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<sup>SK</sup>) or alanine (factor V<sup>SA</sup>). We have also constructed a mutant molecule with this region deleted (factor V<sup>Δ659–666</sup>). The recombinant molecules along with wild-type factor V (factor V<sup>WT</sup>) 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<sup>WT</sup>. Gel electrophoresis analyses of plasma-derived and recombinant mutant prothrombin activation demonstrated delayed cleavage of prothrombin at both Arg<sup>320</sup> and Arg<sup>271</sup> 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<sup>2+</sup> (1, 2). Factor Va can activate prothrombin following sequential cleavages at Arg<sup>271</sup> and Arg<sup>320</sup>, 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<sup>320</sup> produces the active intermediate meizothrombin, while the second cleavage at Arg<sup>271</sup> 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<sup>320</sup> 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<sup>271</sup> (8, 9). Factor Va is required for the specific recognition of prothrombinase by (pro)exosite I of prothrombin (10, 11). Proteolytic elimination of fragment I of prothrombin eliminates the accelerating effect of the membrane surface for initial cleavage at Arg<sup>320</sup> by prothrombinase (12, 13).

Coagulation factor V circulates in plasma at a concentration of 20 nM, as a single-chain inactive precursor (M<sub>r</sub> = 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<sup>709</sup>, Arg<sup>1018</sup>, and Arg<sup>1545</sup> to generate the active cofactor (factor Va) composed of a heavy chain [M<sub>r</sub> = 105000 (A1-A2 domains)] and a light chain [M<sub>r</sub> = 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...
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$^\text{271}$ 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 $R^\text{-thrombin}$ 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.

**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 $R^\text{-thrombin}$ at Arg$^\text{709}$, Arg$^\text{1018}$, and Arg$^\text{1545}$. 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).

**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; Otolurus garnetii, small-eared galago; Bos taurus, cattle; Sus scrofa, pig; Canis lupus familiaris, dog; Myotis lucifugus, little brown bat; Equus caballus, horse; Dassypus 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.
EXPERIMENTAL PROCEDURES

Materials and Reagents. Disopropyl 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 Biotechnol- 

Associate Inc. (Birmingham, AL). l-α-Phosphatidylserine (PS) and l-α-phosphatidylcholine (PC) were obtained from Avanti Polar Lipids (Alabaster, AL). Chemiluminescent reagent ECL and Heparin-Sepharose were obtained from Amersharn- 

Pharmacia Biotech Inc. (Piscataway, NJ). Normal reference 

plasma and the chromogenic substrate H-o-hexahydroxyrosyl- 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 Haematologics Technologies Inc. (Essex Junction, VT). Human factor Xa was purchased from Enzyme Research Labo-

ratories (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 molec-

ular biology and tissue culture reagents, specific primers, and medium were purchased from Gibco, Invitrogen Corp. (Grand Island, NY) or as indicated. Recombinant wild-type pro-

thrombin, prothrombin rMZ-II that has only one cleavage site for factor Xa (i.e., Arg271), and prothrombin rP2-II that has only one cleavage site for factor Xa (i.e., Arg271) 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 chromatogra-

phy (FPLC) as initially described (26). Human factor V monoclonal antibodies (αHFV1c17 and αHFV1c9) 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 SalI 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 manufac-

turer’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 V5K were 5'TGAGGGCTAAATCCGGATGTTAATATCTCCTACCTATGAGGATTTTTGACCTCCAGAAC-3 (sense) and 5'GGATCCTGAGGTT-TCAAAAAATCTCATATGCTTTTCCTCTTTGGGATTCACTATTACCCCTGAACTTCGCCTCA-3 (antisense). Primers for factor V659-663 were 5'AGGGCTGAAATCCGGATGTTAATATCTCCTACCTATGAGGATTTTTGACCTCCAGAAC-3 (sense) and 5'GGATCCTGAGGTT-TCAAAAAATCTCATATGCTTTTCCTCTTTGGGATTCACTATTACCCCTGAACTTCGCCTCA-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 30 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–polyacryl-

amide gel electrophoresis (SDS–PAGE) followed by Western blotting using both monoclonal and polyclonal antibodies. In some instances, factor Va fragments were also visualized follow-

ing 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 mono-

clonal and polyclonal antibodies (31, 32). Immunoreactive fragments were visualized with chemiluminescense. In several instances, recombinant factor V and factor Va fragments obtained follow-

ing activation of the procofactor with thrombin were visualized following staining of the gels with silver as described previ-

ously (33).

Analysis of Prothrombin or Recombinant Mutant Pro-

thrombin Activation and FPR-Meizothrombin Cleavage at Arg271 by Gel Electrophoresis. Prothrombin or recombinant
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 Ca²⁺ 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-methiothrebin cleavage at Arg²⁷¹ 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 Brilliant Blue R and defined by destaining in a methanol/acetic acid/water solution.

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, 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 VaWT 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 (R²).

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 VaD659–663, factor VaK, and factor VaA (Figure 1). Full-length recombinant factor VWT 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 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 Va 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 VaWT 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 (R²).
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 Arg643 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).

The recombinant molecules were first assessed for their clotting activity in a two-stage clotting assay. Thrombin activation of factor VWT resulted in a cofactor with a clotting activity similar to that of the plasma-derived molecule (Table 1). Under similar experimental conditions, factor Va9659–663, factor VaSK, and factor Va5A displayed ∼3.5-, 11-, and 24-fold less clotting activity, respectively, than factor VWT (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 VWT 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 VWT for factor Xa. These results are in 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

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### Table 1: Functional Properties of Various Recombinant Factor Va Molecules

| factor Va species | clotting activity (units/mg) | $x$-fold decrease$^b$ | $K_{D,app}$ (nM)$^c$ | $k_{cat}$ (μM)$^d$ | $k_{cat}$ (min$^{-1}$)$^e$ | $k_{cat}/K_{m}$ ($\times 10^9$ M$^{-1}$ s$^{-1}$) | $x$-fold decrease$^f$ |
|------------------|-----------------------------|-----------------------|----------------------|----------------------|----------------------|----------------------------------|-----------------------|
| factor VaWTPLASMA | 3124 ± 413                  | –                     | 0.67 ± 0.15          | 0.26 ± 0.04          | 2162 ± 55            | 1.40                             | –                     |
| factor VaWT      | 2926 ± 320                  | –                     | 0.81 ± 0.10          | 0.23 ± 0.01          | 2317 ± 67            | 1.70                             | –                     |
| factor Va9659–663 | 825 ± 180                   | 3.5                   | 0.99 ± 0.22          | 0.23 ± 0.03          | 1493 ± 58            | 1.08                             | 1.6                   |
| factor VaSK      | 275 ± 92                    | 11                    | 0.85 ± 0.16          | 0.28 ± 0.03          | 1102 ± 25            | 0.65                             | 2.6                   |
| factor Va5A      | 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 VaWT 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 ($K_{D,app}$) 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. $^d$Kinetic constants were derived directly from the fitted data. $^e$The $k_{cat}$ and $K_{m}$ 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. $^f$The $k_{cat}$ and $K_{m}$ of prothrombinase assembled with saturating concentrations of recombinant factor Va molecules were determined as described in Experimental Procedures.
When compared to the catalytic efficiency of factor Va WT, while factor Va 5K or factor Va 5A has approxi- mately 80% or 80% reduced catalytic efficiency, respectively, when compared to the catalytic efficiency of factor Va WT, while factor Va Δ659–663 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 (kcat/Km) 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 Δ659–663, 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 Δ659–663, 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 Δ659–663 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 Δ659–663 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 prothrombin-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 (Arg271 and Arg320) appear to be affected when prothrombinase is assembled with either factor Va Δ659–663, factor Va 5K, or factor Va 5A. Overall, the data demonstrate that amino acid region 659–663 actively participates in the activation
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 \( \text{rMZ-II} \) or Arg 320 \( \text{rP2-II} \) (Figure 8). Scanning densitometry of the gels shown in Figure 8 demonstrates that cleavage at Arg 271 of rMZ-II is delayed by \( \sim 2 \)-fold when prothrombinase is assembled with either prothrombinase assembled with factor VaWT, factor VaSK, or factor VaAS compared to the rates of rMZ-II cleavage and activation by prothrombinase made with factor VaWT or factor VaPLASMA (Figure 8).
prothrombinase assembled with factor VaWT (Table 2 and Figure 10A). In contrast, very slow prothrombin consumption was observed when rMZ-II was incubated with prothrombinase assembled with factor Va659-663 (Figure 8E). The rate of cleavage of rMZ-II by prothrombinase assembled with factor VaSA is 33-fold slower than the rate of cleavage of rMZ-II by prothrombinase assembled with factor VaWT (Table 2). These data confirm our findings obtained with plasma-derived prothrombin. In particular, the data obtained with factor VaSA and rMZ-II verify the data shown in Figures 5E and 7C obtained with plasma-derived prothrombin and factor VaSA, demonstrating slow cleavage of prothrombin at Arg203 by prothrombinase assembled with factor VaSA. Overall, the data demonstrate that prothrombinase-mediated cleavage at Arg203 in prothrombin is affected by modifications in amino acid region 659–663 of factor Va heavy chain.

The data shown in Figures 9 and 10B and Table 3 demonstrate that the rate of cleavage of rP2-II at Arg271 by prothrombinase assembled with either factor VaΔ659–663, factor VaΔSK, or factor VaSA is very slow compared to the rate of cleavage of rP2-II at Arg203 by prothrombinase assembled with either factor VaWT or factor VaPLASMA. 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 VaΔ659–663, factor VaΔSK, and factor VaSA is impaired compared to cleavage of the recombinant prothrombin molecules by prothrombinase assembled with factor VaWT because of delayed cleavages at both Arg203 and Arg271 resulting in both slow prothrombin consumption and less conversion of meizothrombin to thrombin. In addition, our data show that the rate of cleavage at Arg203 of rP2-II appears to be affected more than the rate of cleavage at Arg271 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 Arg203 (39). To ascertain the effect of the mutations of the factor Va heavy chain on the cleavage of prothrombin at Arg271 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 | rMZ-II |
|--------|-----------------------------|--------|
|        | (initial cleavage at Arg203) | (cleavage at Arg203) |
|        | [mol consumed s⁻¹ (mol of factor Xa)⁻¹] | [mol consumed s⁻¹ (mol of factor Xa)⁻¹] |
| factor Xa | 0.065 ± 0.025 | 0.0235 ± 0.0176 |
| prothrombinase with factor VaPLASMA | 19.0 ± 1.5 | 14.8 ± 0.8 |
| prothrombinase with factor VaWT | 21.8 ± 1.9 | 11.2 ± 0.8 |
| prothrombinase with factor VaΔ659–663 | 7.7 ± 0.5 | 6.6 ± 0.7 |
| prothrombinase with factor VaΔSK | 2.1 ± 0.4 | 2.9 ± 0.8 |
| prothrombinase with factor VaSA | 0.58 ± 0.1² ± 0.18 ± 0.06 | 0.34 ± 0.1 |

²The 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. ³Rate obtained from a time course using 10 nM factor Xa (R² = 0.922). ⁴From the 1 h time course with 20 nM factor VaΔSK and 1 nM factor Xa [R² = 0.985 (Figure 5)]. ⁵From the 3 h time course 20 nM factor VaΔSK and 0.5 nM factor Xa [R² = 0.987 (Figure 7)].
These data are in complete agreement with all the findings presented herein and overall demonstrate that the 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$^{659-663}$ > factor Va$^{5K}$ > factor Va$^{5A}$ with respect to retention of clotting and that prothrombinase assembled with factor Va$^{5K}$, which in turn is a better enzyme than prothrombinase assembled with factor Va$^{5A}$, followed by cleavage at Arg$^{271}$. 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$^{659-663}$ (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.
and factor Va 5A, cleavage at Arg 271 and rP2-II consumption are delayed significantly when prothrombinase was assembled with either factor ValWT. Similarly, experiments using recombinant derived prothrombin showed a delay in prothrombin consumption by prothrombinase assembled ΔVa. Data obtained with plasma-thrombinase assembled with factor VaWT and recombinant molecules are substantially delayed (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 Va5A-mediated activation of prothrombin is associated with little meizothrombin accumulation (Figure 5E). However, efficient initial cleavage at Arg271 by prothrombinase assembled with factor Va5A can be easily detected (Figures 8 and 10A). These data suggest that cleavage of meizothrombin at Arg271 may be faster than the initial cleavage of prothrombin at Arg320. Accumulation of prethrombin-2 (observed in Figures 5E and 7C) indicates that initial cleavage at Arg271 can occur but does not indicate that it must occur first. In fact, the rate of cleavage of prothrombin at Arg320 by prothrombinase assembled with factor Va5A is substantial (Figure 8E) and comparable to the rate of prothrombin cleavage by prothrombinase assembled with factor Va5K (Figure 8D), using rMZ-II as the substrate. On the other
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 WT 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 Va5A could be explained by the fact that the rate of cleavage at Arg 271 is much faster acting on
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 Arg320 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 Arg320, with thrombin formation through the meizothrombin pathway. The data shown in Figures 5–8 demonstrate that prothrombinase assembled with factor Va659–663, factor VaΔ659–663, factor VaΔ659–663, and factor Va5A (under conditions that would promote activation of prothrombin exclusively through the meizothrombin pathway) are impaired in their

### 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 ( cleavage at Arg320 ) [mol consumed s⁻¹ (mol of factor Xa)⁻¹] | FPR-meizothrombin ( cleavage at Arg320 ) [mol of fragment 1-2-A consumed s⁻¹ (mol of factor Xa)⁻¹] |
|--------|---------------------------------------------------------------|--------------------------------------------------------------------------------|
| factor Xa | 0.048 ± 0.012(ab) | 13.5 ± 2.3(c) |
| prothrombinase with factor VaPLASMA | 3.4 ± 0.5 | 42.6 ± 7(d) |
| prothrombinase with factor VaWT | 3.6 ± 0.5 | 52.2 ± 1.4 |
| prothrombinase with factor VaΔ659–663 | NR | 23.5 ± 2.1 |
| prothrombinase with factor Va5K | NR | 20.1 ± 4.3 |
| prothrombinase with factor Va3A | 26.1 ± 2.2 |

*Rates of rP2-II consumption in the presence of wild-type or plasma factor Va and recombinant mutant factor VaΔ659–663, factor Va5K, and factor Va3A 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 Va5K, factor VaΔ659–663, factor Va3A, or factor VaΔ5K 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). *Rate obtained from a time course using 10 nM factor Xa (R² = 0.975). **From ref 20 (R² = 0.97 for both sets of data). ***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 VaΔ659–663. **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 Va5K. **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 Va3A.**

### 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 VaWT, (B) factor VaΔ659–663, (C) factor Va5K, and (D) factor Va3A. 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, 10 min, 15 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.
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 Arg271 of prothrombin. This result was achieved directly from the fitted data. Prothrombinase was assembled with recombinant factor VaWT (□, $R^2 = 0.99$), factor VaΔΔ59–663 (■, $R^2 = 0.99$), factor Va5K (○, $R^2 = 0.98$), and factor VaΔ4A (▲, $R^2 = 0.99$). The resulting numbers representing FPR-meizothrombin consumption are reported in Table 3.

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 Arg271 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 Arg271, is required to actively guide factor Xa through 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 Arg271 or Arg320 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 Arg320 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 Arg320 and Arg271 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 Arg271 has an important effect on the progressiveness 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 Arg271 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 $k_{\text{cat}}$ 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 VaWT or factor VaΔPLASMA. In contrast, the clotting activity of the mutant cofactor molecules is severely impaired (11- and 24-fold decreased clotting activity with factor Va5K and factor Va5A, 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 Va5K and factor Va5A, respectively), with meizothrombin being more stable throughout the activation process when prothrombinase is assembled with factor Va5K as compared to prothrombin activation by prothrombinase assembled with either factor VaWT or factor VaΔPLASMA. 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 VaΔ680–709 (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 VaΔ659–663 or factor Va5K. 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.

**ACKNOWLEDGMENT**

We thank Dr. Ken Mann and Dr. Tom Orfeo from the Department of Biochemistry at the University of Vermont for providing antibodies to factor V, Dr. Michael Nesheim and Dr. Paul Kim from the Department of Biochemistry at Queen’s University for providing cells expressing rMZ-II and rP2-II, and Dr. Kathleen Berker from the Department of Molecular Cardiology at The Cleveland Clinic for helpful advice. We thank Dr. Susan Kennedy-Kalafatis and Dr. Ed Plow for helpful advice and for critical reading of the manuscript.

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