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The Contribution of Amino Acid Region Asp^{695}–Tyr^{698} of Factor V to Procofactor Activation and Factor Va Function*

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There is strong evidence that a functionally important cluster of amino acids is located on the COOH-terminal portion of the heavy chain of factor Va, between amino acid residues 680 and 709. To ascertain the importance of this region for cofactor activity, we have synthesized five overlapping peptides representing this amino acid stretch (10 amino acids each, HC1–HC5) and tested them for inhibition of prothrombinase assembly and function. Two peptides, HC3 (spanning amino acid region 690–699) and HC4 (containing amino acid residues 695–704), were found to be potent inhibitors of prothrombinase activity with IC$_{50}$ values of ~12 and ~10 μM, respectively. The two peptides were unable to interfere with the binding of factor Va to active site fluorescently labeled Glu-Gly-Arg human factor Xa, and kinetic analyses showed that HC3 and HC4 are competitive inhibitors of prothrombinase with respect to prothrombin with K_i values of ~6.3 and ~5.3 μM, respectively. These data suggest that the peptides inhibit prothrombinase because they interfere with the incorporation of prothrombin into prothrombinase. The shared amino acid motif between HC3 and HC4 is composed of Asp^{695}-Tyr-Asp-Tyr-Gln^{699} (DYDYQ). A pentapeptide with this sequence inhibited both prothrombinase function with an IC$_{50}$ of 1.6 μM (with a K_D for prothrombin of 850 nM), and activation of factor V by thrombin. Peptides HC3, HC4, and DYDYQ were also found to interact with immobilized thrombin. A recombinant factor V molecule with the mutations Asp^{695} → Lys, Tyr^{696} → Phe, Asp^{697} → Lys, and Tyr^{698} → Phe (factor V^{2K2F}) was partially resistant to activation by thrombin but could be readily activated by RVV-V activator (factor Va$_{RVV}$^{2K2F}) and factor Xa (factor Va$_{Xa}$^{2K2F}). Factor Va$_{RVV}$^{2K2F} and factor Va$_{Xa}$^{2K2F} had impaired cofactor activity within prothrombinase in a system using purified reagents. Our data demonstrate for the first time that amino acid sequence 695–698 of factor Va heavy chain is important for procofactor activation and is required for optimum prothrombinase function. These data provide functional evidence for an essential and productive contribution of factor Va to the activity of prothrombinase.

The prothrombinase complex, which is composed of the non-enzymatic cofactor factor Va, the enzyme, factor Xa, and the substrate, prothrombin, associated on a cell membrane-surface in the presence of Ca^{2+} ions, is responsible for α-thrombin formation during blood coagulation (1, 2). The prothrombinase complex catalyzes the activation of prothrombin ~300,000 times more efficiently than factor Xa alone (3). The increase in the catalytic efficiency of prothrombinase as compared with factor Xa alone arises from a decrease in the K_m and an increase in the k_{cat} of the enzyme (4–7). The procofactor, factor V, does not interact with the components of prothrombinase. Proteolytic processing of factor V by thrombin at Arg^{709}, Arg^{1018}, and Arg^{1545}, resulting in the production of the active cofactor, factor Va, which consists of a heavy chain (M_r 105,000) and a light chain (M_r 74,000), is required for the interaction of the cofactor with the members of prothrombinase (8–10). In contrast, proteolytic inactivation of factor Va by activated protein C (APC)^1 results in its inactivation because of the inability of the cleaved cofactor to interact with factor Xa and prothrombin (11–16).

Earlier data have demonstrated that, although both chains of factor Va are required for the interaction with factor Xa, only the heavy chain of the cofactor binds prothrombin (14, 15, 17). Cleavage of factor Va by APC at Arg^{506}/Arg^{679} results in a 10-fold decrease in the affinity of the molecule for factor Xa and the elimination of its interaction with prothrombin (13–16). Subsequent cleavage at Arg^{306}, which is lipid-dependent, completely abolishes the ability of the cofactor to interact with factor Xa (12, 13). Prothrombin and thrombin have two distinct electropositive binding exosites (anion binding exosite I (ABE-I) and anion binding exosite II (ABE-II)) that are responsible for the functions of the molecules (18–27). ABE-I has been involved in binding to thrombomodulin (28), fibrinogen (29), heparin cofactor II (30), PAR1 (31), and the COOH-terminal hirudin peptides (32). ABE-II was found to be involved in the interaction with heparin cofactor II (30), protease nexin (33), and antithrombin III (30). Although the involvement of ABE-I

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^{1} The abbreviations used are: APC, activated protein C; DFP, diisopropyl fluorophosphate; PS, L-α-phosphatidylserine; PC, L-α-phosphatidylcholine; PCPS, small unilamellar phospholipids vesicles composed of 75% L-α-phosphatidylcholine and 25% L-α-phosphatidylserine (w/w); DAPA, dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amide; [OG$_{488}$]EGR-hXa, human factor Xa blocked in the active site with glutamylglycinylarginyl chloromethyl ketone labeled with Oregon Green 488; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; ELISA, enzyme-linked immunosorbent assay; PVDF, polyvinylidene difluoride; ABE-I, anion binding exosite I; ABE-II, anion binding exosite II; factor V^{2K2F}, quadruple mutant, recombinant human factor V with the mutations Asp^{695} → Lys, Tyr^{696} → Phe, Asp^{697} → Lys, and Tyr^{698} → Phe; factor Va$_{IIa}$^{2K2F}, quadruple mutant activated with thrombin; factor Va$_{RVV}$^{2K2F}, quadruple mutant activated with RVV-V activator; factor Va$_{Xa}$^{2K2F}, quadruple mutant activated with factor Xa.

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of prothrombin in the productive interaction with factor Va within prothrombinase has been clearly demonstrated (18–21, 34), some data also suggested that ABE-II of the molecule is also involved in the activation of factors V and VIII (22, 23). Proexosite I of prothrombin, which is present in a low affinity state on the molecule, is fully exposed following activation and formation of thrombin, and the affinity for its ligands increases by ∼100-fold (21, 27). The procofactor, factor V, was found to interact with immobilized thrombin through ABE-I but with a lower affinity than factor Va (18). As a consequence, there has been no interaction between prothrombin and factor V reported because the binding sites involved in the interaction between the two molecules are most likely hidden within their core and at least one of the two molecules must be activated and expose a portion or an entire exosite for the binary interaction to occur.

Factor Va is required for the presentation of the substrate (prothrombin) to the enzyme (factor Xa). There is evidence suggesting that incorporation of factor Va into prothrombinase and its interaction with factor Xa and prothrombin, does not significantly alters the catalytic triad of the enzyme (35–39). As a consequence, it has been suggested that, upon interaction with factor Va, factor Xa expresses cryptic exosites for prothrombin, which in turn appear to be largely responsible for the increase in the catalytic efficiency of the enzyme within prothrombinase (35–38). These latter studies were performed with specific inhibitors of factor Xa that interact with the enzyme at precise sites remote from its active site. However, several laboratories have demonstrated that prothrombin and thrombin bind to the isolated heavy chain of the cofactor in a calcium-independent manner through ABE-I (14, 15, 18–21, 40).

Although a binding site for factor Xa has been recently identified on the heavy chain of factor Va (41–43), the specific site(s) on the heavy chain of the cofactor that interact with thrombin and prothrombin remain to be identified. Using various proteolytic enzymes, we and others have shown that the carboxyl-terminal portion of the heavy chain of factor Va (residues 680–709) is responsible for the interaction of factor Va with one or both components of prothrombinase (44–46). This amino acid region, which is highly acidic in nature and contains the tyrosine residues that were previously shown to have the potential to be involved in both procofactor activation by thrombin as well as cofactor function (47, 48), may possess residues that are directly involved in the interaction of the cofactor with positively charged amino acids provided by one of the protein components of prothrombinase. Further, we have recently demonstrated that a binding site for prothrombin is located on the last 13 amino acids of the factor Va heavy chain (46). The present study was undertaken to identify the specific amino acid residues from the acidic COOH-terminal region of factor Va heavy chain that are important for cofactor function and the molecular mechanisms underlying their contribution to prothrombinase function.

EXPERIMENTAL PROCEDURES

Materials, Reagents, and Proteins—Diisopropyl fluorophosphate (DFP), *O*-phenylenediamine dihydrochloride, HEPES, Trizma (Tris base), Coomassie Blue R-250, and factor V-deficient plasma were purchased from Sigma. The secondary 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) were from Avanti Polar Lipids (Alabaster, AL). The chemiluminescent reagent ECL$^+$ and heparin-Sepharose were from Amersham Biosciences. Normal reference plasma and the chromogenic substrate Spectrozyme-TH were from American Diagnostica Inc. (Greenwich, CT). The thromboplastin reagent for the clotting assays was purchased from Organon Teknika Corp. (Durham, NC). Polyethylene glycol (M_r 8000) was purchased from J. T. Baker (Danvers, MA). The fluorescent thrombin inhibitor dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amide (DAPA), N^α-[(acetylthio)acetyl]-Phe-Pro-Arg-thrombin

coupled to agarose through the active site as described (18), RVV-factor V activator, human APC, human factor Xa, human thrombin, human prothrombin, the sheep antifactor V polyclonal antibody, the monoclonal antibody αhFV#1 coupled to Sepharose, and human factor Xa labeled in the active site with Oregon Green 488 ([OG$_{488}$]EGR-hXa) as previously described (49), were from Hematologic Technologies Inc. (Essex Junction, VT). The cDNA for factor V was purchased from American Type Tissue Collection (ATCC 40515 pMT2-V, Manassas, VA). The sequence of this cDNA molecule is identical to the cDNA published by Jenny *et al.* (50). 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, University of Vermont, Burlington, VT) and have been extensively characterized (51–54). Overlapping peptides from the region 680–709 as well as pentapeptide DYDYQ were synthesized in the Biotechnology Core of the Cleveland Clinic Foundation (Cleveland, OH), purified by high performance liquid chromatography (HPLC), and characterized by mass spectrometry as described (42). Human factor V and factor Va were purified and concentrated using methodologies previously described employing the monoclonal antibody αhFV#1 coupled to Sepharose and heparin-Sepharose (42, 55, 56). The cofactor activity of the factor V preparations was measured by a clotting assay using factor V-deficient plasma and standardized to the percentage of control as described (55). Phospholipids vesicles composed of 75% PC and 25% PS (referred to as PCPS vesicles throughout this report) were prepared as previously described (57). The concentration of phospholipids vesicles was determined by phosphorus assay as described earlier and is given as the concentration of inorganic phosphate (58).

Determination of Factor V/Va Clotting Activity of the Recombinant Molecules—Cofactor activity of wild type and mutant molecules was measured in a clotting assay using factor V-deficient plasma prior and after activation by thrombin (15 min, 37 °C) and RVV-V activator (2 h, 37 °C) as described (43, 46). The values were standardized to the percentage of control (55). A linear semi-log graph was constructed using known concentrations of purified factor V (units/ml as a function of clotting time). The assay end point was determined by visualization of the fibrin clot. The activity of the factor V/Va solution (units/ml) was determined by extrapolation from the graph. The concentration of the recombinant molecules was determined by a recently described ELISA (43). Finally, the numbers were combined to obtain the specific activity of the recombinant factor V solutions (units/mg).

Assay Measuring Thrombin Formation—The formation of thrombin was analyzed using the fluorescent thrombin inhibitor DAPA as described (11, 12, 42, 59) using a PerkinElmer LS-50B Luminescence Spectrometer (PerkinElmer Life Sciences, Norwalk, CT) with $\lambda_{ex} = 280$ nm, $\lambda_{em} = 550$ 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"). In all cases peptides were preincubated with factor Va prior to the assay as described in the legend to the figures. The final concentration of factor Va in the mixture was 4 nM with factor Xa at 10 nM, prothrombin at 350 nM, DAPA at 700 nM, in the presence of PCPS vesicles (10 μM). The initial rate of thrombin formation (nM IIa·min^{-1}) was calculated as described (11, 12, 42) during the initial 5–10 s of the reaction. To verify whether the peptides have any effect on the active site of thrombin, control experiments were performed as follows. A given peptide (at 100 μM) was incubated in the assay buffer containing DAPA (700 nM); the base line was monitored for 30 s; thrombin (350 nM) was then added to the mixture, and the fluorescent intensity resulting from the complexation of DAPA with the active site of thrombin was monitored for 60 s. The slope of the reaction measuring thrombin formation in the presence of a given peptide during the first 5 s was calculated and compared with the slope of a reaction obtained in the absence of peptide. It is noteworthy that, under the conditions employed, the thrombin-DAPA interaction occurred rapidly and the calculated slope of the reaction was sensitive to all parameters used. However, multiple titrations of the same reaction using various preparations of thrombin and peptide, demonstrated that the peptides do not have any significant effect on the capabilities of thrombin to interact with DAPA. All experiments were performed in triplicates or as indicated. The concentration of each peptide given in the figures and figure legends is their final concentration in the assay. The data were stored using the software FL WinLab (PerkinElmer Life Sciences) and further analyzed and plotted with the software Prizm (GraphPad, San Diego, CA). In some cases the data

were also analyzed and plotted using DeltaGraph (DeltaPoint, Monterey, 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 (42). Anisotropy measurements were performed in a quartz cuvette under constant stirring (low) with $\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm with a long pass filter (Schott KV-520) in the emission beam. In all cases, the total addition of peptide did not exceed 10% of the volume of the reaction. The concentration of peptide given in the text is the final concentration of the peptide in the assay mixture. The data were stored using the software FL WinLab (PerkinElmer Life Sciences) and further analyzed and plotted with the software Prizm (GraphPad). In some cases the data were also plotted using DeltaGraph (DeltaPoint).

Mutagenesis and Transient Expression of Recombinant Factor V Molecules—A quadruple mutation of factor V, pMT2-FV-Asp^{695} → Lys/Tyr^{696} → Phe/Asp^{697} → Lys/Tyr^{698} → Phe was synthesized by PCR-based method as described recently (43). First, a double mutant FV-Asp^{695} → Lys/Tyr^{696} → Phe was made in a small DNA fragment of the factor V cDNA. The mutagenic primers for this double mutant fragment were 5′-GAGTGATGCTAAGTTTGATTACC-3′ (sense) and 5′-GGTAATCAAACTTAGCATCACTC-3′ (antisense) (underlined letters indicate the mismatch), whereas the outer primers were 5′-CATGGAGTGACC-TTCTCG-3′ (sense) and 5′-TCATCCAGGAGAACC-3′ (antisense). The amplicon was subcloned in the cloning vector pGEM-T, and the nucleotide sequences were verified by DNA sequencing. The plasmid having the double mutation was used as template for synthesizing the quadruple mutant. The mutagenic primers in this case were 5′-GCTAAGT-TTAAGTTCCAGAACAGACTGG-3′ (sense) and 5′-CCAGTCTGTTCT-GGAACTTAAACTTAGC-3′ (antisense). The outer primers were the same sense and antisense outer primers used in the synthesis of first double mutant. The factor V DNA fragment having all four mutations was subcloned into pGEM-T vector and sequenced. Finally, the DNA fragment was removed from the plasmid by digestion with the restriction enzymes Bsu361 and DraIII. Following purification of the insert from the agarose gel, the factor V insert that possessed the mutations was re-ligated into the plasmid pMT2-FV, in which the DNA fragment between the Bsu361 and DraIII restriction sites was removed. The ligated plasmids were transformed into DH5α 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).

Expression of Recombinant Wild Type and Mutant Factor V in Mammalian Cells—COS-7 cells (ATTC, Manassas VA) 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, and pMT2-FV (^{695}KFKF^{698}) were used to transfect into COS-7L cells as recently described (43). Following transfection, cells were washed twice with serum-free medium and 6–10 ml of conditioned media VP-SFM supplemented with 4 mM of L-glutamine were added. After 24 and 48 h, the harvested medium containing recombinant factor V was centrifuged at 4500 rpm at 4 °C to removed insoluble particles. All control media and solutions containing the recombinant factor V molecules were concentrated using centrifugal ultrafiltration (Centricon YM 30,000) (43). The activity and integrity of the molecules was verified before and after thrombin (and/or RVV-V activator) activation by clotting assays using factor V-deficient plasma and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting using both monoclonal and polyclonal antibodies. The concentration of the recombinant molecules was determined by an ELISA recently described by our laboratory (43). 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 to another. 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 Thrombin Formation in a Prothrombinase Assay Using Purified Reagents—All factor V species were activated with thrombin for 15 min at 37 °C, or with RVV-V activator for 2 h at

37 °C as described (43, 46) followed by the addition of DFP. 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 because DFP is readily hydrolyzed in aqueous solution. Factor V was also activated by factor Xa in the presence of phospholipids. Assay mixtures contained PCPS vesicles (20 μM), DAPA (3 μM), various concentrations of recombinant factor Va species, prothrombin (1.4 μM), in 20 mM HEPES, 0.15 M NaCl, 5 mM CaCl$_2$, pH 7.4. The assay was conducted as recently described by measuring thrombin formation by the change in the absorbance of a chromogenic substrate at 405 nm monitored with a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA) (43). The initial rates of thrombin generation under the conditions employed were linear, and in all experiments no more than 10% of prothrombin was consumed during the initial course of the assay. All data were analyzed with the software Prizm (GraphPad).

Direct Binding of the Peptides to Thrombin—Thrombin was immobilized onto agarose through the active site as described (18). Peptide solutions of HC1-HC5, D13R (46), DYDYQ, and P15H (42) were dissolved in water to a given concentration and then diluted in 20 mM HEPES, 0.1 M NaCl, pH 7.4, in a manner that 400 μg was contained in each starting solution. In control experiments it was determined that the maximum amount of peptide that could be retained by the thrombin-agarose column was 400 μg. Because some of the peptides contain aromatic amino acid residues, their concentration was also measured by optical density. Nevertheless, the presence of all peptides including those that do not contain aromatic amino acids (HC1, HC2, and HC5) in the void volume or in the fractions representing the elution of the thrombin-agarose column was verified by LC/MS as detailed below in the Analytical Facility of Cleveland State University.

Mass Spectrometry Instruments and Conditions—The identity of all peptides found in the flow-through or the elution of the thrombin-agarose column was verified by mass spectrometry. Because the peptides were in a buffer solution, we used LC/MS for their identification. In this procedure peptides are first separated from the salt content of the buffer using an HPLC system (HP 1100, HPLC gradient system, Agilent Technologies, Palo Alto, CA) with a C18 column (1 mm × 15 cm, GraceVydac, Hesperia, CA) with buffers A (0.3% acetic acid in water) and B (0.3% acetic acid in acetonitrile). The elution of the column was monitored with a Micromass Quatro II ESI-Triple Quadrupole Mass Spectrometer (Waters, Milford, MA). The data were collected using a Compaq Professional Work station AP200 (Hewlett-Packard, Palo Alto, CA) and analyzed by the software MassLynx version 3.3 (Waters).

Gel Electrophoresis and Western Blotting—SDS-PAGE analyses were performed using 4–12% gradient gels according to the method of Laemmli (60). In several experiments, proteins were transferred to polyvinylidene difluoride (PVDF) membranes according to the method described by Towbin *et al.* (61). After transfer to nitrocellulose, factor V heavy and light chain(s) were detected using the appropriate monoclonal and polyclonal antibodies (43, 51–54). Immunoreactive fragments were visualized with chemiluminescence.

RESULTS

Inhibition of Prothrombinase Function by Synthetic Peptides from the Carboxyl-terminal Portion of Factor Va Heavy Chain—There is increasing evidence that region 680–709 of the heavy chain of factor Va is important for cofactor activity (44–46). This hirudin-like region containing functionally important tyrosine residues (47, 48) was proposed to provide an important site for the productive interaction with proexosite I of prothrombin (20, 21, 34). To explicitly identify the amino acid residues from this region that are important for cofactor activity, we have synthesized five overlapping peptides (10 amino acids each) spanning the entire region of interest (HC1–HC5, Fig. 1). Each synthetic peptide, except the first and the last, has 5 amino acids in common with the preceding and the following peptide of the series.

Under the conditions employed, two peptides, HC3, and HC4 (spanning amino acid regions 690–699 and 695–704 of factor Va heavy chain, respectively) inhibited prothrombinase when used at 100 μM (Fig. 2*A*). Fig. 2*A* also shows a positive control peptide, D13R, recently shown to interfere with prothrombin incorporation into prothrombinase (46) and a negative control peptide, P15H, that has no effect on cofactor activity under the

[680]KMHD*R*LEPEDEESDADYDYQN*R*LAAALGIR[709]

HC1   KMHDRLEPED
HC2   LEPEDEESDA
HC3   EESDADYDYQ
HC4   DYDYQNRLAA
HC5   NRLAAALGIR

FIG. 1. **Peptides from amino acid region 680–709 of factor V.** Overlapping peptides (10 residues each) from the COOH-terminal portion of the heavy chain of human factor Va are shown (amino acid region 680–709, HC1–HC5). The arginines are identified (*bold* and *underlined*). For the easy reading of this report and the identification of the position of several important amino acids, Lys[680] and Arg[709] are identified as the beginning and the end, respectively, of the sequence of interest.

conditions employed (42). Control experiments also demonstrated that the peptides do not interfere with the capability of thrombin to interact with DAPA (data not shown). All other peptides from region 680–709 had no significant effect on prothrombinase activity. Overall, the data also show that amino acid sequences 680–689 and 700–709, which together represent ~67% of the entire amino acid sequence studied, do not appear to have any major effect on prothrombinase function under the conditions employed.

A titration of the inhibition of prothrombinase by peptides HC3 and HC4 is shown in Fig. 2*B*. The data demonstrated that HC3 and HC4 inhibit prothrombinase activity with similar IC_{50} values of 12 μM (Fig. 2*B*, *filled triangles*) and 10 μM (Fig. 2*B*, *filled circles*), respectively. In the presence of 100 μM HC3 and HC4, complete inhibition of prothrombinase function was observed (Fig. 2*B*). Thus, both peptides show similar inhibitory potential. A control pentadecapeptide from the middle portion of the heavy chain of factor Va (P15H, Ref. 42) had no effect on prothrombinase function at similar concentrations under the conditions employed (Fig. 2*B*, *filled squares*).

We next tested HC3 and HC4 for their ability to interfere with the fluorescence anisotropy of a preformed complex composed of membrane-bound [OG_{488}]EGR-hXa-human factor Va as described (42, 43). No significant decrease in the anisotropy of [OG_{488}]EGR-hXa was detected following incubation of the preformed complex with increasing concentrations of either HC3 or HC4, even in the presence of high concentrations of peptide (300 μM, data not shown). The data demonstrate that peptides HC3 and HC4 do not interfere with the high affinity interaction between factor Va and factor Xa on the membrane surface. The peptides must thus impair another function of the cofactor within prothrombinase.
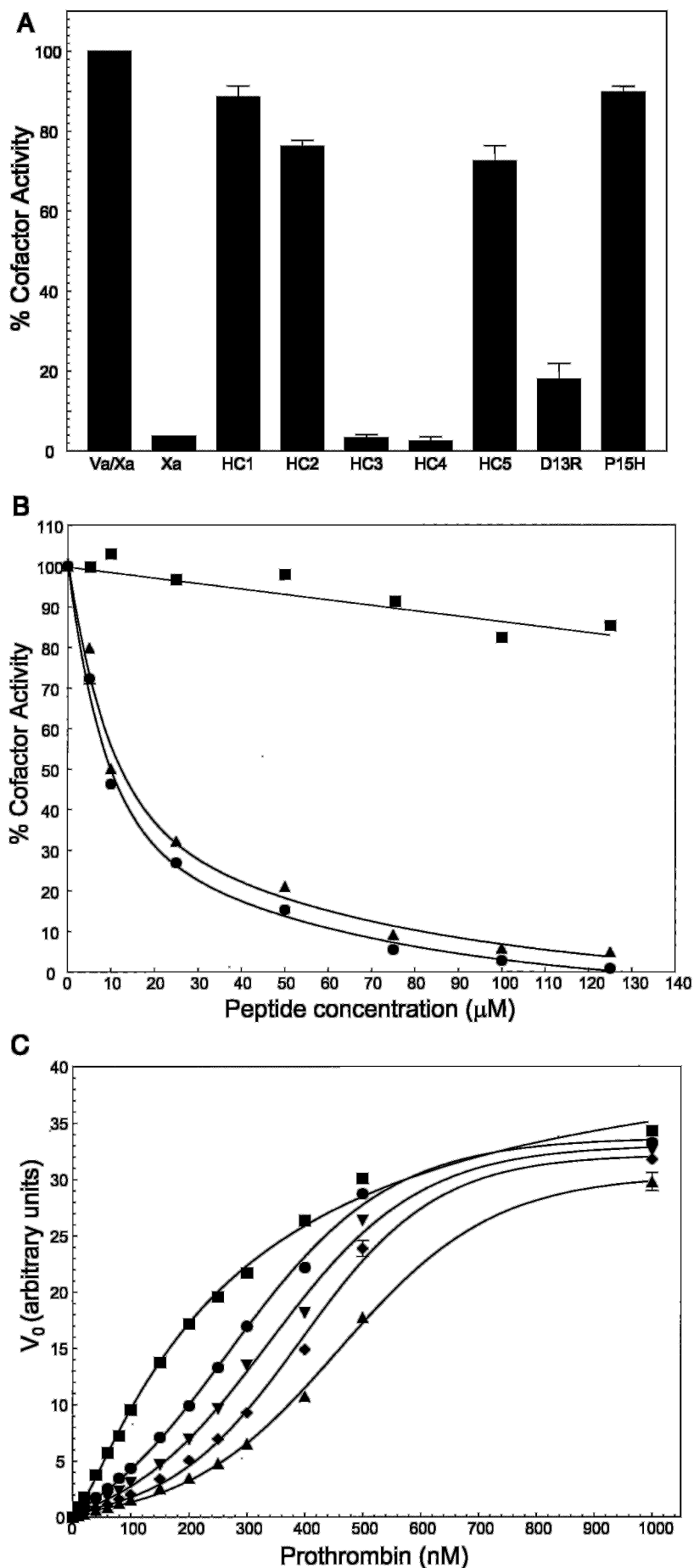
The mechanism of inhibition of prothrombinase by the hirudin-like peptides HC3 and HC4 was addressed by investigating the effect of the peptides on the kinetic parameters of the enzymatic complex (K_m and V_{max}). For the sake of simplicity, only the results obtained with HC4 are shown (Fig. 2*C*). The data demonstrated that under the conditions employed and in the presence of increasing concentrations of peptide, all reactions tend toward the same asymptotic value, which is the V_{max} of the reaction. The calculated values of the V_{max} of prothrombinase remained approximately unchanged (~960 ± 27 nM IIa/min), whereas the $K_{0.5}$ of the enzymatic reactions increased. These results suggest a competitive type of inhibition (see Refs. 62–64). Examination of the data shown in Fig. 2*C* demonstrated a sigmoidal shape of the graphs, which became more pronounced as the concentration of HC4 was increased (from 0.5 μM (*filled circles*) to 2 μM (*filled triangles*)). These data represent a competitive inhibition mechanism, where only free substrate (prothrombin) can produce thrombin in the presence of prothrombinase (62). According to this model, HC4 binds

prothrombin in competition with the binding of prothrombin to prothrombinase (membrane-bound factor Va-factor Xa) (62). The K_i of prothrombinase inhibition by HC3 obtained from the value of the IC_{50} derived from Fig. 2*B* using the value of the K_m (0.4 μM) determined in Fig. 2*C* (*filled squares*) was 6.3 μM, whereas the K_i for prothrombinase inhibition by HC4 was determined to be 5.3 μM. These values represent the K_D of HC3 and HC4 for their interaction with prothrombin. A similar inhibition pattern of prothrombinase function was observed by Anderson *et al.* (21) who used a peptide containing twelve amino acids from the COOH-terminal tail of hirudin. Interestingly, the peptide, which has an amino acid composition similar to HC3 and HC4, with multiple acidic amino acids and a tyrosine, inhibited prothrombinase with a K_i of 3 μM. Overall the data demonstrate that the hirudin-like peptides HC3 and HC4 do not interfere with the binding of factor Va to factor Xa but rather impair prothrombinase activity by inhibiting the direct interaction of the cofactor with prothrombin. An interference of the peptides with the membrane binding properties of the cofactor must be excluded, because we and others have demonstrated that a factor Va molecule lacking a portion or the entire acidic COOH-terminal peptide from the heavy chain binds to the lipid bilayer with affinity similar to that of the purified intact plasma cofactor (44–46).

Function of the Amino Acid Sequence Common to HC3 and HC4—The common amino acid motif between HC3 and HC4 consists of amino acid residues Asp[695]-Tyr-Asp-Tyr-Gln[699] (DYDYQ) (50). A peptide with this sequence was found to be a potent inhibitor of prothrombinase function with an IC_{50} of 1.6 μM (Fig. 3*A*). Complete inhibition of prothrombinase by the pentapeptide occurred at 40 μM. Kinetic analyses of prothrombinase inhibition by DYDYQ similar to the analyses described for HC4 and shown in Fig. 2*C* revealed similar sigmoidal tracings in the presence of increasing concentrations of inhibitor (Fig. 3*B*). The sigmoidal nature of the curves and the $K_{0.5}$ increased with increasing inhibitor concentration (100 nM (*filled circles*) to 500 nM (*open squares*)) while the V_{max} of the reaction remained approximately unchanged. Under the conditions employed, the K_i for inhibition of prothrombinase by the pentapeptide, which is the K_D for its interaction with prothrombin, was determined to be 850 nM. The data suggest that the hirudin-like amino acid motif Tyr[695]–Gln[699] from the COOH-terminal portion of the heavy chain of factor Va represents a binding site for prothrombin within prothrombinase. Our results verify the data found with HC4 and are consistent with the kinetic model of inhibition of prothrombinase shown in Scheme I. According to this model, the true inhibitor of the enzymatic reaction is the prothrombin-DYDYQ complex, which competes with free prothrombin for binding to prothrombinase (62).

Because peptide DYDYQ is composed of acidic amino acids and most likely interacts with the positively charged amino acids from ABE-I of prothrombin as previously suggested (20, 21, 34), it is possible that the peptide also binds to thrombin and inhibits activation of factor V. We have thus preincubated thrombin with 100 μM pentapeptide and tested the capacity of the mixture to activate single-chain plasma factor V. Under the conditions employed, the pentapeptide is a potent inhibitor of factor V activation by thrombin (Fig. 4, *lanes 2–7*) because it impairs cleavage at Arg[709] (Fig. 4*B*), which is the first required step during the sequential activation of human factor V (8–10, 50). It is noteworthy that a delay in cleavage at Arg[709] and generation of the heavy chain of the cofactor by the pentapeptide was observed with highly purified, single-chain factor V only. When using partially activated preparations of factor V, no delay in the generation of the heavy chain in the presence of

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FIG. 2. **Inhibition of prothrombinase function**. *Panel A*, inhibition of thrombin formation by the peptides. Peptides were incubated with factor Va as described under "Experimental Procedures" at a fixed concentration (100 μM). The percentage of factor Va cofactor activity was calculated by comparing the activity of prothrombinase in the presence of a given peptide 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 control peptide represents a pentadecapeptide from the middle portion of factor Va heavy chain (P15H; Ref. 42). D13R is a peptide recently shown to interfere with the factor Va-prothrombin interaction (46). *Panel B*, titration of HC3 and HC4. Increasing concentrations of HC3 (*filled triangles*), HC4 (*filled circles*), and P15H (*filled squares*) were preincubated with factor Va and assayed for prothrombinase activity as described under "Experimental Procedures." HC3 represents amino acid sequence 690–699 of factor Va heavy chain; HC4 contains sequence 695–704 of human factor Va heavy chain; P15H represents amino acid sequence 337–351 from the middle portion of human factor Va heavy chain (42). The data represent the average of the results found in three independent experiments. The concentration of each peptide given on the *x* axis represents its final concentration in the prothrombinase mixture. *Panel C*, kinetic analyses of prothrombin activation in the presence of HC4. The data are plotted as V_0 (initial velocity, in arbitrary units) as a function of increasing prothrombin concentration in the presence of increasing concentrations of HC4. The *lines* drawn represent the best fit through the points with an R^2 of 0.99. The concentrations of HC4 used in the experiments are as follows: control, no peptide (*filled squares*), 0.5 μM peptide (*filled circles*), 1 μM peptide (*filled inverted triangles*), 1.5 μM peptide (*filled diamonds*), and 2 μM peptide (*filled triangles*). The data represent the average of the results found in three independent experiments. The apparent inhibition constant (K_i) reported in the text is the value calculated from the formula: $IC_{50} = K_i(1 + S_0/K_m)$ (62), where K_m is the Michaelis-Menten constant of the reaction in the absence of inhibitor (0.4 μM), S_0 is the concentration of prothrombin used (350 nM), and IC_{50} is the half-maximal inhibition of prothrombinase by HC4 (*panel B*, 10 μM).

FIG. 3. **The effect of peptide DYDYQ on factor Va function.** *Panel A*, titration of prothrombinase activity in the presence of increasing concentrations of peptide DYDYQ. Increasing concentrations of DYDYQ (*filled squares*) were preincubated with factor Va and assayed for prothrombinase activity as described under "Experimental Procedures" and in the legend to Fig. 2. The *inset* shows the progress of the reaction in the presence of 0–5 μM pentapeptide and allows for a more precise calculation of the IC$_{50}$ for prothrombinase inhibition, which in turn is necessary for the calculation of the K_i of the pentapeptide. The latter number represents K_D of the inhibitor for its interaction with prothrombin (see Scheme I and Ref. 62). The data represent the average of the results found in three independent experiments. The concentration of peptide given on the *x* axis represents its final concentration in the prothrombinase mixture. *Panel B*, kinetics of inhibition of prothrombinase by peptide DYDYQ. The data are plotted as V_0 (initial velocity, in arbitrary units) as a function of increasing prothrombin concentration in the presence of increasing concentrations of peptide DYDYQ. The *lines* drawn represent the best fit through the points with an R^2 of 0.99. The concentrations of DYDYQ used in the experiments are as follows: control, no peptide (*filled squares*), 100 nM peptide (*filled circles*), 200 nM peptide (*filled inverted triangles*), 300 nM peptide (*filled diamonds*), 400 nM peptide (*filled triangles*), and 500 nM peptide (*open squares*). The data represent the average of the results found in three independent experiments. The apparent inhibition constant (K_i) reported in the text was calculated as described in the legend to Fig. 2C using an IC$_{50}$ of 1.6 μM (Fig. 3A). The kinetic constants calculated from the data presented in this graph were used in Scheme I.

$$\text{Va-L} + \text{Xa-L} \rightleftarrows \text{(Va-Xa)-L} + \text{II-L} \underset{\rightleftarrows}{\overset{K_s}{}} \text{(Va-Xa-II)-L} \underset{\rightleftarrows}{\overset{K_s{}^*}{}} [\text{(Va-Xa-II)-L}]^* \underset{\rightleftarrows}{\overset{k_{cat}}{}} \text{(Va-Xa)-L} + \text{IIa}$$

$$+$$

$$\text{DYDYQ}$$

$$\updownarrow K_D = 850 \text{ nM}$$

$$\text{(Va-Xa)-L} + \text{(II-DYDYQ)-L} \rightleftarrows [\text{(Va-Xa)-(II-DYDYQ)}]\text{-L}$$

$$K_{m(obs)} = 0.39 \pm 0.02 \ \mu\text{M} \cong K_s \qquad \frac{V_{max(obs)}/E_T}{K_{m(obs)}} = 1 \times 10^7 \ \text{M}^{-1}\text{s}^{-1}$$

$$\frac{V_{max(obs)}}{E_T} = 4 \pm 0.1 \ \text{s}^{-1}$$

SCHEME I. **Kinetic model for inhibition of prothrombinase.** Activation of prothrombin by prothrombinase is a multistep pathway (3–7). The initial bimolecular interaction responsible for enzyme formation on the membrane surface (*L*), is mediated by exosites from factor Va and factor Xa optimally exposed upon their interaction with the lipid surface. This complex interacts with membrane-bound prothrombin (K_S) followed by docking of the scissile bonds in the active site of prothrombinase ($K_S{}^*$). The last step of the reaction results in thrombin formation. The inhibitor (DYDYQ) interacts with prothrombin ($K_D \sim 0.85 \ \mu$M) in competition with the binding of prothrombin to prothrombinase ($K_S \sim 0.4 \ \mu$M) (62).

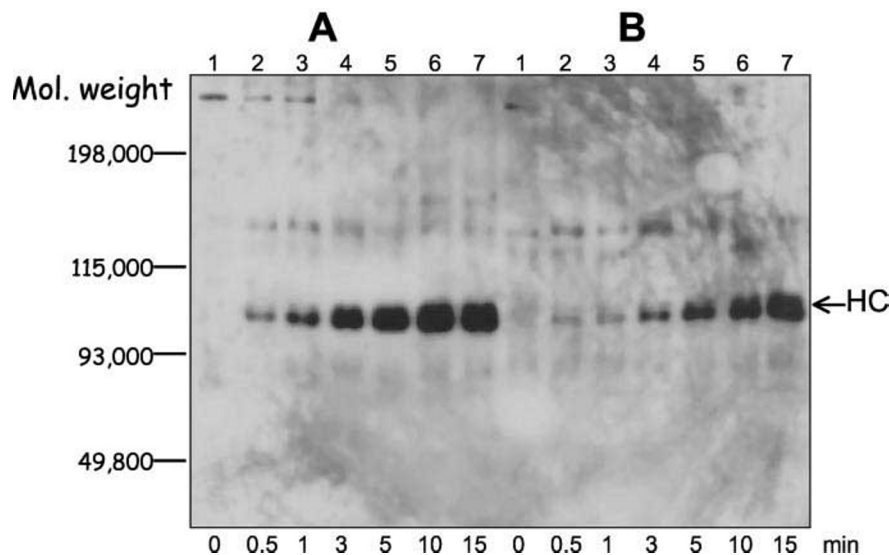*Thrombin and Prothrombin Interactive Site on Factor V/Va*

FIG. 4. **The effect of peptide DYDYQ on factor V activation.** *Panel A*, activation of factor V by thrombin alone. Factor V (250 nM) was incubated with thrombin (1 nM). *Panel B*, thrombin (1 nM) was preincubated with 100 μM peptide DYDYQ and the mixture was added to factor V (250 nM). At selected time intervals, aliquots of the mixtures were removed, mixed with 2% SDS, heated for 5 min at 90 °C, and analyzed on a 4–12% SDS-PAGE followed by immunoblotting. Fragments were identified following staining with monoclonal antibody αHFV#17, which recognizes an epitope between amino acid residues 307–506 of factor V and chemiluminescence as described previously (51–54). *Lane 1* in *panels A* and *B* depicts aliquots of the mixture withdrawn from the reaction before the addition of thrombin or thrombin/peptide mixture, whereas *lanes 2–7* show aliquots of the reaction mixture withdrawn at 30 s, 1 min, 3 min, 5 min, 10 min, and 15 min following the addition of thrombin alone or of thrombin/peptide mixture. The time of incubation is shown at the *bottom* of the figure. Position of the molecular size markers is indicated at *left*.

the pentapeptide was observed. In contrast, using these latter preparations, a slower disappearance of the single chain factor V molecule was apparent (data not shown). These data suggest that the pentapeptide impairs cleavage at Arg[709] on the intact procofactor only. These data also suggest that other exosites for thrombin may be available on the factor V molecule as suggested (18–21). These exosites appear to be involved in cleavage at Arg[709] by thrombin, albeit less efficiently. Overall the data depicted in Figs. 3 and 4 together with the data shown in Fig. 2 demonstrate that the acidic amino acid stretch Asp[695]–Gln[699] located at the carboxyl-terminal part of the factor Va heavy chain appears to have a dual function; it provides an exosite for prothrombin docking within prothrombinase, and it also serves as an interactive site for thrombin necessary for optimum rates of cleavage at Arg[709] and activation of the procofactor. These results are in complete agreement with data on record demonstrating inhibition of factor V activation by thrombin by the hirudin-derived peptide, hirudin[54–65] (18).

Direct Interaction of Hirudin-like Peptides from Factor Va Heavy Chain with Thrombin-Agarose—To ascertain that the peptides identified thus far to be inhibitory of prothrombinase activity contain a binding site for thrombin, the interaction of all peptides with active site-immobilized thrombin was studied in chromatographic experiments and is shown in Fig. 5. The control peptide, P15H (42), did not interact with thrombin, as demonstrated by its elution in the void volume of the thrombin-agarose column (Fig. 5, *filled diamonds*). All other peptides containing the hirudin-like motif DYDYQ were eluted from the column with high salt buffer (Fig. 5, HC3 (*filled squares*), HC4 (*filled triangles*), and DYDYQ (*filled circles*)). Two other peptides that could not be identified by the absorbance at 280 nm because they do not contain aromatic amino acids, but containing acidic amino acids and having the potential to interact with ABE-I or ABE-II of thrombin (HC1 and HC2, Fig. 1), were present in the flow-through of the thrombin-agarose column and detected by LC/MS (Fig. 5, *inset, panels A* and *B*). Fig. 5C shows that peptide HC5 containing amino acid residues 700–709 does not interact with thrombin-agarose. The data demon-

strate that not any randomly selected acidic amino acid sequence can interfere with the binding of factor Va to the anionic binding exosite(s) of thrombin and strongly suggest that amino acid sequence Asp[695]–Gln[699] contained in three of the seven peptides tested is a specific amino acid motif, which represents a binding site for thrombin.

Expression and Activation of Recombinant Human Factor V Molecules—In view of all these findings, we used recombinant technology to assess the contribution of 4 of the 5 amino acid residues identified above on both factor V activation and factor Va cofactor function. Two charge reversal and two conservative mutations were introduced into sequence 695–698 of factor V. We have thus prepared a quadruple mutant factor V molecule with the mutations Asp[695] → Lys, Tyr[696] → Phe, Asp[697] → Lys, and Tyr[698] → Phe (factor V[2K2F]). Recombinant wild type factor V and factor V[2K2F] were expressed in COS-7L cells, and their concentrations were determined using the ELISA recently developed in our laboratory (43).

The recombinant molecules were first screened for clotting activity and the results are shown in Table I. Wild type factor V had a specific activity of 145 units/mg. Activation of the wild type molecule by thrombin or RVV-V activator resulted in cofactors with similar clotting activities (497 and 570 units/mg, respectively; Table I). The quadruple mutant (factor V[2K2F]) was unable to promote clotting under the conditions employed. Factor V[2K2F] was also unable to promote clotting following activation by thrombin (factor Va[IIa][2K2F]) and/or RVV-V activator (factor Va[RVV][2K2F]). The two cofactor molecules had an activity analogous to the activity of the media collected from mock-transfected cells (<0.2% of the clotting activity of the wild type molecules activated under similar conditions; Table I). These data demonstrate that, once activated, factor Va[IIa][2K2F] and factor Va[RVV][2K2F] are deficient in their clotting activity.

We next investigated the ability of the recombinant molecules to be assembled into prothrombinase using an assay employing purified reagents and a chromogenic substrate that measures thrombin generation. Because the assay is conducted with limiting factor Va concentrations (0.5 nM), any intrinsic
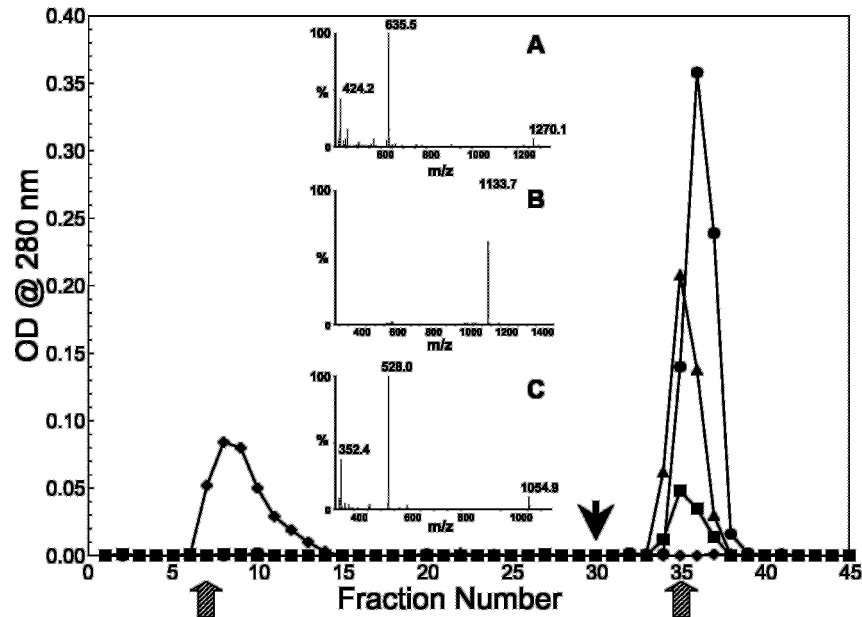
FIG. 5. **Direct interaction of hirudin-like peptides from the COOH terminus of factor Va heavy chain with thrombin-agarose.** Small scale chromatography of hirudin-like peptides was performed on a 2.5-ml thrombin-agarose as described (18). Each run represents 400 μg of peptide. This amount of peptide was determined to saturate the specific sites of thrombin of the column used (2.5 ml). Elution was performed with 2 M NaCl and was started at the point indicated by the *arrow* (fraction 30). The presence of the peptides in the corresponding fractions was monitored by absorbance at 280 nm (shown on the *y* axis) and by LC/MS (*inset*). Results show HC3 (*filled squares*), HC4 (*filled triangles*), DYDYQ (*filled circles*), and P15H (*filled diamonds*) monitored by the absorbance at 280 nm. *Inset*, mass spectrometry analysis of the peptides that could not be monitored by absorbance at 280 nm (*i.e.* HC1, HC2, and HC5). These peptides were treated similarly to all other peptides and fractions of similar size were collected. Aliquots from fractions 7 and 35 from each separate experiment were submitted to LC/MS analysis as described under "Experimental Procedures" (depicted by the *hatched arrows* at the *bottom* of the chromatogram). The results from tube 7 from three chromatograms are depicted. The data are presented as percentage of intensity of the signal as a function of the mass of the peptide divided by the charge (*m/z*). *Inset panel A*, HC1 ($M_{r(calculated)} = 1268$); *inset panel B*, HC2 ($M_{r(calculated)} = 1132$); *inset panel C*, HC5 ($M_{r(calculated)} = 1053$). The spectrum of HC1 has two major peaks: at 635.5 and at 424, and one minor peak at 1270 (~1268 + 1). The peak at 635.5 represents the peptide (mass/charge) with two positive charges ([M + 2H]$^+$ *i.e.* [(1268 + 2 = 1270)]/2 = 635, whereas the peak at 424 represents HC1 with three positive charges ([M + 3H]$^{2+}$ *i.e.* [(1268 + 3 = 1271)]/3 = 424. *Inset panel B* shows HC2 with one positive charge (1132 + 1 = 1133). HC5 spectrum has two major peaks: one at 528 and one at 352. The peak at 528 represents the peptide with two positive charges ([M + 2H]$^+$ *i.e.* [(1053 + 2 = 1055)]/2 = 527.5, whereas the other peak represents HC5 with three positive charges ([M + 3H]$^{2+}$ *i.e.* [(1053 + 3 = 1056)]/3 = 352. The minor peak at 1054 represents the peptide with one positive charge. It is noteworthy that, in gas phase, the number of protons attached to each peptide depends on the number (quantity) of basic residues contained in each peptide. HC1 contains 1 lysine and 1 arginine, HC2 does not contain any basic residues, whereas HC5 has 2 arginines (see Fig. 1). Thus, HC1 and HC5 are more likely to bind two or three protons than HC2. Consequently the molecule with one proton is the major species observed in the mass spectrum of HC2 (*inset panel B*).

TABLE I
Clotting activity of various recombinant factor V species

Wild type and mutant factor V molecules were assayed for clotting activity as described under "Experimental Procedures."

Factor V species[a]	Clotting time	Activity	Specific activity
	s	*units/ml*	*units/mg*
Media from mock transfected cells	67.6 ± 1.1		
Wild type FV	32.1 ± 1.2	0.12	145
Wild type FV$_{IIa}$	20.5 ± 0.6	0.44	497
Wild type FV$_{RVV}$	18.8 ± 0.5	0.47	570
Factor V^{2K2F}	66.7 ± 1.3	0.0046	5.6
Factor Va$_{IIa}$^{2K2F}	68.7 ± 4.8	0.0042	5.1
Factor Va$_{RVV}$^{2K2F}	73.1 ± 4	0.002	2.4

[a] All factor V species were assayed for clotting activity at 2.5 nM. Factor Va$_{IIa}$, wild type factor V activated with thrombin; factor Va$_{RVV}$, wild type factor V activated with RVV.

deficiency in the activity of the complex reflects the inability of the recombinant mutant molecule to act as a cofactor in prothrombinase. Fig. 6A shows the results obtained following incubation of the procofactors with thrombin. The data demonstrate that the wild type recombinant factor Va molecule displays normal cofactor activity (~1800 mOD/min, *filled squares*) under the conditions employed and is composed of heavy and light chains (Fig. 6A, *inset*, *lane 1*). Under similar experimental conditions, factor V^{2K2F} activation by thrombin was impaired. The *inset* in Fig. 6A (*lane 2*) shows that, although there was some generation of heavy and light chains of

mutant factor Va following a 15-min incubation with thrombin, considerable amounts of high molecular weight material remained on top of the gel. Furthermore, the factor V^{2K2F}/Va$_{IIa}$^{2K2F} mixture had no cofactor activity when compared with the wild type factor Va molecule (≤20 mOD/min); its cofactor activity was similar to the activity of factor Xa alone. The activity of the factor V^{2K2F}/Va$_{IIa}$^{2K2F} solution within prothrombinase remained essentially the same even when 10 and 50 times more total protein was used (Fig. 6A, 5 nM (*filled inverse triangles*) and 25 nM (*filled diamonds*)). The slow increase in the activity of the factor V^{2K2F}/Va$_{IIa}$^{2K2F} solution after 2 min of incubation in the reaction mixture is a consequence of the slow activation of the molecule during the course of the assay by factor Xa and confirms that the mutant procofactor cannot be efficiently activated by thrombin. Slow activation of factor V during the course of the prothrombinase assay by factor Xa and/or thrombin generated *in situ* was previously observed when studying prothrombinase activity in the presence of unactivated factor V (12, 65).

The results shown in Fig. 6 (*B* and *C*) demonstrate that activation of the wild type molecule by RVV-V activator (Fig. 6B, *filled squares*) or by factor Xa (65) (Fig. 6C, *filled squares*) results in cofactors with activities similar to those of the thrombin-activated molecule (Fig. 6A, *filled squares*). However, under similar experimental conditions, factor Va$_{RVV}$^{2K2F} and factor Va$_{Xa}$^{2K2F} have similar but still impaired cofactor activities within prothrombinase (Fig. 6, *B* (*filled triangles*) and *C* (*filled*
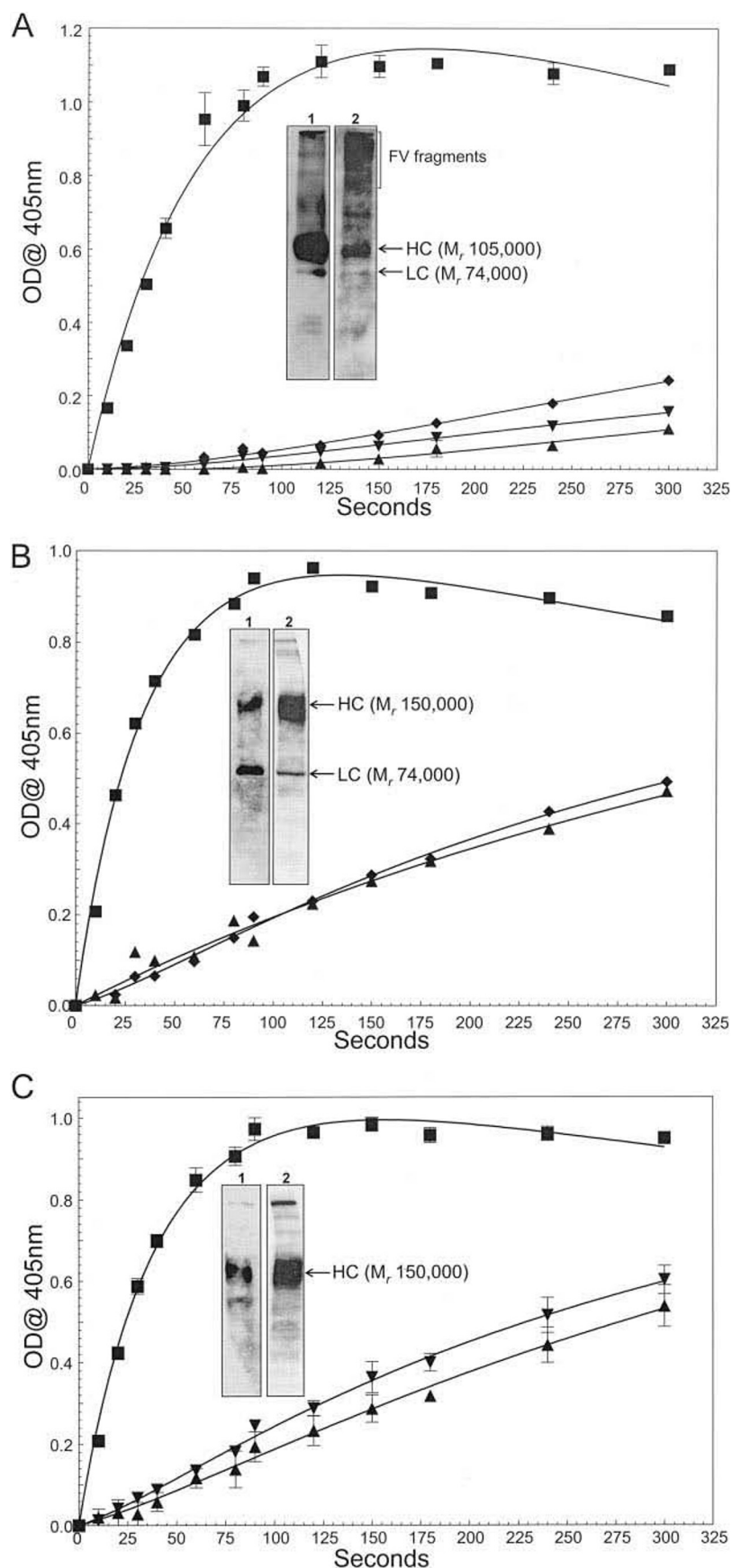
FIG. 6. **Comparison of cofactor activities of recombinant factor V molecules following activation by thrombin, RVV-V activator, and factor Xa.** The cofactor activities of various recombinant factor Va molecules were measured in an assay system using purified reagents and employing Spectrozyme-TH to probe for thrombin generation as previously described (43) and detailed under "Experimental Procedures." Initial rates of prothrombin activation were determined at ambient temperature in the presence of 5 nM factor Xa. *Panel A*, activation by thrombin. The factor V species were activated by thrombin (10 nM, 15 min at 37 °C). The subunit composition of the thrombin

triangles)). Prothrombinase activity does not increase with increasing cofactor concentration (Fig. 6, *B* (*filled diamonds*) and *C* (*filled inverse triangles*)). Although the activity of these cofactors within prothrombinase is ~6-fold higher than the cofactor activity obtained with the thrombin activated solution of factor V^{2K2F}/Va_{IIa}^{2K2F}, factors Va_{RVV}^{2K2F} and Va_{Xa}^{2K2F} have ~22-fold less cofactor activity than the wild type molecule activated under similar experimental conditions. It is noteworthy that prolonged incubation of factor V^{2K2F} with thrombin resulted in a cofactor molecule (factor Va_{IIa}^{2K2F}) composed of heavy and light chains with activity similar to that for factors Va_{RVV}^{2K2F} and Va_{Xa}^{2K2F} (data not shown). Altogether the data shown in Fig. 6 demonstrate that: 1) factor V^{2K2F} is impaired in its activation by thrombin, and 2) the activated mutant cofactor molecules (factor Va_{RVV}^{2K2F} and factor Va_{Xa}^{2K2F}) are impaired in their intrinsic function within prothrombinase, most likely because of impaired interaction with prothrombin. These data underline the importance of amino acid region Asp^{695}–Tyr^{698} from factor V and verify a previously postulated hypothesis suggesting that the binding site for thrombin responsible for facilitating cleavage at Arg^{709} of factor V may also be involved in the interaction of factor Va with the substrate of prothrombinase, thus regulating prothrombin activation (19).

DISCUSSION

In the present report, we have shown that amino acids 695–698 of factor Va heavy chain are crucial for both factor Va cofactor activity and cleavage of factor V by thrombin at Arg^{709} and activation. These 4 amino acids are part of the acidic COOH-terminal portion of factor Va heavy chain (amino acid residues 680–709). This entire sequence is not conserved among species, with only 7 amino acids being identical (7/30~ 23%) (50, 66–68). By mutating residues 695–698, we have obtained a recombinant factor V molecule, which is impaired in its activation by thrombin and is deficient in its clotting activity. This molecule has also impaired cofactor activity in a prothrombinase assay using purified reagents and saturating concentrations of factor Xa. It is remarkable that we obtained such a dramatic effect on both factor V activation and cofactor function, by merely changing 4 amino acids among the 2196 residues in factor V. However, a similar dramatic reduction of clotting and intrinsic tenase activity as well as a decrease in thrombin cleavage efficiency were observed when several important tyrosine residues adjacent to thrombin-activating cleavage sites were mutated to phenylalanine in recombinant human factor VIII (69). Further, as in the present study, the level of decrease in cofactor activity when using purified re-

agents did not match the considerable loss in clotting activity (69). Earlier data also demonstrated that: 1) factor V sulfation at several tyrosine residues located at the carboxyl-terminal portion of its heavy chain is required for both activation of the procofactor by thrombin and full procoagulant activity of factor Va (47, 48), and 2) several non-homologous peptides corresponding to amino acid sequences from natural thrombin inhibitors and substrates were found to inhibit thrombin activity without blocking its active site because of an interaction with a specific exosite of the enzyme (70–73). The common theme between all these findings, which is also illustrated by our data, was an acidic amino acid sequence containing the amino acid motif Asp-Tyr that is required for tyrosyl-sulfotransferases (72). Additionally, sulfation of specific hirudin-derived peptides on tyrosine residues, adjacent to acidic amino acids, resulted in a significant increase in the affinity of the peptides for ABE-I of thrombin (20, 21, 74). In fact, the acidic region preceding Tyr^{696} and Tyr^{698} in factor V satisfies all the criteria for sulfation and may be required for proper attachment and function of tyrosyl sulfotransferases (72, 75–77). Thus, the amino acid motif DYDYQ described herein appears to be a good substrate for sulfation and can also mediate a productive interaction with ABE-I of thrombin as previously suggested (20, 21, 47, 48). It is important to note that, although the amino acid motif DYQ is conserved among species, the preceding 2 amino acids of this sequence vary considerably among them (50, 66–78). Because it has been shown that there is a difference in prothrombinase efficiency when mixing bovine prothrombin with human prothrombinase as compared with activation of human prothrombin by human prothrombinase (21), we can speculate that, although the amino acid sequence DYDYQ of human factor Va provides a binding site for the human prothrombin molecule, the first 2 amino acids of this motif may be required for species specificity recognition. However, it must be also noted that the possibility that region DYDYQ of the cofactor modulates a remote portion of factor Va, which in turn is responsible for the interaction of factor Va with thrombin and prothrombin, cannot be excluded by our findings.

We have shown that a 42-amino acid peptide (N42R) from the middle portion of the factor Va heavy chain (representing residues 307–351 of factor V) produces a cofactor effect on factor Xa, increasing the catalytic efficiency of the enzyme by severalfold. Similarly, a 9-amino acid peptide (AP4′, residues 323–331) and a 5-amino acid peptide (E5A, residues 323–327) from N42R also generated a cofactor effect when incubated with factor Xa alone. Nonetheless, the effect of all these pep-

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activated species was also analyzed on a 4–12% SDS-PAGE, followed by transfer to PVDF membranes and immunostaining with monoclonal antibodies αHFV_{HC}#17 and αHFV_{LC}#9 (*inset*). *Lane 1*, wild type factor V following the incubation with thrombin; *lane 2*, factor V^{2K2F} following incubation with thrombin under similar experimental conditions. The cofactor activities of various factor Va species are depicted as follows: *filled squares*, wild type recombinant factor Va (0.5 nM); *filled triangles*, factor V^{2K2F}/Va^{2K2F} solution (0.5 nM); *filled inverted triangles*, factor V^{2K2F}/Va^{2K2F} solution (5 nM); *filled diamonds*, factor V^{2K2F}/Va^{2K2F} solution (25 nM). The data represent the average of the results found in three independent experiments. *HC* and *LC* represent the heavy (M_r 105,000) and light (M_r 74,000) chains of the cofactor, respectively. *Panel B*, activation by RVV-V activator. The recombinant factor V species were activated with RVV-V activator (6 nM, 2 h at 37 °C) as recently described (46). The subunit composition of the RVV-activated species was also analyzed on a 4–12% SDS-PAGE after reduction with 2% β-mercaptoethanol followed by transfer to PVDF membranes and immunostaining with monoclonal antibodies αHFV_{HC}#17 and αHFV_{LC}#9 (*inset*). *Lane 1*, wild type factor V following incubation with RVV; *lane 2*, factor V^{2K2F} following incubation with RVV. The cofactor activities of various recombinant factor Va species are depicted as follows: *filled squares*, wild type recombinant factor Va (0.5 nM); *filled triangles*, factor Va_{RVV}^{2K2F} (0.5 nM); *filled diamonds*, factor Va_{RVV}^{2K2F} (25 nM). The data represent the average of the results found in two independent experiments. *HC* and *LC* represent the heavy (M_r 150,000) and light (M_r 74,000) chains of the RVV-activated cofactors, respectively. *Panel C*, activation by factor Xa. The factor V species were activated with factor Xa (5 nM, 20 min in the presence of 20 μM PCPS vesicles at 37 °C) as described (65). The amount of factor Xa brought in the assay from the activation mixtures was accounted for in the calculation of the final concentration of factor Xa (5 nM final concentration). The subunit composition of the factor Xa-activated species was also analyzed on a 4–12% SDS-PAGE after reduction with 2% β-mercaptoethanol, followed by transfer to PVDF membranes and immunostaining with monoclonal antibodies αHFV_{HC}#17 and αHFV_{LC}#9 (inset). The cofactor activities of various factor Va species are depicted as follows: *filled squares*, wild type recombinant factor Va (0.5 nM); *filled triangles*, factor Va_{Xa}^{2K2F} (0.5 nM); *filled inverted triangles*, factor Va_{Xa}^{2K2F} (5 nM). The data represent the average of the results found in three independent experiments. *HC* represents the heavy chain (M_r 150,000) of the factor Xa-activated cofactors. Upon prolonged exposure of the immunoblots, the M_r 105,000 heavy chain of the cofactor was also apparent. In all *insets*, the mutant molecules were consistently overloaded on the gels to identify any abnormal fragments and/or migration.

Thrombin and Prothrombin Interactive Site on Factor V/Va

tides on the enzyme was not as pronounced as with intact factor Va (41–43). All these peptides contain a portion or the entire binding site of factor Va heavy chain for factor Xa. Thus, binding of the cofactor or of its isolated binding domains to factor Xa most likely results in the exposure of specific binding exosites on the enzyme necessary for prothrombin docking as suggested (35–38, 49, 78). However, the magnitude of the effect observed on factor Xa was severalfold smaller in the presence of the peptides when compared with the effect produced by the entire factor Va molecule. Although the extent of the cofactor effect on factor Xa may be dependent on the size of the molecule and/or the multiple points of contact from factor Va that participate to the binding to the enzyme, it is also possible that expression of the hidden exosite for prothrombin on factor Xa may not be enough alone to account for the dramatic increase in the catalytic efficiency of factor Xa within prothrombinase when compared with factor Xa alone.

Although the critical role of factor Va for timely and specific prothrombin activation by prothrombinase has been long established, the molecular mechanism by which factor Va accelerates the catalytic efficiency of factor Xa upon prothrombinase assembly remains an enigma. Several studies based on experiments using either inhibitors of prothrombinase that interact with factor Xa at sites remote from its active site or active site-inhibited thrombin have offered a litany of arguments in favor of the hypothesis that incorporation of factor Va into prothrombinase only results in the exposure of cryptic exosites on factor Xa that facilitate its interaction with prothrombin. Because complete inhibition of prothrombinase occurred when using these competitive inhibitors without any interference with the active site of the enzyme, it was also concluded that the exposed cryptic exosites on factor Xa alone, following its interaction with factor Va, may also account for the substrate specificity of prothrombinase (35–38, 49, 78). However, several laboratories have independently demonstrated that factor Va heavy chain interacts directly with prothrombin through ABE-I and probably ABE-II (14, 15, 18–23, 34, 79). These latter conclusions are also supported by the fact that no interaction between bovine prothrombin and factor Xa could be detected in the absence of factor Va (80). A direct interaction between factor Va and active site-labeled meizothrombin on the membrane surface has also been demonstrated (81). More recently, the direct involvement of ABE-I of prethrombin-1 (prothrombin molecule lacking the Gla and Kringle-1 domains) with factor Va has been demonstrated (34). These latter studies showed that Arg^{62} and Lys^{65} of the B chain of thrombin (Arg^{67} and Lys^{70} chymotrypsin numbering; Refs. 34 and 82) were the major contributing amino acid residues from ABE-I of prothrombin to prothrombinase activity (34). All these data suggest that a significant conformational transition of the proteinase domain of the prothrombin molecule occurs upon its interaction with factor Va. It was thus hypothesized that factor Va may be at least partially responsible for the rearrangement of the prothrombin structure allowing exposure of hidden or nonoptimally exposed proteolytic sites required for efficient substrate catalysis. This extensive molecular rearrangement of prothrombin for efficient catalysis at Arg^{320} was also suggested following the determination of the crystal structure of prethrombin 2 (83). Our data put in the context of the literature suggest that the extensive binding exosite for prothrombin that provides for prothrombinase specificity and is responsible for the correct docking of the substrate in the active site of the enzyme, is most likely provided by amino acids belonging to both the carboxyl-terminal portion of factor Va heavy chain and factor Xa. Complete inhibition of either one separately will result in the loss of the catalytic efficiency of prothrombinase.

However, it is important to underline that there is not enough evidence overall to conclude that ABE-I of prothrombin interacts exclusively with the DYDYQ motif from factor Va heavy chain. It is possible that the extended surface spanning ABE-I is also responsible for the interaction of the substrate with the cryptic exosite from factor Xa exposed upon its interaction with factor Va. Thus, in all cases (*i.e.* whether ABE-I interacts exclusively with factor Va heavy chain, exclusively with the cryptic exosite from factor Xa, or with both), it would appear that the role of ABE-I in prothrombinase is dependent on the incorporation of factor Va into the complex as suggested (18, 21, 34). Overall our data underline the importance of factor Va for the specificity involved in substrate recognition and cleavage by prothrombinase and together with data on record demonstrate that: 1) factor Va binds factor Xa (receptor effect); 2) following binding a conformational transition of the enzyme occurs, exposing a portion of the binding exosite(s) for prothrombin (effector effect on factor Xa); and 3) the extended and contiguous prothrombin binding exosite within prothrombinase is completed by a portion of the heavy chain of the cofactor (effector effect on prothrombinase). The latter is required to achieve rates of prothrombin generation observed with prothrombinase. Our data thus define the cofactor, factor Va, as being the impresario of prothrombinase, orchestrating the spatial rearrangement of substrate and enzyme, which in turn are necessary for specific and efficient catalysis.

Finally, it is important to note that several studies utilizing high resolution x-ray crystal structures of coagulation and fibrinolytic enzyme complexes have suggested that, although the active site geometry of the enzyme component from several procoagulant and fibrinolytic complexes does not appear to be altered upon incorporation of the corresponding protein cofactor into the complex, docking of a hidden cleavage site of the substrate into the active site cleft of the enzyme following binding of the cofactor molecule to the substrate appeared to promote enzymatic specificity and optimum catalysis because the cofactor provided an extended and specific binding surface for the substrate (84–86). Our results are in complete agreement with these findings, and provide for the first time a functional demonstration for cofactor-directed catalysis of an enzymatic complex.

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