-type factor Va. However, the activity of factor Va^{MI} was ~3-fold lower than the activity of factor Va^{FF} and was always consistently lower than factor Va^{FF} in several other experiments using various concentrations of factor Xa.

In preliminary experiments, using several preparations of the double mutant (factor Va$^{FF/MI}$), we have observed that the rate of thrombin generation by this mutant, under the conditions employed above (0.5 nM factor Va with 5 nM factor Xa), could not be distinguished from the rate of thrombin generation by factor Xa alone (Fig. 7, *open diamonds*, ~32 mOD/min) or from the rate of thrombin generation by factor Xa in the presence of the media collected from mock-transfected cells (Fig. 7, *stars*, 36 mOD/min). We have thus decided to measure thrombin generation by prothrombinase in the presence of increasing concentrations of factor Va$^{FF/MI}$ (Fig. 7, 1 nM (*open squares*), 2 nM (*open triangles*), and 5 nM (*open inverted triangles*) with

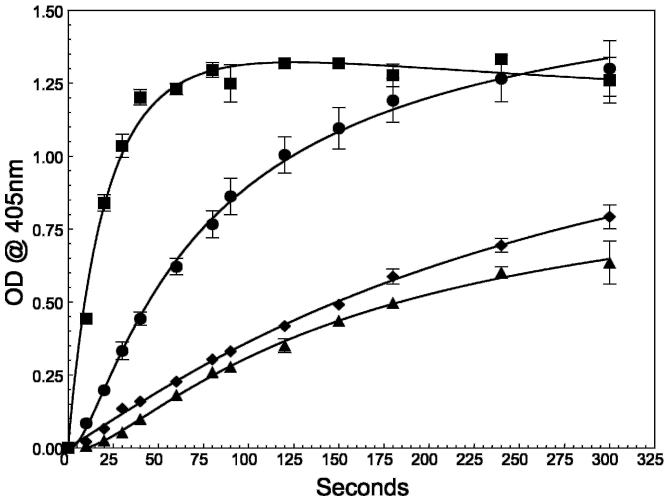

FIG. 8. **Contribution of the light chain of factor Va to the binding to factor Xa.** The cofactor activities of the various factor Va molecules were measured following activation by thrombin in an assay system using purified reagents and employing Spectrozyme-TH to probe for thrombin generation. Initial rates of prothrombin activation were determined at ambient temperature using 5 nM factor Xa and factor Va species as indicated under "Experimental Procedures." The various factor Va species are depicted as follows. *Filled squares*, wild type factor Va (1 nM); *filled circles*, factor Va^{FF} (1 nM); *filled diamonds*, factor Va^{MI} (1 nM); *filled triangles*, factor $Va^{FF/MI}$ at 10 nM. The data represent the average of the results found in three independent experiments.

factor Xa constant at 5 nM. The data obtained showed a small but significant increase in the catalytic efficiency of factor Xa in the presence of increasing concentrations of the double mutant, up to 90 mOD/s in the presence of 5 nM factor $Va^{FF/MI}$. A direct comparison between wild type factor Va and the double mutant revealed a 284-fold decrease in cofactor activity of factor $Va^{FF/MI}$ (5100 mOD·min^{-1}·nM^{-1} for the wild type and 18 mOD·min^{-1}·nM^{-1} for factor $Va^{FF/MI}$). These data demonstrate that even in the presence of stoichiometric concentrations (Fig. 7, *open inverted triangles*) factor $Va^{FF/MI}$ is a poor cofactor for factor Xa.

It has been established that binding sites from both chains of the cofactor contribute to the interaction of the cofactor with factor Xa (11, 21–24). In the experiment shown in Fig. 7, we have assessed the contribution of factor Va to prothrombinase at limited cofactor concentration. The experimental conditions were thus designed to make prothrombinase assembly sensitive to any alterations in the factor Va molecule. However, if the binding site from the light chain depends on the binding of the heavy chain, by increasing the concentration of the cofactor molecule (with factor Xa kept constant at 5 nM) we do not expect to observe an increase in the rate of thrombin generation when using the mutant molecules. In contrast, if the binding site on the light chain can increase the catalytic efficiency of factor Xa, independently of the binding site located on the heavy chain, by increasing the concentration of the cofactor, an increase in the catalytic efficiency of all mutants will be observed.

A direct comparison between factor Va^{FF} and factor Va^{MI} (1 nM mutant cofactor with 5 nM factor Xa) demonstrated that the activity of factor Va^{FF} was 780 mOD/min, whereas the activity of factor Va^{MI} was 200 mOD/min (Fig. 8, *filled circles* and *filled diamonds*, respectively). These data, together with the data shown in Fig. 7, verify our previous conclusion that under similar experimental conditions, the absence of amino acids Glu^{330} and Val^{331} has a more profound effect on cofactor function than the absence of amino acids Glu^{323} and Tyr^{324}. In the presence of 10 nM factor $Va^{FF/MI}$, the rate of thrombin formation was ~180 mOD/min (Fig. 8, *filled triangles*); this rate is 25-fold smaller than the rate of thrombin generation in the presence of wild type recombinant factor Va (~4500 mOD/min, Fig. 8, *filled squares*). However, when comparing prothrombinase activity per nM enzyme used, and assuming a 1:1 stoichiometry between factor Va and factor Xa, the activity of the double mutant when used at 10 nM (5 nM prothrombinase) was 36 mOD·min^{-1}·nM^{-1}, whereas the activity of the wild type molecule was 4500 mOD·min^{-1}·nM^{-1} (1 nM prothrombinase). These data show a 125-fold decrease in activity of the double mutant when used at saturating concentrations compared with the wild type molecule. Altogether, our data suggest that whereas the remaining binding site for factor Xa on the light chain of the cofactor may be able to partially compensate for the absence of residues Glu^{323} and Tyr^{324}, the absence of amino acids Glu^{330} and Val^{331} from the heavy chain of the recombinant molecule appears more difficult to overcome. However, absence of all 4 amino cannot be efficiently overcome by the binding site(s) on the light chain, resulting in a factor Va molecule with severely impaired cofactor activity. Thus, the binding site(s) from the light chain of the molecule can increase factor Xa activity independently of the binding site from the heavy chain as previously suggested (23).

The fact that an increase in the concentration of factor $Va^{FF/MI}$ is required to obtain a weak cofactor effect demonstrates 1) a weakened interaction of the mutant cofactor with factor Xa and 2) that discrete amino acids from both chains of factor Va must interact with factor Xa in order to achieve the dramatic increase in the catalytic efficiency of prothrombinase when compared with factor Xa alone; replacement of the specific amino acids with residues containing a different functional group results in the elimination of the contribution of those amino acids to the activity of prothrombinase. It is noteworthy that our data do not exclude the contribution of another portion of the heavy chain to the interaction with factor Xa as suggested earlier (49, 50). However, amino acids Glu^{323}, Tyr^{324}, Glu^{330}, and Val^{331} from the heavy chain of factor Va appear to be critical for the interaction of the cofactor with the enzyme and contribute a major binding site for factor Xa.

DISCUSSION

By using a systematic peptide approach and employing both protein chemistry and molecular biology approaches, we have demonstrated that amino acids Glu^{323}, Tyr^{324}, Glu^{330}, and Val^{331} located on the A2 domain of factor V are crucial for expression of factor Va cofactor activity. The region 323–331 of the heavy chain of factor Va containing these 4 amino acid residues was conserved throughout evolution, being 100% identical between species (17, 51–53). It is not yet known if the same amino acid residues are responsible for both the cofactor and effector functions of factor Va or different amino acids are involved in the two processes.

Factor Xa alone possesses the catalytic machinery for cleavage and activation of prothrombin; however, its interaction with factor Va and a membrane surface are required to obtain a physiologically relevant rate for catalysis. Membrane-bound factor Xa alone activates prothrombin following two sequential cleavages: Arg^{271} followed by cleavage at Arg^{320} (54–56). These cleavages proceed through the intermediate prethrombin 2 and produce α-thrombin and the activation fragment, fragment 1·2. The interaction of factor Va with factor Xa and its incorporation into the prothrombinase complex alters the order and the rate of peptide bond cleavages, resulting in the formation of an active intermediate, meizothrombin (first cleavage at Arg^{320}), followed by the formation of fragment 1·2 and α-thrombin

(cleavage at Arg^{271}) (7, 54–56). The factor Va effect on factor Xa, besides reversing the order of cleavages during prothrombin activation, is also manifested by the increase in the overall k_{cat} of the prothrombin, activating reaction by 3,000-fold. The dramatic effect of factor Va on the catalytic machinery of factor Xa is yet to be understood at the molecular level. A recent study using recombinant mutant prothrombin molecules has shown that whereas the catalytic efficiency of factor Xa for cleavage at Arg^{320} is increased by ~20,000-fold in the presence of factor Va, the catalytic efficiency of the enzyme for cleavage at the Arg^{271} is only increased by 453-fold following binding to the cofactor (57). However, the overall catalytic efficiency of the assembled prothrombinase for cleavage at each site separately was similar. Thus, cleavage at Arg^{320} largely benefits from the interaction of the cofactor with factor Xa when compared with cleavage at Arg^{271} (57). As a consequence, it appears that incorporation of factor Va into prothrombinase results in the rearrangement of the prothrombin molecule in such a manner that cleavages at Arg^{320} and Arg^{271} by the factor Va-factor Xa complex independently become more favorable for catalysis by prothrombinase than by membrane-bound factor Xa. This rearrangement of the prothrombin molecule upon its incorporation into the complex and its interaction with factor Va and factor Xa was previously suggested following the determination of the x-ray crystal structure of prethrombin 2 (58).

The binding of factor Va to factor Xa involves both chains of the cofactor (11, 22–24). Whereas the binding site on the light chain of factor Va remains to be identified, the binding site on the heavy chain of the cofactor has been the object of intense investigation. Because APC and plasmin inactivate the cofactor following limited proteolysis of the heavy chain and dissociation of a portion of the molecule (25–28), several studies have attempted to identify amino acid regions from the cofactor that are important for its interaction with factor Xa. The regions studied were usually located around the inactivating cleavage sites of the cofactor or comprised an entire region located between two cleavage sites. A binding site for factor Xa was reported within amino acid residues 493–506 of the heavy chain (49, 50). We have previously suggested that a binding site for factor Xa is located within amino acid region 307–348 of the heavy chain of factor Va (28). We have next focused on this region of the cofactor, because our findings suggested that whereas cleavage of the heavy chain by plasmin at Arg^{348} is of no consequence for the activity of the cofactor, cleavage by APC at Arg^{306} or by plasmin at Lys^{309}, Lys^{310}, and Arg^{313}, which are all membrane-dependent, resulted in rapid and complete inactivation of factor Va (26, 28). Thus, this region of factor Va, which most likely undergoes conformational rearrangement(s) upon binding of the cofactor to a cell surface at the place of vascular injury, contains several amino acids that are important for its incorporation into prothrombinase. Using overlapping peptides from the region 307–351, we have recently identified a nonapeptide containing amino acid residues 323–331 (AP4′) that represents a binding site for factor Xa. We have further shown that the peptide interferes with the binding of factor Va to $[OG_{488}]$-EGR-hXa and can induce a "cofactor" effect in the absence of factor Va, increasing the catalytic efficiency of the enzyme by 4-fold when used at high concentrations (29). In the present study, the use of overlapping pentapeptides from AP4′ led to the identification of 4 amino acids located at its extremities that are required for its overall activity. Recombinant molecules mutated at the specific sites confirmed our findings.

The measure of the factor Va cofactor effect on factor Xa when using the recombinant factor Va molecules and an assay measuring α-thrombin generation is usually determined by the extent of the increase in the rate of α-thrombin formation. Thus, the experiments using recombinant molecules cannot distinguish between binding and effector function of factor Va. The identification of the 4 amino acids described herein and their putative function within the factor Va molecule was based purely on the experiments using synthetic peptides. The experiments using recombinant molecules were only confirmatory and can only attest to their importance within the overall structure of the cofactor. Our data show that whereas peptide A5V was able to interfere with the fluorescent anisotropy of $[OG_{488}]$-EGR-hXa, E5A had no significant effect on the fluorescent anisotropy of the enzyme. In contrast, whereas E5A alone was able to increase the catalytic efficiency of the enzyme toward prothrombin, peptide A5V was unable to produce a similar effect. Since a perturbation of the signal of membrane-bound $[OG_{488}]$-EGR-hXa (positive or negative) is usually correlated with direct binding (4, 5, 29, 30), overall the data suggest that whereas amino acids Glu^{323} and Tyr^{324} alone may contribute to the cofactor effect of factor Va (*i.e.* increase in the catalytic efficiency of factor Xa), residues Glu^{330} and Val^{331} may be responsible for the high affinity interaction between the cofactor and the enzyme on the membrane surface.

The reporter group Oregon Green 488 is attached to a chloromethyl ketone, which is covalently linked to the histidine located in the active site of the enzyme (59, 60). Its intensity is only perturbed in the presence of peptide A5V, which, when tested alone, does not have any effect on the catalytic efficiency of the enzyme. In contrast, peptide E5A, which increases the activity of factor Xa toward prothrombin, does not have any significant effect on the fluorescent anisotropy of the probe attached to the active site of the enzyme. Thus, the changes in the structure of factor Xa induced by E5A are different from the changes induced on the molecule by A5V. Using recombinant molecules, we have validated the data obtained with synthetic peptides, and we have shown that under similar experimental conditions, factor Va^{FF} had consistently higher cofactor activity than factor Va^{MI}, but both recombinant molecules had impaired cofactor activity when compared with the wild type cofactor. Thus, under any conditions employed, the binding site on the light chain together with any remaining binding site from the heavy chain is more efficient in increasing factor Xa catalytic activity in the presence of amino acid residues Glu^{330} and Val^{331} than in the presence of amino acids Glu^{323} and Tyr^{324}. However, lack of all 4 amino acids results in a factor Va molecule (factor $Va^{FF/MI}$) that is severely deficient in its cofactor activity.

In the activity assays described here, the exact concentration of each recombinant molecule used was of crucial importance for the validation of our experiments, since impaired activity of the recombinant molecules could be explained by the wrong concentration used in the assay. An alternative explanation for the decreased activities of the mutant factor Va molecules could also be the fact that the recombinant molecules have altered conformation, due to the change in the amino acid side chain, which will ultimately impair their cofactor function. Finally, the reason for impaired cofactor activity could be the lack of the critical amino acids required for proper cofactor function. In order to eliminate the first two possibilities, since the latter explanation is the ultimate desired effect of our experiments, the concentration of each molecule prior to all assays was calculated using an ELISA recently developed in our laboratory. All molecules were recognized with similar affinities by the detecting antibodies, and SDS-PAGE analysis did not reveal any electrophoretic abnormalities between normal plasma factor Va and the recombinant factor Va molecules.

Further, we have made three conservative and one nonconservative mutation in such a manner that the entire conformation of the recombinant factor V molecules possesses a tertiary conformation as close as possible to the wild type factor Va molecule. Since the cofactor activity of the recombinant wild type factor Va molecule was similar to the activity of the plasma-derived cofactor, we must conclude that 1) the concentrations of the recombinant factor Va molecules obtained from the ELISA were accurate, and 2) the recombinant molecules were properly folded. As a conclusion, impaired cofactor effect of the mutant molecules is the consequence of the mutations. All of these findings can be offered as arguments against generalized conformational change phenomena resulting in impaired cofactor activity. Further, several studies using recombinant factor V and factor V mutants have been performed by various investigators using methodologies similar to ours (45–48). No problems with the conformation of the recombinant proteins have yet been reported. Thus, any difference observed in the cofactor activities of the recombinant mutant molecules is a direct consequence of the specific mutations. Altogether, our data would suggest that amino acids Glu^{323}, Tyr^{324}, Glu^{330}, and Val^{331} are critical for the high affinity interaction of factor Va with factor Xa on the membrane surface and provide a reasonable starting point for a systematic investigation of the amino acids that play crucial role for the expression of cofactor activity. Finally, it is important to note that the possibility that the mutant molecules have a weakened chain-chain interaction because of the mutations resulting in a weaker interaction between factor Va and factor Xa could not be excluded by our studies.

As stated earlier, our data do not exclude the presence of another binding site for factor Xa on the heavy chain of the cofactor. The existence of a binding site for the enzyme was suggested between amino acid residues 493 and 506 (49, 50). A peptide containing these amino acids was found to inhibit prothrombinase activity in a system using purified reagents with an IC_{50} of 3 μM. Kinetic analyses of the inhibition pattern suggested a mixed type of inhibition. The inhibitory peptide also interfered with the amidolytic activity of the enzyme toward a chromogenic substrate when used at high concentrations and was also found to interact with prothrombin. Thus, it was not clear if the inhibitory peptide inhibited factor Xa or prothrombin interaction with factor Va or if the peptide interfered with the binding of the cofactor to the phospholipid bilayer. Further, when the inhibitory peptide was amidated at the COOH terminus or when a glutamine was substituted for an arginine at the carboxyl terminus, its inhibitory activity was lost (49, 50). It is thus possible that Arg^{506} from factor Va or its surrounding amino acids may also provide for an interactive site with factor Xa, albeit this interaction with the enzyme may be secondary to the interaction of factor Xa with the peptide containing amino acid residues 323–331.

In conclusion, our data identify amino acid residues Glu^{323}, Tyr^{324}, Glu^{330}, and Val^{331} from the factor Va heavy chain to be essential for expression of optimum factor Va cofactor function. A synthetic peptide containing these amino acids that will inhibit cofactor activity will have the same effect as inhibiting factor Xa and/or α-thrombin directly and may thus be a potent anticoagulant with few or no side effects, since it is unlikely that this peptide will interfere with the functions of any other protein of the blood clotting cascade.

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