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Contribution of Amino Acid Region 659−663 of Factor Va Heavy Chain to the Activity of Factor Xa within Prothrombinase[†,‡]

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ABSTRACT: Factor Va, the cofactor of prothrombinase, is composed of heavy and light chains associated noncovalently in the presence of divalent metal ions. The COOH-terminal region of the heavy chain contains acidic amino acid clusters that are important for cofactor activity. In this work, we have investigated the role of amino acid region 659−663, which contains five consecutive acidic amino acid residues, by site-directed mutagenesis. We have generated factor V molecules in which all residues were mutated to either lysine (factor V^{5K}) or alanine (factor V^{5A}). We have also constructed a mutant molecule with this region deleted (factor $V^{\Delta 659-663}$). The recombinant molecules along with wild-type factor V (factor V^{WT}) were transiently expressed in mammalian cells, purified, and assessed for cofactor activity. Two-stage clotting assays revealed that the mutant molecules had reduced clotting activities compared to that of factor Va^{WT}. Kinetic analyses of prothrombinase assembled with the mutant molecules demonstrated diminished k_{cat} values, while the affinity of all mutant molecules for factor Xa was similar to that for factor Va^{WT}. Gel electrophoresis analyses of plasma-derived and recombinant mutant prothrombin activation demonstrated delayed cleavage of prothrombin at both Arg^{320} and Arg^{271} by prothrombinase assembled with the mutant molecules, resulting in meizothrombin lingering throughout the activation process. These results were confirmed after analysis of the cleavage of FPR-meizothrombin. Our findings provide new insights into the structural contribution of the acidic COOH-terminal region of factor Va heavy chain to factor Xa activity within prothrombinase and demonstrate that amino acid region 659−663 from the heavy chain of the cofactor contributes to the regulation of the rate of cleavage of prothrombin by prothrombinase.

The proteolytic conversion of prothrombin to thrombin is catalyzed by the prothrombinase complex composed of the enzyme, factor Xa, and the cofactor, factor Va, assembled on a membrane surface in the presence of Ca^{2+} (*1*, *2*). Factor Xa alone can activate prothrombin following sequential cleavages at Arg^{271} and Arg^{320}, yielding the transient inactive intermediate prethrombin-2. However, the interaction of factor Va with factor Xa on a membrane or cell surface in the presence of divalent metal ions and formation of the prothrombinase complex results in the reversal of the order of cleavage and a 300000-fold increase in the catalytic efficiency of factor Xa for thrombin generation. A first cleavage of prothrombin by prothrombinase at Arg^{320} produces the active intermediate meizothrombin, while the second cleavage at Arg^{271} produces thrombin (*3*−*6*). Thrombin and prothrombin contain two positively charged binding regions [anion binding exosite I (ABE-I) and anion binding exosite II (ABE II)] that are crucial for protein function. Initial cleavage of prothrombin at Arg^{320} by prothrombinase, which is absolutely factor Va-dependent, entirely exposes (pro)exosite I (*7*) and is responsible for the partial formation of thrombin's active site. Complete formation of the active site of thrombin requires cleavage of meizothrombin at Arg^{271} (*8*, *9*). Factor Va is required for the specific recognition of prothrombinase by (pro)exosite I of prothrombin (*10*, *11*). Proteolytic elimination of fragment 1 of prothrombin eliminates the accelerating effect of the membrane surface for initial cleavage at Arg^{320} by prothrombinase (*12*, *13*).

Coagulation factor V circulates in plasma at a concentration of 20 nM, as a single-chain inactive precursor (M_r = 330000) consisting of three subdomains in A1-A2-B-A3-C1-C2 order (Figure 1). The molecule is activated by thrombin following sequential cleavages at Arg^{709}, Arg^{1018}, and Arg^{1545} to generate the active cofactor (factor Va) composed of a heavy chain [M_r = 105000 (A1-A2 domains)] and a light chain [M_r = 74000 (A3-C1-C2 domains)]. The two chains are associated via noncovalent bonds in the presence of divalent metal ions (*14*). The light chain contains the domains that interact with the cell membrane surface at the place of vascular injury, while the heavy chain possesses specific amino acid motifs that are involved in the formation and function of the prothrombinase complex. We have demonstrated

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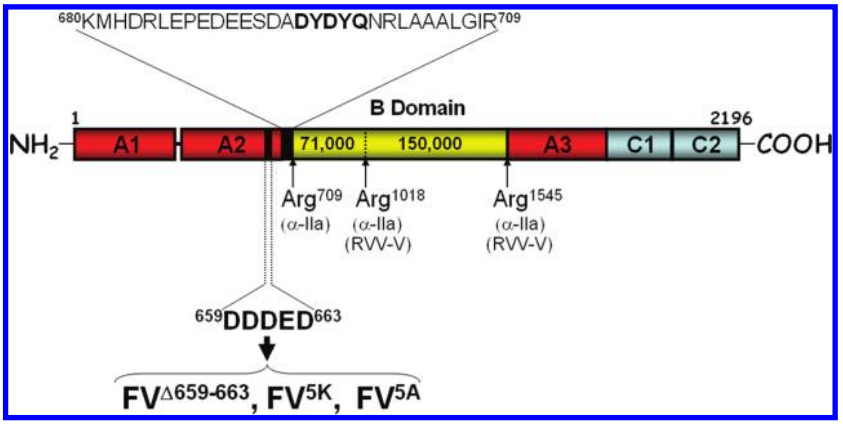

FIGURE 1: Factor V structure and mutant molecules. The procofactor, factor V, is composed of three A domains (red), a connecting B region (yellow), and two C domains (blue). Factor Va is generated following three sequential cleavages of factor V by α-thrombin at Arg^{709}, Arg^{1018}, and Arg^{1545}. The mutations within the acidic, hirudin-like COOH-terminal region of the heavy chain (amino acid residues 659−663) are indicated together with the designation of the recombinant mutant factor V molecules created and used throughout this work. The acidic region 680−709, recently shown to be involved in factor Va cofactor function, is also illustrated (*20*).

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                        656   660         670         680         690         700         710
                         |     |          |           |           |           |           |
Humf5.Pep               CIP--DDDEDSYEI--FEPPESTVMATRKMHDRLEPEDEESDADYDYQNRLAAALGIRS
chimpf5_pep_            CIP--DDDEDSYEI--FEPPESTVMATRKMHDRLEPEDEESDADYDYQNRLAAALGIRS
orangf5tot_pep_         CIP--DDDEDSYEI--FEPPESTVMATRKMHDRLEPEDEESDADYDYQNRLAAALGIRS
nomaleucf5_pep_         CIP--DDDEDSYEI--FEPPESTVMATRKMHDRLEPEDEESDADYDYQSRLAAALGIRS
macacaf5.pep_           CIT--DDDEDSYEI--FEPPESTVIATRKMHDPLETEDEESDTDYDYQSRLAAALGIRS
papiohamaf5_pep_        CIT--DDDEDSYEI--FEPPESTVIATRKMHDRLETEDEEGDTDYDYQSRLAAALGIRS
calljaccf5.pep_         CIR--DDYEDSYEI--YEPLESSVTATRKMHTPSENEDEESDADYDYQTRLASALGIRS
otogarf5.pep_           CIL--DDGDNSYEI--YEPPSFTPMETRKMHDFPDYEDEETKIEDYYQYMLASEFGIRS
Bovf5.Pep               CIR--NDDDDSYEI-IYEPSGSTAMTTKKIHDSSE-IEDENDADSDYQDELALILGLRS
pigf5.pep_              CIR--DDDEDSYEI-IYEPSSSTTLTTRKMHDSSENKEEENDDEYDYQDLLASVLGIRS
dogf5.pep_              CIR--DDYEDSYEI-IYESLAPTVMTTRKMRDSPEDNGDENDADYDYQNNLASWLGIRS
myotlucif5_pep_         CNR--NDENEEYEFEIYKPPPSTPMTTRKVHEFPENQGEIDETEDDYDSHLASLYGIRS
horsef5_pep_            CIL--DDGEGSYEF-IFQPPESTAITTRKMHDSSENSNEEFDADYDYQNRLASLFGIRS
dasnovf5_pep_           CIR-DYDDEDSYQI-IYEHKTSSTMDTRKMHDSSEDKSEMDDTDSDYQDTLASLLGIRS
elephf5.pep_            CIR--DDYEGSYEI-MYGPPTSIPMDTRKMHDSLENKSGEDATEYDYQDSLASSLGIRS
dipordif5_pep_          CNRNDDDNDDSY-I--YKPVESTVMETRKMRYSAENEQEEDDNESDYQDELATSLGIRS
rabbitf5_pep_           CNR--DDDDDSYEI--YQPPTSSPIDTRKMRDSSENRDEEYDAEYDYQNSLASSLGIRS
caviaf5_pep_            CIG--DDDEDSYKI-YAPPEVSTPMDVRKIKFPSENEHEEINPDDDYQDDLASALGLRS
Musf5.Pep               CNR-DYDNEDSYEI-YEPP-APTSMTTRRIHDSLENEFGIDNEDDDYQYLLASSLGIRS
ratf5_pep_              CNRNDDDEDSYEI-YQPL-EPTSMTTRKIHDSVENDFGIENEDDDYQYELASTLGIRS
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FIGURE 2: Comparison of the acidic COOH-terminal amino acid sequences 659−663 and 695−698 from factor Va heavy chain among species (numbering from the human molecule, top sequence). Sequences were derived from various database sources, such as GenBank and the NCBI Trace Archive. The acidic amino acid sequences of interest are shown in bold, together with amino acids 679 and 680 of the cofactor that represent a specific inactivating activated protein C cleavage site. The following species are included (from top to bottom): *Homo sapiens*, human; *Pan troglodytes*, chimpanzee; *Pongo pygmaeus*, orangutan; *Nomascus leucogenys*, white-cheeked gibbon; *Macaca mulatta*, rhesus monkey; *Papio hamadryas*, hamadryas baboon; *Callithrix jacchus*, white-tufted-ear marmoset; *Otolemur garnettii*, small-eared galago; *Bos taurus*, cattle; *Sus scrofa*, pig; *Canis lupus familiariz*, dog; *Myotis lucifugus*, little brown bat; *Equus caballus*, horse; *Dasypus novemcinctus*, nine-banded armadillo; *Loxodonta africana*, African elephant; *Dipodomys ordii*, Ord's kangaroo rat; *Oryctolagus cuniculus*, rabbit; *Cavia porcellus*, domestic guinea pig; *Mus musculus*, western European house mouse; *Rattus norvegicus*, Norway rat.

that a binding site for factor Xa is contained within the heavy chain of the cofactor (*15*, *16*), and we have recently shown that two residues from the central portion of the heavy chain of factor Va (amino acid residues 334 and 335) are crucial for cofactor function (*17*).

The COOH-terminal region of factor Va heavy chain is involved in the interaction with prothrombin (*18*, *19*). We have recently demonstrated that a factor Va molecule lacking the last 30 amino acids from the carboxyl-terminal end of the heavy chain (amino acid residues 680−709) displayed a reduction in clotting activity and a delay in prothrombin consumption, leading to the accumulation of the intermediate meizothrombin during prothrombin activation. Prothrombinase assembled with the same mutant also demonstrated an increased k_{cat} for prothrombin activation when compared to that of prothrombinase assembled with wild-type factor Va (*20*). We have established that this acidic region is essential for optimal expression of cofactor activity because it promotes a productive interaction with prothrombin regulating the rate of cleavage at Arg^{271} by prothrombinase (*20*−*23*). This region of the cofactor is highly acidic in nature and contains several tyrosine residues that have been shown to be involved in factor V activation by α-thrombin and proper cofactor function (*24*). This part of the molecule and, more precisely, the DYDYQ motif (amino acid residues 695−699) are highly conserved in 20 different mammal species (Figure 2). The heavy chain of the cofactor contains another cluster of acidic amino acids (DDDED, amino acid region 659−663) that may also be involved in the regulation of prothrombinase activity. This region is also conserved among species, a likely indicator of its physiological significance (Figure 2). This study was undertaken to evaluate the importance of amino acid region 659−663 of the factor Va heavy chain for the assembly and function of the prothrombinase complex. Our results show that this region exerts a profound and unexpected effect on prothrombin activation by prothrombinase.

EXPERIMENTAL PROCEDURES

Materials and Reagents. Diisopropyl fluorophosphate (DFP), *O*-phenylenediamine (OPD) dihydrochloride, and Coomassie Blue R-250 were purchased from Sigma (St. Louis, MO). Factor V-deficient plasma was from Research Protein Inc. (Essex Junction, VT). Secondary anti-mouse and anti-sheep IgG coupled to peroxidase was purchased from Southern Biotechnology Associates Inc. (Birmingham, AL). L-α-Phosphatidylserine (PS)[1] and L-α-phosphatidylcholine (PC) were obtained from Avanti Polar Lipids (Alabaster, AL). Chemiluminescent reagent ECL^+ and Heparin-Sepharose were obtained from Amersham-Pharmacia Biotech Inc. (Piscataway, NJ). Normal reference plasma and the chromogenic substrate H-D-hexahydrotyrosyl-alanyl-arginyl-*p*-nitroanilide diacetate (Spectrozyme-TH) were purchased from American Diagnostica Inc. (Greenwich, CT). RecombiPlasTin used in the clotting assays was purchased from Instrumentation Laboratory Co. (Lexington, MA). The reversible fluorescent α-thrombin inhibitor dansylarginine-*N*-(3-ethyl-1,5-pentanediyl) amide (DAPA), human α-thrombin, human prothrombin, and active site-blocked human meizothrombin [obtained following digestion of prothrombin with the purified component from the venom of the snake *Echis carinatus* as described previously (*25*), FPR-meizothrombin] were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Human factor Xa was purchased from Enzyme Research Laboratories (South Bend, IN). Human factor V cDNA was obtained from American Type Tissue Collection (ATCC# 40515 pMT2-V, Manassas, VA). All restriction enzymes were obtained from New England Biolabs (Beverly, MA). All molecular biology and tissue culture reagents, specific primers, and medium were purchased from Gibco, Invitrogen Corp. (Grand Island, NY) or as indicated. Recombinant wild-type prothrombin, prothrombin rMZ-II that has only one cleavage site for factor Xa (i.e., Arg^{320}), and prothrombin rP2-II that has only one cleavage site for factor Xa (i.e., Arg^{271}) were prepared and purified as previously described (*26*) and provided by M. Nesheim (Queen's University, Kingston, ON). Alternatively, cells stably transfected with the rMZ-II cDNA provided by M. Nesheim were grown and the media collected as described previously (*26*). rMZ-II was purified to homogeneity by fast liquid chromatography (FPLC) as initially described (*26*). Human factor V monoclonal antibodies ($\alpha HFV_{HC}17$ and $\alpha HFV_{LC}9$) used for immunoblotting experiments and monoclonal antibody αHFV1 coupled to Sepharose used to purify plasma and recombinant factor V molecules were provided by K. G. Mann (Department of Biochemistry, University of Vermont, Burlington, VT).

Mutagenesis and Transient Expression of Recombinant Factor V Molecules. The factor V cDNA consists of a 6909 bp fragment inserted into the pMT2 mammalian expression vector at the *Sal*I site. Mutant factor V molecules consisting of point mutations and various deletions of the COOH-terminus of the heavy chain were synthesized using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The mutagenic primers used for the deletions were 58 bp primers on the sense and antisense strands of the recombinant factor V molecule. The primers for factor V^{5K} were 5′-TGAGGCTGAAATTCAGGGATGTTAAATGTATCCCAAAGAAGAAGAAAAAGTCATATGAGATTTTTGAACCTCCAGAATC-3′ (sense) and 5′-GATTCTGGAGGTTCAAAAATCTCATATGACTTTTTCTTCTTCTTTGGGATACATTTAACATCCCTGAATTTCAGCCTCA-3′ (antisense). Primers for factor $V^{659-663}$ were 5′-AGGCTGAAATTCAGGGATGTTAAATGTATCCCATCATATGAGATTTTTGAACCTC-3′ (sense) and 5′-GAGGTTCAAAAATCTCATATGATGGGATACATTTAACATCCCTGAATTTCAGCCT-3′ (antisense). Primers for factor V^{5A} were 5′-GCTGAAATTCAGGGATATTAAATGTATCCCAGCGGCGGCGGCGGCGTCATATGAGATTTTTGAACCTCCAGA-3′ (sense) and 5′-TCTGGAGGTTCAAAAATCTCATATGACGCCGCCGCCGCCGCTGGGATACATTTAACATCCCTGAATTTCAGC-3′ (antisense). The mutagenized primers were transformed into competent *Escherichia coli* cells, and positive ampicillin-resistant clones were selected to screen for mutants. Wild-type factor V and mutant factor V clones were cultured and isolated using the PureLink Quick Plasmid miniprep kit (Invitrogen, Carlsbad, CA). The incorporation of the mutations into the cDNA was verified by DNA sequence analysis, using factor V-specific primers. Transfection and harvesting of the media were performed as described in detail by our laboratory (*16*, *27*). All media containing the recombinant factor V molecules were concentrated using the Vivaflow 50 Complete System (Vivascience AG, Hannover, Germany) according to the manufacturer's instructions. All recombinant factor V molecules were purified according to the detailed protocol previously described by our laboratory (*27*). The concentration of the recombinant proteins was determined by an enzyme-linked immunosorbent assay (ELISA) as previously described (*16*, *28*). The activity and integrity of the recombinant molecules were verified before and after activation with thrombin 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. In some instances, factor Va fragments were also visualized following staining with silver.

Gel Electrophoresis and Western Blotting. SDS−PAGE analyses of recombinant factor V molecules were performed using 4 to 12% gradient gels according to the method of Laemmli (*29*). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes according to the method described by Towbin et al. (*30*). After the transfer to PVDF, factor V heavy and light chain(s) were detected using the appropriate monoclonal and polyclonal antibodies (*31*, *32*). Immunoreactive fragments were visualized with chemiluminescence. In several instances, recombinant factor V and factor Va fragments obtained following activation of the procofactor with thrombin were visualized following staining of the gels with silver as described previously (*33*).

Analysis of Prothrombin or Recombinant Mutant Prothrombin Activation and FPR-Meizothrombin Cleavage at Arg^{271} by Gel Electrophoresis. Prothrombin or recombinant

[1]Abbreviations: PS, L-α-phosphatidylserine; PC, L-α-phosphatidylcholine; PCPS vesicles, small unilamellar phospholipid vesicles composed of 75% PC and 25% PS (w/w); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; factor V^{WT}, wild-type recombinant human factor V; factor $Va_{IIa}{}^{WT}$, wild-type recombinant human factor V activated with α-thrombin; factor $V^{\Delta 659-663}$, recombinant human factor V missing amino acid residues 659−663; factor $Va_{IIa}{}^{\Delta 659-663}$, recombinant human factor V missing amino acid residues 659−663 activated with α-thrombin; factor V^{5K}, recombinant human factor V with the ^{659}DDDED^{663} → KKKKK mutations; factor $Va_{IIa}{}^{5K}$, recombinant human factor V with the ^{659}DDDED^{663} → KKKKK mutations activated with α-thrombin; factor V^{5A}, recombinant human factor V with the ^{659}DDDED^{663} → AAAAA mutations; factor $Va_{IIa}{}^{5A}$, recombinant human factor V with the ^{659}DDDED^{663} → AAAAA mutations activated with α-thrombin; factor $V_{IIa}{}^{PLASMA}$, plasma-derived human factor V activated with α-thrombin.

mutant prothrombin molecules (1.4 μM) were incubated with PCPS vesicles (20 μM), DAPA (50 μM), and factor Va (10−20 nM) in the presence of 5 mM Ca^{2+} in 20 mM Tris and 0.15 M NaCl (pH 7.4). The reaction was initiated upon addition of factor Xa (0.5−1 nM) at room temperature over the time course indicated in the figure legends. Aliquots (50 μL) from the reaction mixture were removed at selected time intervals (as indicated in the figure legends), diluted into 2 volumes of 0.2 M glacial acetic acid, and concentrated using a Centrivap concentrator attached to a Centrivap cold trap (Labconco, Kansas City, MO). The dried samples were dissolved in 0.1 M Tris base (pH 6.8), 1% SDS, and 1% β-mercaptoethanol, heated for exactly 75 s at 90 °C, mixed, and subjected to SDS−PAGE using 9.5% gels prepared according to the method of Laemmli (*29*); 6 μg of protein per lane was applied. FPR-meizothrombin cleavage at Arg^{271} was assessed in a similar manner using 12% SDS−PAGE. Protein bands were visualized following staining with Coomassie Brilliant Blue R and destained by diffusion in a methanol/acetic acid/water solution.

Scanning Densitometry of SDS−PAGE and Calculation of the Rate of Prothrombin Consumption. Scanning densitometry of the gels was performed as described previously (*34*). Briefly, the stained gels were scanned and imported into the UN-SCAN-IT gel (Silk Scientific, Orem, UT). The numbers were normalized to the initial concentration of prothrombin (1.4 μM) and adjusted for the capability of each fragment to be stained by Coomassie as described previously (*35*). Following scanning densitometry, the data representing prothrombin consumption as a function of time (seconds) were subsequently plotted using nonlinear regression analysis and the equation representing a first-order exponential decay using Prism (GraphPad, San Diego, CA). The apparent first-order rate constant, k (inverse seconds), obtained directly from the fitted data was divided by the molar concentration of factor Xa used in each experiment. The number obtained was subsequently multiplied by the starting concentration of prothrombin. The final numbers reported throughout this work characterizing the effect of the mutations on plasma-derived prothrombin consumption by prothrombinase assembled with various recombinant factor Va molecules represent the moles of prothrombin consumed per mole of factor Xa per second for a given experiment. The consumption rates reported here are representative of experiments performed at least in triplicate using two to four different preparations of recombinant proteins. All constants reported in Tables 2 and 3 were extracted directly from the fitted data. The tables report the standard deviation for each measurement. In the figure legends, we also provide the goodness of fit to the equation representing a first-order exponential decay (R^2).

Measurement of Rates of Thrombin Formation in a Prothrombinase Assay. All factor V molecules, both recombinant and plasma, were activated with human α-thrombin as described previously (*36*). The assay verifying the activity of the recombinant molecules was conducted as described by measuring thrombin formation by the change in the absorbance of a chromogenic substrate at 405 nm (Spectrozyme-TH) monitored with a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA) (*16*, *22*). Absorbance at 405 nm was compared with a standard curve prepared daily using purified thrombin (0−50 nM).

For the functional calculation of the apparent dissociation constants (K_{Dapp}) between the recombinant factor Va molecules and factor Xa, experiments were performed in the presence of a limited concentration of factor Xa (15 pM) and varying concentrations of factor Va (between 30 pM and 10 nM). Throughout the experiments, the assumption was that n was the number of moles of factor Xa bound per mole of factor Va at saturation; throughout this study, $n = 1$, and the stoichiometry of the factor Va−factor Xa interaction was fixed at 1. The initial rate of thrombin formation was calculated, and the data were analyzed and plotted using nonlinear regression analysis and Prism (GraphPad) according to the one-binding site model. Dissociation constants reported here were extracted directly from the fitted data.

The assay using purified reagents and verifying the cofactor activity of the recombinant factor V molecules for prothrombin activation was conducted under conditions where all factor Xa was saturated with factor Va, as described by measuring α-thrombin formation by the change in the absorbance of a chromogenic substrate at 405 nm (Spectrozyme-TH, 0.4 mM) (*16*). All factor V molecules were activated with thrombin as described previously (*21*, *22*). Because we wanted to prevent the possibility that differences between prothrombinase assembled with factor Va^{WT} and prothrombinase assembled with the three recombinant mutant factor Va molecules may be attributed to subtle differences in the K_{Dapp} of factor Va for factor Xa, which would result in a smaller amount of prothrombinase formed, all experiments were conducted under conditions where factor Xa was saturated with factor Va. Knowing the K_{Dapp} of each factor Va species for factor Xa, we calculated the amount necessary to saturate factor Xa using the quadratic equation described in the literature (*17*, *37*, *38*) before each experiment. The absorbance was monitored with a Thermomax microplate reader and compared to that of a α-thrombin standard prepared daily using purified plasma-derived α-thrombin. The data were analyzed and plotted using nonlinear regression analysis and Prism according to the Henri Michaelis−Menten equation. Kinetic constants provided here were extracted directly from the fitted data. In addition, in the figure legends, we report the goodness of fit to the Henri Michaelis−Menten equation (R^2).

RESULTS

Transient Expression and Activation of Recombinant Factor V Molecules. To assess the importance of amino acid region 659−663 from the heavy chain of factor Va, we constructed three mutant molecules. We prepared recombinant factor $V^{\Delta 659-663}$, factor V^{5K}, and factor V^{5A} (Figure 1). Full-length recombinant factor V^{WT} and the mutant molecules were expressed in mammalian cells and purified by immunoaffinity chromatography as previously described in detail by our laboratory (*27*). All recombinant mutant molecules were activated with thrombin. Figure 3 illustrates a typical quality control procedure performed prior to all experiments. Panels A−C show the subunit composition of the recombinant molecules before and after activation by thrombin following staining with silver. It is noteworthy that in all experiments we compare wild-type recombinant and plasma factor V to assess if the minute amounts of impurities present in our recombinant factor V preparations interfere with cofactor activity. All data obtained with purified wild-type recombinant factor Va are equivalent and comparable to the data obtained with a cofactor molecule purified from pooled normal plasma (Table 1). For the record, our data obtained with purified recombinant wild-type factor Va are also

comparable to the results previously obtained with recombinant mutant factor Va molecules used in conditioned media (*16*, *28*). Thus, the minimal amounts of impurities present in our preparations in this study do not interfere with cofactor activity. In addition, all recombinant factor V molecules that are purified employing immunoaffinity chromatography using a monoclonal antibody to factor V are fully active using the experimental protocol described here [as demonstrated by additional experiments using gel electrophoresis and a polyclonal antibody to factor V (not shown)]. As shown in Figure 3, following incubation with thrombin no single chain factor V was apparent on the gels (most obvious in panel C, lane 2), while fragments with the expected molecular weights representing the heavy and light chain of the cofactor appeared. Both of these observations are consistent with the conclusion that recombinant factor V molecules were fully active using the conditions described in Experimental Procedures. Control experiments demonstrated that further incubation with thrombin does not result in increased cofactor activity. In contrast, prolonged incubation of the recombinant procofactor molecules with thrombin results in partial inactivation because of cleavage of the heavy chain by thrombin at Arg^{643} as previously demonstrated (*27*). As a consequence, the differences in activity observed between the wild-type or plasma cofactor molecules and the recombinant mutant factor Va molecules are due to the specific mutations. SDS−PAGE analyses followed by immunoblotting with specific monoclonal antibodies to the heavy and light chain of the cofactor demonstrate that the mutant recombinant proteins are intact and composed of heavy and light chains that migrated in accord with their expected molecular weights (panel D).

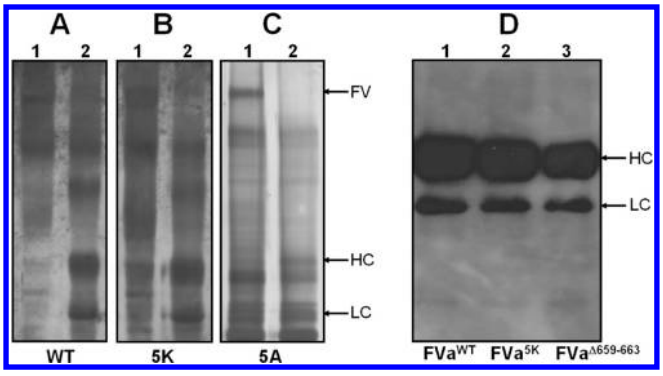

FIGURE 3: Electrophoretic analyses of wild-type factor V and recombinant factor V molecules. Factor V^{WT}, factor $V^{\Delta 659-663}$, factor V^{5K}, and factor V^{5A} were activated with thrombin as described in Experimental Procedures using anti-factor V immunoaffinity chromatography and analyzed by SDS−PAGE followed by staining with silver (A−C): lane 1, factor V prior to activation by thrombin; lane 2, recombinant molecules following incubation with thrombin. (D) Following SDS−PAGE and transfer to a PVDF membrane, immunoreactive fragments were detected with monoclonal antibodies $\alpha HFVa_{HC}17$ (recognizing an epitope on the heavy chain of the cofactor between amino acid residues 307 and 506) and $\alpha HFVa_{HC}9$ (recognizing the light chain). At the right of all panels, the positions of factor V and the heavy and light chains of factor Va are shown.

The recombinant molecules were first assessed for their clotting activity in a two-stage clotting assay. Thrombin activation of factor V^{WT} resulted in a cofactor with a clotting activity similar to that of the plasma-derived molecule (Table 1). Under similar experimental conditions, factor $Va^{\Delta 659-663}$, factor Va^{5K}, and factor V^{5A} displayed ~3.5-, 11-, and 24-fold less clotting activity, respectively, than factor Va^{WT} (Table 1). These data demonstrate that amino acid region 659−663 is important for the expression of optimal factor Va clotting activity.

Kinetic Analyses of Recombinant Factor Va Molecules. We next examined the capability of the recombinant factor Va molecules to bind factor Xa and to assemble in prothrombinase using an assay employing purified reagents and a chromogenic substrate to probe for thrombin generation. The assay was performed under conditions of limiting factor Xa concentrations while the concentration of recombinant factor Va molecules was varied. Table 1 provides the results of the kinetic studies. The data demonstrate that under the experimental conditions used, factor Va^{WT} has a similar affinity for the enzyme, factor Xa, as its plasma counterpart. Likewise, the three recombinant mutant factor Va molecules have similar affinities for plasma-derived factor Xa that are indistinguishable from the affinity of factor Va^{WT} for factor Xa. These results are in complete accord with our recent findings (*20*) and demonstrate that the acidic hirudin-like amino acid regions from the COOH-terminal portion of the heavy chain of factor Va do not participate in the interaction of the cofactor with plasma-derived factor Xa (Table 1).

We subsequently evaluated the effect of the mutations on the K_m and k_{cat} of prothrombinase. The raw data are displayed in Figure 4, while the kinetic constants for each set of titration derived directly from the fitted data are reported in Table 1. Under the experimental conditions employed, the mutations had

Table 1: Functional Properties of Various Recombinant Factor Va Molecules

factor Va species	clotting activity (units/mg)[a]	*x*-fold decrease[b]	K_{Dapp} (nM)[c]	K_m (μM)[d]	k_{cat} (min^{-1})[d,e]	k_{cat}/K_m ($\times 10^8$ M^{-1} s^{-1})	*x*-fold decrease[f]
factor Va^{PLASMA}	3124 ± 413	−	0.67 ± 0.15	0.26 ± 0.04	2162 ± 55	1.40	−
factor Va^{WT}	2926 ± 320	−	0.81 ± 0.10	0.23 ± 0.01	2317 ± 67	1.70	−
factor $Va^{\Delta 659-663}$	823 ± 180	3.5	0.99 ± 0.22	0.23 ± 0.03	1493 ± 58	1.08	1.6
factor Va^{5K}	275 ± 92	11	0.85 ± 0.16	0.28 ± 0.03	1102 ± 25	0.65	2.6
factor Va^{5A}	120 ± 30	24	0.84 ± 0.10	0.30 ± 0.04	512 ± 20	0.28	6.1

[a]Two-stage clotting assays of recombinant factor V molecules was performed as described in Experimental Procedures. [b]The *x*-fold decrease is the ratio of the clotting activity of factor Va^{WT} compared to the clotting activity of the recombinant mutant factor Va molecules. [c]Apparent dissociation constants of recombinant factor Va for plasma-derived factor Xa (K_{Dapp}) were determined as described in Experimental Procedures at a limiting factor Xa concentration (15 pM) according to the binding model assuming one binding site using Prism. Dissociation constants were derived directly from the fitted data. [d]The K_m and k_{cat} of prothrombinase assembled with saturating concentrations of recombinant factor Va molecules were determined as described in Experimental Procedures according to the Michaelis−Menten equation using Prism. Kinetic constants were derived directly from the fitted data. [e]$k_{cat} = V_{max}$/[enzyme] (in the presence of factor Va); the enzyme concentrations of prothrombinase (factor Xa−factor Va complex on the membrane surface in the presence of Ca^{2+}) were calculated on the basis of the equations previously described in detail in the literature (*37*, *38*). Under the conditions employed, the prothrombinase concentration was 10 pM. [f]The *x*-fold decrease is the ratio of the second-order rate constant (k_{cat}/K_m) of prothrombinase assembled with factor Va^{WT} to the second-order rate constant of prothrombinase assembled with the recombinant mutant factor Va molecules.

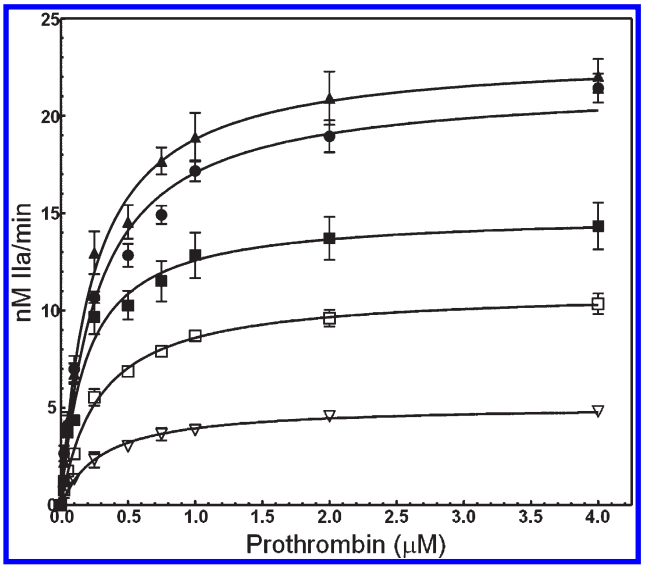

FIGURE 4: Determination of the kinetic parameters of prothrombinase assembled with the various recombinant factor Va species. Thrombin generation experiments were conducted as described in Experimental Procedures by varying the substrate concentration (from 25 nM to 4 μM) in the presence of 10 pM factor Xa. Factor V^{WT}, factor V^{PLASMA}, factor $V^{\Delta 659-663}$, factor V^{5K}, and factor V^{5A} were activated with thrombin as described in Experimental Procedures. The solid lines represent the nonlinear regression fits of the data using Prism according to the Henri Michaelis−Menten equation ($V_o = V_{max}$[prothrombin]/K_m + [prothrombin]) to yield the K_m and k_{cat} ($k_{cat} = V_{max}/E_{total}$, where E_{total} is the concentration of prothrombinase) (Table 1). Prothrombinase was assembled with recombinant factor Va^{WT} [20 nM (▲); $R^2 = 0.95$], factor Va^{PLASMA} [20 nM (●); $R^2 = 0.96$], factor $Va^{\Delta 659-663}$ [20 nM (■): $R^2 = 0.94$], factor Va^{5K} [20 nM (□); $R^2 = 0.97$], and factor Va^{5A} [20 nM (▽); $R^2 = 0.94$]. The data for factor Va^{WT}, factor Va^{PLASMA}, factor $Va^{\Delta 659-663}$, and factor V^{5K} show the average results obtained from at least three different titrations with three different preparations of purified proteins. The data for factor Va^{5A} are representative of six different titrations using five different preparations of recombinant protein. Kinetic constants reported in Table 1 were extracted directly from the fitted data.

no significant effect on the K_m of the reaction, while the catalytic efficiencies of prothrombinase assembled with the three mutant recombinant cofactor molecules were decreased compared to the catalytic efficiency of prothrombinase assembled with factor Va^{WT} (Figure 4 and Table 1). The data show that prothrombinase assembled with factor Va^{5K} or factor Va^{5A} has approximately ~50 or ~80% reduced catalytic efficiency, respectively, when compared to the catalytic efficiency of factor Va^{WT}, while factor $Va^{\Delta 659-663}$ has a ~40% reduced catalytic efficiency compared to the value obtained for prothrombinase assembled with factor Va^{WT}. Comparison of the second-order rate constant between prothrombinase assembled with the recombinant mutant cofactor molecules (k_{cat}/K_m) and the second-order rate constant obtained with prothrombinase assembled with factor Va^{WT} or factor Va^{PLASMA} demonstrates that the turnover number for prothrombinase assembled with the mutant molecules is 40−80% reduced (Table 1). The inability of prothrombinase assembled with the mutant cofactor molecules to function optimally coupled to the lack of an effect of the mutations on the dissociation constant of the recombinant mutant cofactor molecules for plasma factor Xa can be explained by the inability of factor Xa within prothrombinase to efficiently convert prothrombin to thrombin because of weakened productive collisions between the enzyme and prothrombin. Overall, the data demonstrate that amino acid region 659−663 of factor Va heavy chain is involved in the activation of prothrombin by factor Xa. However, it is important to note that while the cofactor molecules have a significant deficiency in clotting activity their deficiency in promoting efficient cleavage of prothrombin when assembled in prothrombinase, as assessed by an assay using saturating concentrations of factor Va and a chromogenic substrate to assess for thrombin activity appears to be less pronounced.

Visualization of the Activation Pathway. The findings obtained thus far indicate that the rate of one or both of the two prothrombin activation cleavages is impaired when prothrombinase is assembled with a cofactor molecule mutated in amino acid region 659−663 of the heavy chain. To improve our understanding of the reason for the deficiency in prothrombin cleavage by prothrombinase assembled with the recombinant mutant cofactor molecules, we have studied prothrombin activation by gel electrophoresis. The results demonstrate a delay in prothrombin activation by prothrombinase assembled with either factor $Va^{\Delta 659-663}$, factor Va^{5K}, or factor Va^{5A} as compared to the activation of prothrombin by prothrombinase assembled with either factor Va^{PLASMA} or factor Va^{WT} (Figure 5A−E). Scanning densitometry of the gels shown in Figure 5 demonstrated 3-, 10-, and ~40-fold delays in prothrombin consumption by prothrombinase assembled with factor $Va^{\Delta 659-663}$, factor Va^{5K}, and factor Va^{5A}, respectively, compared to the consumption of prothrombin assembled with factor Va^{WT} or factor Va^{PLASMA} (Figure 6 and Table 2). The levels of decreased prothrombin consumption observed correlates very nicely with the results observed in the clotting assay (Table 1). Prothrombin consumption by prothrombinase assembled with factor Va^{5A} was most severely impaired with little meizothrombin visible during the activation process (Figures 5E). In contrast, when prothrombin is activated by prothrombinase assembled with either factor $Va^{\Delta 659-663}$ or factor Va^{5K}, there is persistence (lingering) of meizothrombin throughout the activation process. Scanning densitometry of the gels shown in panels C and D of Figure 5 demonstrated a peak for meizothrombin at 120 s when prothrombin is activated by prothrombinase assembled with factor Va^{WT}, while a peak for meizothrombin is detected at 200 or 360 s when prothrombin is activated by prothrombinase assembled with either factor $Va^{\Delta 659-663}$ or factor Va^{5K}, respectively (not shown). Overall, the data strongly suggest that the integrity of amino acid region 659−663 is required for optimum rates of prothrombin activation.

To improve our understanding of the effect of prothrombinase assembled with factor Va^{5A} on prothrombin activation, we compared prothrombin activation by factor Xa alone or by membrane-bound factor Xa, or by prothrombinase assembled with factor Va^{5A} over a 3 h time period (Figure 7A−D). The data demonstrate that very little thrombin is produced by factor Xa alone (panel A) or by membrane-bound factor Xa (panel B). In the presence of prothrombinase assembled with factor Va^{5A} (panel C), both intermediates, meizothrombin and prethrombin-2, are observed. Scanning densitometry of the gel shown in Figure 7C demonstrated a rate of prothrombin consumption by prothrombinase assembled with factor Va^{5A} similar to the rate observed in Figure 5D (Table 2). Altogether, these data are in complete accord with the findings obtained in the functional assays, and the combined data suggest that both prothrombin activation cleavages (Arg^{320} and Arg^{271}) appear to be affected when prothrombinase is assembled with either factor $Va^{\Delta 659-663}$, factor Va^{5K}, or factor Va^{5A}. Overall, the data demonstrate that amino acid region 659−663 actively participates in the activation

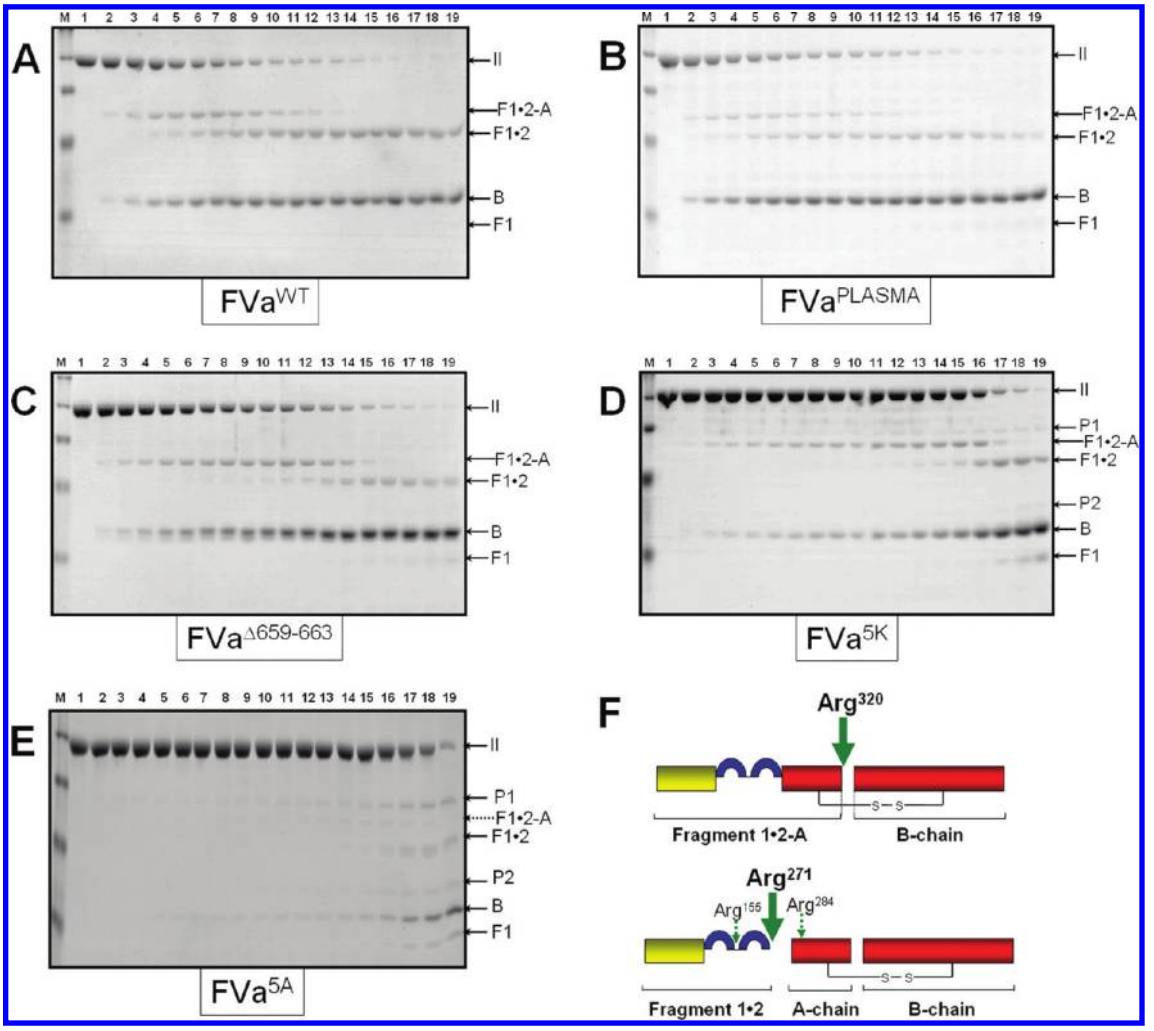

FIGURE 5: Analysis of the activation of plasma-derived prothrombin by prothrombinase assembled with various mutant factor Va molecules. Plasma-derived prothrombin (1.4 μM) was incubated in different mixtures with PCPS vesicles (20 μM) and (A and B) prothrombinase assembled with either factor Va^{WT} [final concentration of 20 nM, 96.6% factor Xa saturation (A)] or factor Va^{PLASMA} [final concentration of 20 nM, 95.6% factor Xa saturation (B)] as described in Experimental Procedures, (C) prothrombinase assembled with factor $Va^{\Delta 659-663}$ (final concentration of 20 nM, 95.1% factor Xa saturation), (D) prothrombinase assembled with factor Va^{5K} (final concentration of 20 nM, 95.7% factor Xa saturation), and (E) prothrombinase assembled with factor Va^{5A} (final concentration of 30 nM, 97.2% factor Xa saturation). The reactions were started by the addition of factor Xa (1 nM). At selected time intervals, aliquots of the reaction mixtures were withdrawn and treated as described in Experimental Procedures. Lane M contained the molecular weight markers (from top to bottom): 98000, 64000, 50000, and 36000, respectively. Lanes 1−19 contained samples from the reaction mixture before (0 min) the addition of factor Xa and 20 s, 40 s, 60 s, 80 s, 100 s, 120 s, 140 s, 160 s, 180 s, 200 s, 220 s, 240 s, 5 min, 6 min, 10 min, 20 min, 30 min, and 60 min, respectively, after the addition of factor Xa. (F) Meizothrombin pathway. Schematic structure of fragments derived following prothrombin activation by prothrombinase via two sequential cleavages: initial cleavage at Arg^{320} followed by cleavage at Arg^{271}. Prothrombin is composed of an NH_2-terminal domain containing 10 γ-carboxyglutamic acid residues (yellow), two kringle domains (blue), and a COOH-terminal serine protease domain (red). The prothrombin-derived fragments identified on the gels are shown as follows: II, prothrombin (amino acid residues 1−579); F1·2-A, fragment 1·2-A chain (amino acid residues 1−320); F1·2, fragment 1·2 (amino acid residues 1−271); B, B chain of α-thrombin (amino acid residues 321−579); F1, fragment 1 (amino acid residues 1−155). Secondary cleavages at Arg^{155} and Arg^{284} (dotted green arrows) are produced by either thrombin or membrane-bound factor Xa. The factor Va species used for the reconstitution of prothrombinase are shown under each panel.

of prothrombin by prothrombinase, and that the nature of the mutations in this region has a differential effect on the pathway and rate of prothrombin activation by prothrombinase.

Activation of Recombinant Mutant Prothrombin by Prothrombinase Assembled with Mutant Factor Va Molecules. The data obtained thus far in the study of plasma-derived prothrombin activation by prothrombinase assembled with the mutant cofactor molecules demonstrate that (1) meizothrombin lingers throughout the time course when prothrombin is activated by prothrombinase assembled with the mutant cofactor molecules and (2) the appearance of the B chain of thrombin is also delayed. To verify which cleavage in prothrombin is specifically affected by each of the modifications in amino acid region 659−663 of factor Va heavy chain, we used prothrombin molecules that cannot be cleaved at either Arg^{271} [rMZ-II (Figure 8)] or Arg^{320} [rP2-II (Figure 9)] (*26*).

Our findings demonstrate a significant difference between the rates of activation of rMZ-II by prothrombinase assembled with either factor $Va^{\Delta 659-663}$, factor Va^{5K}, or factor Va^{5A} as compared to the rates of rMZ-II cleavage and activation by prothrombinase made with factor Va^{WT} or factor Va^{PLASMA} (Figure 8). Scanning densitometry of the gels shown in Figure 8 demonstrates that cleavage at Arg^{320} of rMZ-II is delayed by ~2-fold when prothrombinase is assembled with factor $Va^{\Delta 659-663}$ and 4-fold when prothrombinase is assembled with factor Va^{5K} compared to cleavage of the recombinant mutant prothrombin molecule by

prothrombinase assembled with factor Va^{WT} (Table 2 and Figure 10A). In contrast, very slow prothrombin consumption was observed when rMZ-II was incubated with prothrombinase assembled with factor Va^{5A} (Figure 8E). The rate of cleavage of rMZ-II by prothrombinase assembled with factor Va^{5A} is 33-fold slower than the rate of cleavage of rMZ-II by prothrombinase assembled with factor Va^{WT} (Table 2). These data confirm our findings obtained with plasma-derived prothrombin. In particular, the data obtained with factor Va^{5A} and rMZ-II verify the data shown in Figures 5E and 7C obtained with plasma-derived prothrombin and factor Va^{5A}, demonstrating slow cleavage of prothrombin at Arg^{320} by prothrombinase assembled with factor Va^{5A}. Overall, the data demonstrate that prothrombinase-mediated cleavage at Arg^{320} in prothrombin is affected by modifications in amino acid region 659−663 of factor Va heavy chain.

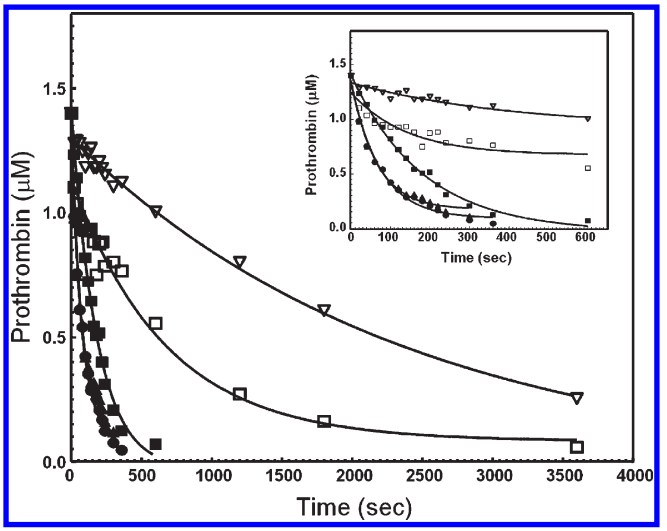

FIGURE 6: Analysis of prothrombin consumption by prothrombinase assembled with recombinant factor Va molecules. The gels shown in Figure 5 were scanned, and prothrombin consumption was recorded as described in Experimental Procedures. Following scanning densitometry, the numbers were normalized to the initial concentration of prothrombin (1.4 μM). The data representing prothrombin consumption (micromolar) as a function of time (seconds) were subsequently plotted using nonlinear regression analysis according to the equation representing a first-order exponential decay using Prism (GraphPad). Prothrombinase was assembled with recombinant factor Va^{WT} [(▲) $R^2 = 0.99$], factor Va^{PLASMA} [(●) $R^2 = 0.99$], factor $Va^{\Delta 659-663}$ [(■) $R^2 = 0.99$], factor Va^{5K} [(□) $R^2 = 0.94$], and factor Va^{5A} [(▽) $R^2 = 0.98$]. The apparent first-order rate constant, k (inverse seconds), obtained directly from the fitted data was divided by the molar concentration of factor Xa used in each experiment. The number obtained was subsequently multiplied by the starting concentration of prothrombin. The resulting numbers representing prothrombin consumption are listed in Table 2. The inset represents the progress of the reaction during the first 10 min.

The data shown in Figures 9 and 10B and Table 3 demonstrate that the rate of cleavage of rP2-II at Arg^{271} by prothrombinase assembled with either factor $Va^{\Delta 659-663}$, factor Va^{5K}, or factor Va^{5A} is very slow compared to the rate of cleavage of rP2-II at Arg^{271} by prothrombinase assembled with either factor Va^{WT} or factor Va^{PLASMA}. However, it is important to note that while under the conditions described in the legend of Figure 9, prothrombin was slowly consumed by prothrombinase assembled with the mutant cofactor molecules (Table 3), and a rate of prothrombin consumption could not be obtained from the gels shown in panels C−E. Overall, the data shown in Figures 5−10 demonstrate that mutations in acidic amino acid segment 659−663 of factor Va differentially affect the rate of cleavage of the two activating cleavage sites of prothrombin by prothrombinase. These data unambiguously demonstrate that this portion of factor Va heavy chain plays a role in controlling the catalytic efficiency of factor Xa within prothrombinase for prothrombin activation.

Activation of FPR-Meizothrombin by Prothrombinase Assembled with Mutant Factor Va Molecules. Analysis of the results obtained thus far with plasma-derived and recombinant prothrombin suggests that activation of the molecules by prothrombinase assembled with factor $Va^{\Delta 659-663}$, factor Va^{5K}, and factor Va^{5A} is impaired compared to cleavage of the recombinant prothrombin molecules by prothrombinase assembled with factor Va^{WT} because of delayed cleavages at both Arg^{320} and Arg^{271} resulting in both slow prothrombin consumption and less conversion of meizothrombin to thrombin. In addition, our data show that the rate of cleavage at Arg^{271} of rP2-II appears to be affected more than the rate of cleavage at Arg^{320} of rMZ-II, by prothrombinase assembled with the mutant cofactor molecules. It has been shown that a change in the conformation of meizothrombin (ratcheting) is associated with cleavage of prothrombin at Arg^{320} (*39*). To ascertain the effect of the mutations of the factor Va heavy chain on the cleavage of prothrombin at Arg^{271} following the transition that occurs after cleavage at

Table 2: Rate of Activation of Native Plasma-Derived Prothrombin and Recombinant Mutant rMZ-II in the Presence of Prothrombinase Assembled with Various Recombinant Factor Va Species

enzyme	plasma-derived prothrombin[a] (initial cleavage at Arg^{320}) [mol consumed s^{-1} (mol of factor Xa)$^{-1}$]	rMZ-II[a] (cleavage at Arg^{320}) [mol consumed s^{-1} (mol of factor Xa)$^{-1}$]
factor Xa	0.065 ± 0.025	0.0235 ± 0.017[b]
prothrombinase with factor Va^{PLASMA}	19.0 ± 1.5	14.8 ± 0.8
prothrombinase with factor Va^{WT}	21.8 ± 1.9	11.2 ± 0.8
prothrombinase with factor $Va^{\Delta 659-663}$	7.7 ± 0.5	6.6 ± 0.7
prothrombinase with factor Va^{5K}	2.1 ± 0.4	2.9 ± 0.8
prothrombinase with factor Va^{5A}	0.58 ± 0.1[c]/0.18 ± 0.06[d]	0.34 ± 0.1

[a]The rates of plasma-derived prothrombin and recombinant mutant prothrombin rMZ-II consumption were obtained following scanning densitometry of gels shown in Figures 5 and 8, respectively. The final rate of prothrombin consumption in the presence of prothrombinase assembled with various factor Va species was extracted following plotting of the data according to the equation representing a first-order exponential decay as described in Experimental Procedures (Figures 6 and 10A). The apparent first-order rate constants were obtained directly from the fitted data. [b]Rate obtained from a time course using 10 nM factor Xa ($R^2 = 0.922$). [c]From the 1 h time course with 20 nM factor Va^{5A} and 1 nM factor Xa [$R^2 = 0.985$ (Figure 5)]. [d]From the 3 h time course 20 nM factor Va^{5A} and 0.5 nM factor Xa [$R^2 = 0.987$ (Figure 7)].

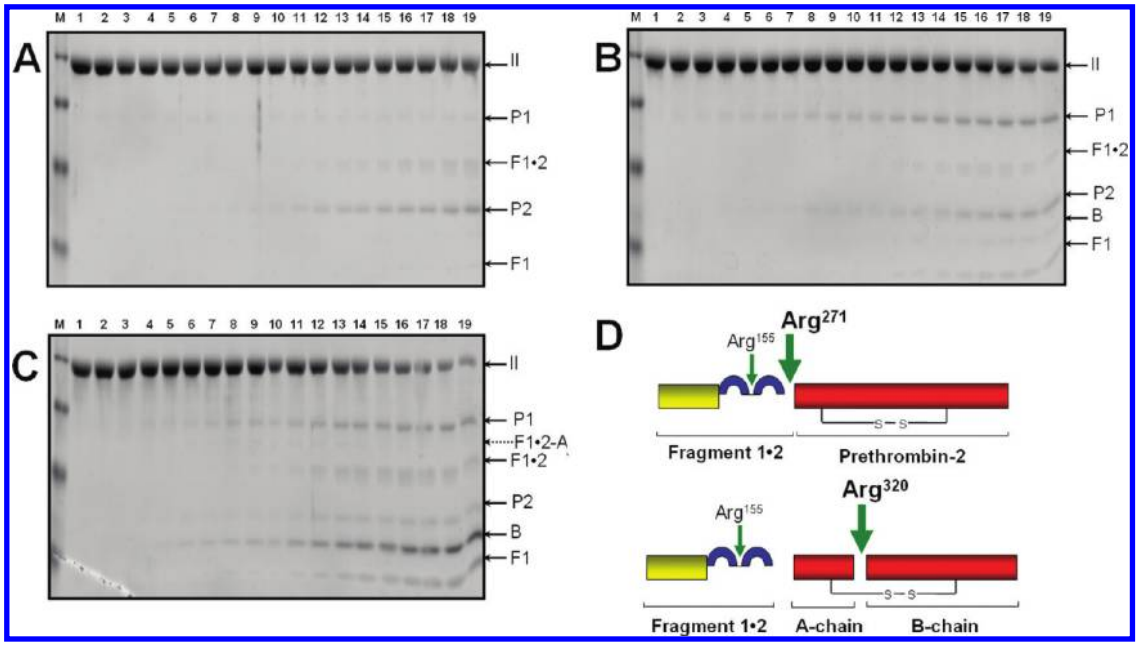

FIGURE 7: Analysis of the activation of plasma-derived prothrombin by prothrombinase assembled with factor Va^{5A} over an extended time period. (A) Plasma-derived prothrombin (1.4 μM) was incubated with factor Xa (10 nM). (B) Plasma-derived prothrombin (1.4 μM) was incubated with PCPS vesicles (20 μM) and factor Xa (0.5 nM). (C) Plasma-derived prothrombin (1.4 μM) was incubated with PCPS vesicles (20 μM), factor Xa (0.5 nM), and factor Va^{5A} (final concentration of 20 nM, 96% factor Xa saturation). All reactions were started by the addition of factor Xa. At selected time intervals, aliquots of the reaction mixtures were withdrawn and treated as described in Experimental Procedures. Lane M contained the molecular weight markers (from top to bottom): 98000, 64000, 50000, and 36000, respectively. Lanes 1−19 contained samples from the reaction mixture before (0 min) the addition of factor Xa and 30 s, 60 s, 3, min, 5 min, 7 min, 10 min, 12 min, 15 min, 20 min, 25 min, 45 min, 60 min, 75 min, 90 min, 105 min, 120 min, 150 min, and 180 min, respectively, after the addition of factor Xa. (D) Schematic representation of fragments derived following prothrombin activation by prothrombinase via two sequential cleavages: initial cleavage at Arg^{271} followed by cleavage at Arg^{320}. The prothrombin-derived fragment identified on the gels in addition to the fragments described in Figure 5F is P2, prethrombin-2 (amino acid residues 272−579).

Arg^{320}, we compared the rate of cleavage of FPR-meizothrombin by prothrombinase assembled with either factor Va^{WT} or the recombinant mutant cofactor molecules (Figure 11). The data demonstrate a delay in cleavage of FPR-meizothrombin at Arg^{271} by prothrombinase assembled with factor $Va^{\Delta 659-663}$ (panel B), factor Va^{5K} (panel C), or factor Va^{5A} (panel D) as compared to the same reaction catalyzed by prothrombinase assembled with factor Va^{WT} (panel A). Quantitative scanning densitometry of fragment 1·2-A present on the gels shown in Figure 11 demonstrated an ~2-fold delay in the cleavage of FPR-meizothrombin at Arg^{271} by prothrombinase assembled with all mutant cofactor molecules, compared to cleavage at Arg^{271} by prothrombinase assembled with factor Va^{WT} (Figure 12 and Table 3). However, the rates for cleavage of FPR-meizothrombin at Arg^{271} are 2-fold faster than the rate of cleavage of FPR-meizothrombin by factor Xa alone. Thus, while mutations in hirudin-like region 659−663 of factor Va have a differential effect on the rate of cleavage at Arg^{320}, deletion or substitutions of amino acid residues within the same region have a similar effect on prothrombinase, namely, considerably impeding the acceleration of the rate of cleavage at Arg^{271} of meizothrombin attributed to the interaction of factor Va with factor Xa (*3*, *40*). These data are in complete agreement with all the findings presented herein and overall demonstrate that the ^{659}DDDED^{663} amino acid sequence from the factor Va heavy chain is part of a group of amino acids that regulate the rate of thrombin formation during activation of prothrombin by prothrombinase.

DISCUSSION

The data presented here using recombinant proteins demonstrate for the first time that the acidic region composed of amino acids 659−663 located at the COOH-terminus of factor Va heavy chain is important for coordinated activation of prothrombin by prothrombinase, resulting in timely thrombin formation at the place of vascular injury. To the best of our knowledge, this is the first time that the role of this specific amino acid region of the cofactor has ever been investigated. Our data demonstrate the order factor $Va^{\Delta 659-663}$ > factor Va^{5K} > factor Va^{5A} with respect to retention of clotting and that prothrombinase assembled with factor $Va^{\Delta 659-663}$ is a better enzyme than prothrombinase assembled with factor Va^{5K}, which in turn is a better enzyme than prothrombinase assembled with factor Va^{5A} for prothrombin activation. These data strongly suggest a differential effect of the mutations within amino acid region 659−663 of factor Va heavy chain on the rate of the two prothrombin activating cleavage sites. It is noteworthy that our data strongly suggest that deletion of a portion of factor Va to test its overall function within the cofactor molecule by functional assays alone, without analyzing the pathway to thrombin formation, does not represent optimal experimental conditions, required to obtain a definite answer. Our findings assign an important physiological role to the acidic hirudin-like region 659−663 from the COOH-terminus of factor Va heavy chain for efficient prothrombin activation. Namely, the ^{659}DDDED^{663} amino acid sequence is part of a group of amino acids that regulate the rate of cleavage of prothrombin by prothrombinase at both Arg^{320} and Arg^{271}. This acidic pentapeptide together with the ^{695}DYDYQ^{699} sequence (*20*, *22*, *23*) are both located on the surface of the factor Va molecule (*41*) and are conserved among species (Figure 2), attesting to their physiological significance. Because both amino acid sequences have no effect on the direct interaction between factor Va and factor Xa, our findings are consistent with the

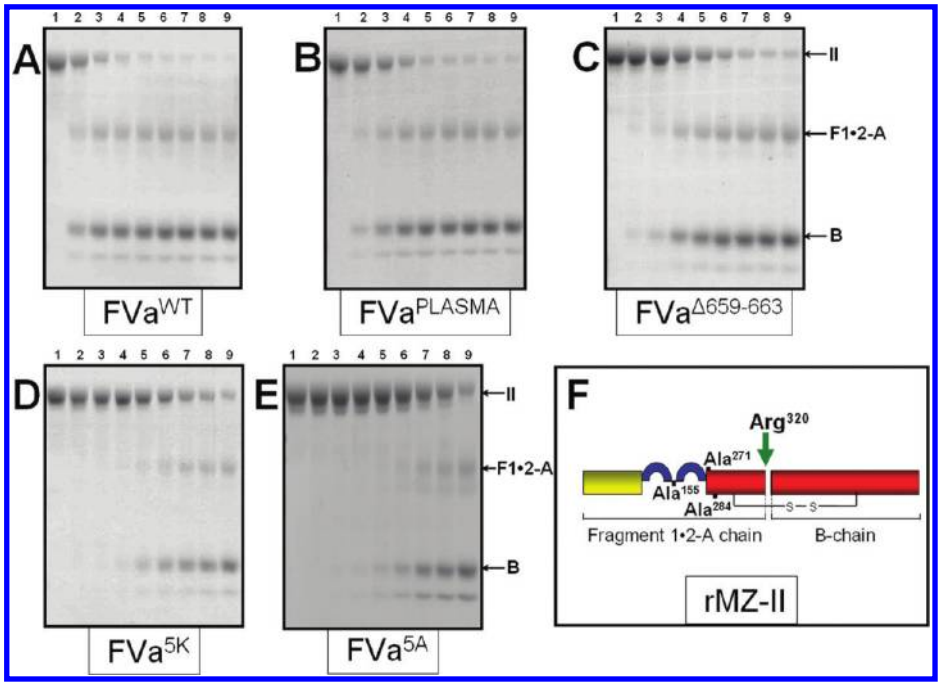

FIGURE 8: Electrophoretic analyses of the activation of rMZ-II by prothrombinase assembled with mutant factor Va molecules. rMZ-II was incubated in different mixtures with PCPS vesicles, DAPA, and various factor Va molecules. The reaction was started by the addition of factor Xa (0.5 nM), and the samples were treated as detailed in Experimental Procedures. The gels were scanned and quantified as detailed in Experimental Procedures. Lanes 1−9 contained samples of the reaction mixture following incubation of prothrombinase with rMZ-II before (lane 1) and after incubation for 1, 3, 5, 10, 20, 45, 60, and 120 min with factor Xa, respectively: (A) prothrombinase assembled with factor Va^{WT} (20 nM), (B) prothrombinase assembled with plasma-derived factor Va (20 nM), (C) prothrombinase assembled with factor $Va^{\Delta 659-663}$ (20 nM), (D) prothrombinase assembled with factor Va^{5K} (20 nM), and (E) prothrombinase assembled with factor Va^{5A} (20 nM). (F) Schematic of the recombinant prothrombin molecule used (rMZ-II). This molecule is a triple mutant with only one cleavage site for prothrombinase remaining at Arg^{320}. Positions of prothrombin-derived fragments are indicated at the right as detailed in the legends of Figures 5 and 7. The time courses were extended for up to 2 h for the titration of each prothrombin molecule, and the data were used to calculate the consumption rate in each experiment (illustrated in Figure 10A and reported in Table 2). The factor Va species used for the reconstitution of prothrombinase are shown under each panel.

interpretation that these acidic amino acid sequences contribute significantly to the macromolecular substrate recognition of prothrombin by prothrombinase and demonstrate that factor Va indeed regulates the activity of factor Xa within the enzymatic complex.

Factor $Va^{\Delta 659-663}$, factor Va^{5K}, and factor Va^{5A} exhibited a significant decrease in clotting activity compared to the activity of factor Va^{WT}. While factor $Va^{\Delta 659-663}$ has 3.5-fold less clotting activity than the wild-type cofactor molecule, factor Va^{5A} has 24-fold less clotting activity than factor Va^{WT}. Gel electrophoresis analyses demonstrated that the recombinant molecules are impaired in their clotting activity because of the inability of prothrombinase assembled with the recombinant cofactor molecules to generate thrombin efficiently. Data obtained with plasma-derived prothrombin showed a delay in prothrombin consumption when prothrombinase was assembled with either factor $Va^{\Delta 659-663}$, factor Va^{5K}, or factor Va^{5A}, compared to plasma-derived prothrombin activation by prothrombinase assembled with factor Va^{WT}. Similarly, experiments using recombinant mutant prothrombin molecules demonstrate that while cleavage at Arg^{320} and rMZ-II consumption are delayed significantly when prothrombinase is assembled with factor $Va^{\Delta 659-663}$, factor Va^{5K}, and factor Va^{5A}, cleavage at Arg^{271} and rP2-II consumption are significantly inhibited compared to cleavage of rMZ-II and rP2 by prothrombinase assembled with factor Va^{WT}. In contrast, cleavage at Arg^{271} of FPR-meizothrombin is comparable with prothrombinase assembled with all recombinant mutant molecules and approximately 2-fold slower than cleavage of the same molecule by prothrombinase assembled with factor Va^{WT}. It is important to note that the maximum increase in the rate of cleavage of meizothrombin at Arg^{271} by prothrombinase assembled with factor Va^{PLASMA} or factor Va^{WT} as compared to cleavage of the molecule by factor Xa alone is 4-fold (*3*, *40*). This fact is clearly illustrated by our findings shown in Table 3 where the rate of FPR-meizothrombin consumption by factor Xa alone is approximately 4-fold slower that the rate of FPR-meizothrombin consumption by prothrombinase assembled with factor Va^{WT}. Thus, a 2-fold decrease in the rate of FPR-meizothrombin consumption by prothrombinase assembled with the mutant molecules represents a significant decrease in the activity of prothrombinase (i.e., 50% of the maximum effect observed).

Our findings demonstrate that acidic amino acid region 659−663 from factor Va is not involved in the interaction of the cofactor with factor Xa. In addition, we show that (in prothrombinase) factor Va^{5A}-mediated activation of prothrombin is associated with little meizothrombin accumulation (Figure 5E). However, efficient initial cleavage at Arg^{320} by prothrombinase assembled with factor Va^{5A} can be easily detected (Figures 8 and 10A). These data suggest that cleavage of meizothrombin at Arg^{271} may be faster than the initial cleavage of prothrombin at Arg^{320}. Accumulation of prethrombin-2 (observed in Figures 5E and 7C) indicates that initial cleavage at Arg^{271} can occur but does not indicate that it must occur first. In fact, the rate of cleavage of prothrombin at Arg^{320} by prothrombinase assembled with factor Va^{5A} is substantial (Figure 8E) and comparable to the rate of prothrombin cleavage by prothrombinase assembled with factor Va^{5K} (Figure 8D), using rMZ-II as the substrate. On the other

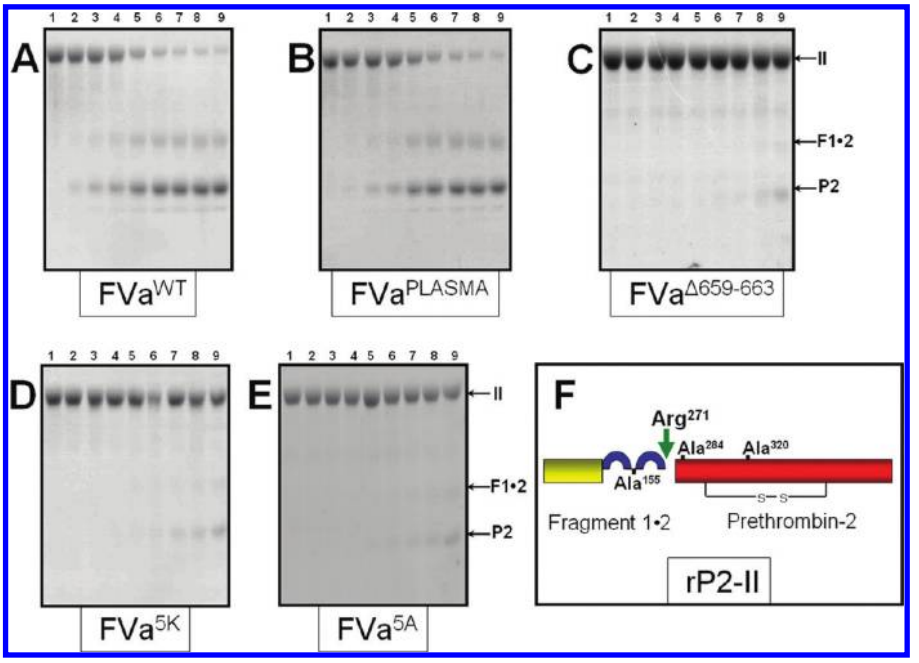

FIGURE 9: Electrophoretic analyses of the activation of rP2-II by prothrombinase assembled with mutant factor Va molecules. rP2-II was incubated in different mixtures with PCPS vesicles, DAPA, and various factor Va molecules. The reaction was started by the addition of factor Xa (0.5 nM), and the samples were treated as detailed in Experimental Procedures. The gels were scanned and quantified as detailed in Experimental Procedures. Lanes 1−9 contained samples of the reaction mixture following incubation of prothrombinase with rP2-II before (lane 1) and after incubation for 1, 3, 5, 10, 20, 45, 60, and 120 min with factor Xa, respectively: (A) prothrombinase assembled with factor Va^{WT} (20 nM), (B) prothrombinase assembled with plasma-derived factor Va (20 nM), (C) prothrombinase assembled with factor $Va^{\Delta 659-663}$ (20 nM), (D) prothrombinase assembled with factor Va^{5K} (20 nM), and (E) prothrombinase assembled with factor Va^{5A} (20 nM). (F) Schematic of the recombinant prothrombin molecule used (rP2-II). This molecule is a triple mutant with only one cleavage site for prothrombinase remaining at Arg^{271}. Positions of prothrombin-derived fragments are indicated at the right as detailed in the legends of Figures 5 and 7. The time courses were extended for up to 2 h for each prothrombin molecule, and the data were used to calculate the consumption rate in each experiment (illustrated in Figure 10B and reported in Table 3). The factor Va species used for the reconstitution of prothrombinase are shown under each panel.

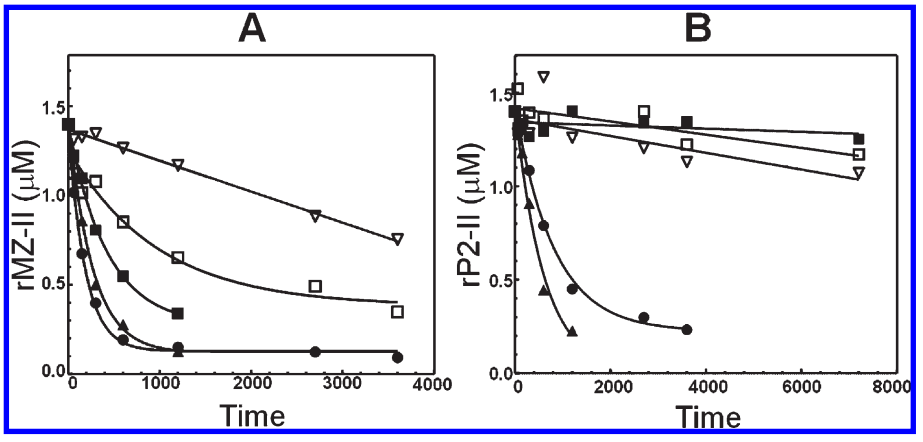

FIGURE 10: Analysis of recombinant prothrombin consumption by prothrombinase assembled with recombinant factor Va molecules. The gels shown in Figures 8 and 9 were scanned, and prothrombin consumption was recorded as described in Experimental Procedures. Following scanning densitometry, the data representing recombinant mutant prothrombin consumption as a function of time (seconds) were plotted using nonlinear regression analysis according to the equation representing a first-order exponential decay using Prism (GraphPad) as described in the legend of Figure 6. Prothrombinase was assembled with recombinant factor Va^{WT} (▲), factor Va^{PLASMA} (●), factor $Va^{\Delta 659-663}$ (■), factor Va^{5K} (□), and factor Va^{5A} (▽). The apparent first-order rate constant, k (inverse seconds), was obtained directly from the fitted data. The resulting numbers representing recombinant mutant prothrombin consumption are reported in Tables 2 and 3. Panel A shows the data obtained following cleavage of rMZ-II: factor Va^{WT}, $R^2 = 0.99$; factor Va^{PLASMA}, $R^2 = 0.99$; factor $Va^{\Delta 659-663}$, $R^2 = 0.99$; factor Va^{5K}, $R^2 = 0.99$; and factor Va^{5A}, $R^2 = 0.98$. The time course was conducted over a period of 2 h; however, for the purpose of graphing, only the data points for the first hour are shown. (B) shows the data obtained following cleavage of rP2-II: factor Va^{WT}, $R^2 = 0.99$; and factor Va^{PLASMA}, $R^2 = 0.98$.

hand, all of the mutants seem profoundly impaired to a similar extent with respect to promoting cleavage at Arg^{271} using substrate rP2-II (Figure 9). Therefore, factor Va^{5A} does not seem to be very exceptional compared to the other mutants with respect to preferred cleavage sites. The data obtained with prothrombinase assembled with factor Va^{5A} and the recombinant prothrombin mutants instead support the usual pathway through meizothrombin, with a preference for initial cleavage after Arg^{320}. Consequently, the combined data suggest that failure of meizothrombin to accumulate during activation of prothrombin by prothrombinase assembled with factor Va^{5A} could be explained by the fact that the rate of cleavage at Arg^{271} is much faster acting on

Table 3: Rates of Activation of Recombinant Prothrombin rP2-II and Plasma-Derived FPR-Meizothrombin in the Presence of Prothrombinase Assembled with Various Recombinant Factor Va Species

enzyme	rP2-II[a] (cleavage at Arg^{271}) [mol consumed s^{-1} (mol of factor Xa)$^{-1}$]	FPR-meizothrombin[a] (cleavage at Arg^{271}) [mol of fragment 1·2-A consumed s^{-1} (mol of factor Xa)$^{-1}$]
factor Xa	0.048 ± 0.012[b]	13.5 ± 2.3[c]
prothrombinase with factor Va^{PLASMA}	3.4 ± 0.5	42.6 ± 7[c]
prothrombinase with factor Va^{WT}	3.6 ± 0.5	52.2 ± 14
prothrombinase with factor $Va^{\Delta 659-663}$	NR[d]	23.5 ± 2.1
prothrombinase with factor Va^{5K}	NR[e]	20.1 ± 4.3
prothrombinase with factor Va^{5A}	NR[f]	26.1 ± 2.2

[a]Rates of rP2-II consumption in the presence of wild-type or plasma factor Va and recombinant mutant factor $Va^{\Delta 659-663}$, factor Va^{5K}, and factor Va^{5A} were measured following scanning densitometry of gels shown in Figure 9. Calculation of the apparent first-order rate constant was achieved as detailed in Experimental Procedures. The rate of fragment 1·2-A consumption in the presence of prothrombinase assembled with factor Va^{WT}, factor $Va^{\Delta 659-663}$, factor Va^{5K}, or factor Va^{5A} was measured following scanning densitometry of the gels shown in Figure 11 and calculation of the apparent first-order rate constant as detailed in Experimental Procedures. The rates of rP2-II and fragment 1·2-A consumption were extracted from the fitted data (shown in Figures 10B and 12, respectively). [b]Rate obtained from a time course using 10 nM factor Xa ($R^2 = 0.975$). [c]From ref *20* ($R^2 = 0.97$ for both sets of data). [d]No rate could be obtained. The concentration of rP2-II varied from 1400 nM at time zero to 1250 nM following a 2 h incubation with prothrombinase assembled with factor $Va^{\Delta 659-663}$. [e]No rate could be obtained. The concentration of rP2-II varied from 1400 nM at time zero to 1200 nM following a 2 h incubation with prothrombinase assembled with factor Va^{5K}. [f]No rate could be obtained. The concentration of rP2-II varied from 1400 nM at time zero to 1100 nM following a 2 h incubation with prothrombinase assembled with factor Va^{5A}.

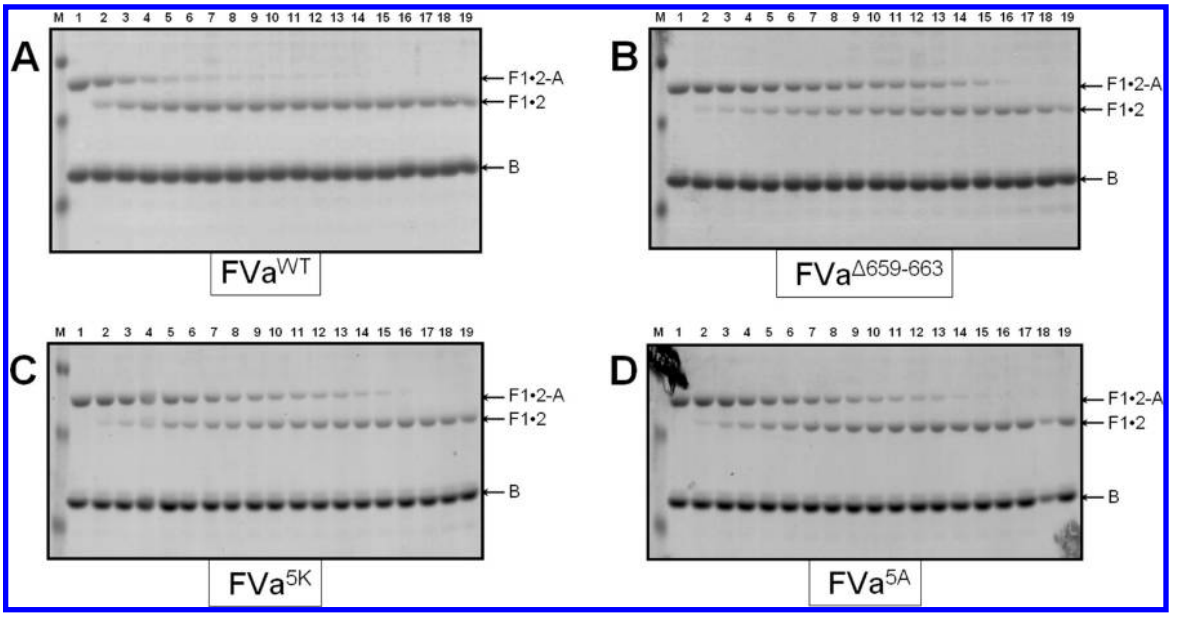

FIGURE 11: Gel electrophoresis analyses for cleavage of FPR-meizothrombin. FPR-meizothrombin (1.4 μM) was incubated in different mixtures with PCPS vesicles (20 μM) and factor Va as described in the legend of Figure 5. The reactions were started by the addition of factor Xa, and the samples were further treated, scanned, and quantified as detailed in Experimental Procedures: (A) factor Va^{WT}, (B) factor $Va^{\Delta 659-663}$, (C) factor Va^{5K}, and (D) factor Va^{5A}. Lane M contained the molecular weight markers (from top to bottom): 50000, 36000, and 22000, respectively. Lanes 1−19 contained samples from the reaction mixture before (0 min) the addition of factor Xa and 20 s, 40 s, 60 s, 80 s, 100 s, 120 s, 140 s, 160 s, 180 s, 200 s, 220 s, 240 s, 5 min, 6 min, 10 min, 20 min, 30 min, and 60 min, respectively, after the addition of factor Xa. The prothrombin-derived fragments are shown as detailed in the legend of Figure 5. The recombinant factor Va species used for the reconstitution of prothrombinase are shown under each panel.

meizothrombin than on prothrombin or rP2-II. Overall, our data strongly suggest that the deficiency of all recombinant mutant factor Va molecules in directing efficient prothrombin consumption by factor Xa within prothrombinase is due to the inability of the mutant cofactors to provide a productive interaction of the enzyme with prothrombin as previously demonstrated (*7*, *10*, *18*, *42*, *43*) because of the lack of the acidic segment composed of amino acids 659−663.

Initial cleavage of prothrombin at Arg^{320} by prothrombinase and generation of meizothrombin is absolutely dependent on the incorporation of factor Va into the enzymatic complex and its interaction with factor Xa and prothrombin on a membrane surface. It is thus assumed that once factor Va binds to factor Xa on the procoagulant membrane surface, the complex will interact with an exosite on prothrombin that in turn will facilitate cleavage at Arg^{320} exclusively (*44*, *45*). Therefore, from the published data, it has been inferred for some time that the productive docking of prothrombinase on prothrombin, under conditions where factor Va is in large excess over factor Xa [conditions mimicking the propagation phase of coagulation (*46*)], will result in fast and efficient cleavage at Arg^{320}, with thrombin formation through the meizothrombin pathway. The data shown in Figures 5−8 demonstrate that prothrombinase assembled with factor $Va^{\Delta 659-663}$, factor Va^{5K}, and factor Va^{5A} (under conditions that would promote activation of prothrombin exclusively through the meizothrombin pathway) are impaired in their

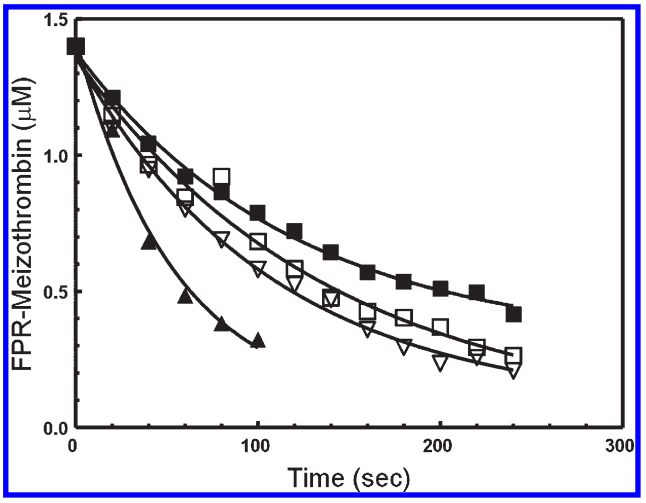

FIGURE 12: Analysis of FPR-meizothrombin consumption by prothrombinase assembled with recombinant factor Va molecules. The gels shown in Figure 11 were scanned, and FPR-meizothrombin consumption was recorded as described in Experimental Procedures. Following scanning densitometry, the data representing fragment 1·2-A consumption as a function of time (seconds) were plotted using nonlinear regression analysis according to the equation representing a first-order exponential decay using Prism (GraphPad) as described in the legend of Figure 6. The apparent first-order rate constant, k (inverse seconds), was obtained directly from the fitted data. Prothrombinase was assembled with recombinant factor Va^{WT} [(▲) R^2 = 0.99], factor $Va^{\Delta 659-663}$ [(■) R^2 = 0.99], factor Va^{5K} [(□) R^2 = 0.98], and factor Va^{5A} [(▽) R^2 = 0.99]. The resulting numbers representing FPR-meizothrombin consumption are reported in Table 3.

ability to support the initial cleavage of prothrombin at Arg^{320}. The data shown in Figures 9−12 also demonstrate that prothrombinase assembled with the mutant recombinant factor Va molecules are also impaired in their ability to cleave at Arg^{271} of either rP2-II or FPR-meizothrombin. These results placed in the context of the literature demonstrate that upon its incorporation into prothrombinase factor Va, rather than providing an exosite for prothrombin necessary for exclusive cleavage at Arg^{320}, is required to actively guide factor Xa though the activation process. We must thus conclude that depending on the quality and concentration of factor Va during the initiation of clot formation, once prothrombin has engaged its exosite(s) on prothrombinase, the enzyme will cleave the substrate at either Arg^{320} or Arg^{271} with optimal rates (*47*). As a consequence, it appears that it is not the extended exosite for prothrombin on prothrombinase per se that promotes acceleration of the rate of prothrombin activation by specifically directing the initial cleavage at Arg^{320} and meizothrombin formation, but it is rather the incorporation of factor Va into the complex that produces a rearrangement of the components of the enzymatic complex, resulting in an increase in the rate of cleavage at both Arg^{320} and Arg^{271} by factor Xa.

The effects observed in our study with the mutant cofactor molecules are significant and comparable when using clotting assays and when studying prothrombin activation. It has been well established that cleavage of prothrombin at Arg^{271} has an important effect on the progressive formation of the active site of thrombin (*8*, *48*), and that meizothrombin has a higher amidolytic activity than thrombin toward several chromogenic substrates usually employed to assess thrombin esterase activity (*25*, *49*−*51*). In addition, it has been demonstrated following analysis of the crystal structure of meizothrombin, that ABE-II of meizothrombin has not yet been exposed, because it is covered by fragment 2 (*9*). This fact alone explains the poor clotting activity of meizothrombin because ABE-II is part of the binding site of thrombin for fibrinogen and is required for optimal rates of fibrin formation during blood clotting. ABE-II is exposed following cleavage of meizothrombin at Arg^{271} and release of fragment 2. We show that the catalytic activity of prothrombinase when assembled with the mutant cofactor molecules is only moderately affected by the mutations, when the activity of factor Va is measured in an assay using a chromogenic substrate to assess thrombin activity. Indeed, the k_{cat} and second-order rate constants of prothrombinase assembled with the mutant factor Va molecules are approximately 20−60% of that of prothrombinase assembled with factor Va^{PLASMA} or factor Va^{WT}. In contrast, the clotting activity of the mutant cofactor molecules is severely impaired (11- and 24-fold decreased clotting activity with factor Va^{5K} and factor V^{5A}, respectively), and gel electrophoresis experiments combined with densitometric analyses reveal a similar and significant delay in prothrombin activation (10- and 38-fold decreased rates of prothrombin consumption with prothrombinase assembled with factor Va^{5K} and factor V^{5A}, respectively), with meizothrombin being more stable throughout the activation process when prothrombinase is assembled with factor Va^{5K} as compared to prothrombin activation by prothrombinase assembled with either factor Va^{WT} or factor Va^{PLASMA}. The findings presented here are entirely consistent with earlier findings obtained with plasma-derived proteins (*52*−*54*), recent findings obtained with recombinant proteins (*42*), and our data (*20*, *21*). Explicitly, when prothrombinase is assembled with a factor Va molecule possessing a heavy chain that is truncated at the acidic hirudin-like COOH-terminal region, a discrepancy is observed between the activity of factor Va measured by the clotting assay and the activity of the cofactor measured in an assay using a chromogenic substrate to assess thrombin activity. This discrepancy is not due to the experimental conditions used since when using purified reagents and a chromogenic substrate to measure thrombin activity, our experimental conditions were chosen carefully to mimic similar conditions obtained physiologically during clotting [7 pmol of prothrombinase formed at clotting time (*55*) compared to 10 pmol of prothrombinase used in the experiments used to calculate the kinetic constants (Figure 4 and Table 1)]. The data provided here and in our recent manuscript detailing the properties of factor $V^{\Delta 680-709}$ (*20*) explain these paradoxical findings. Because we have studied plasma-derived prothrombin, rMZ-II, rP2-II, and FPR-meizothrombin activation by gel electrophoresis, we can conclude that meizothrombin lingers throughout the activation of prothrombin by prothrombinase assembled with factor $Va^{\Delta 659-663}$ or factor Va^{5K}. The excess meizothrombin present in the assays, while having poor clotting activity, can compensate for the lack of thrombin activity because of its increased amidolytic activity toward chromogenic substrates (*49*, *50*) that are usually employed to assess thrombin activity, thus creating the false impression that the mutations have a minimal effect on prothrombinase activity. As a consequence, and because factor Va is devoid of enzymatic activity, when recombinant mutant factor Va molecules are being studied, drawing conclusions from activity assays only, without visualizing the pathway to thrombin generation, would lead to incorrect interpretations with regard to the functional contribution of a given amino acid sequence from factor Va on the rates of the specific prothrombin activating cleavages. We therefore conclude that following the activity of various recombinant mutant factor Va molecules by clotting

assays, gel electrophoresis analyses, and assays using chromogenic substrates is by no means redundant and prevents oversimplification that in turn might lead to flawed conclusions.

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