Trp^{2063} and Trp^{2064} in the Factor Va C2 Domain Are Required for High-Affinity Binding to Phospholipid Membranes but Not for Assembly of the Prothrombinase Complex†

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ABSTRACT: Interactions between factor Va and membrane phosphatidylserine (PS) regulate activity of the prothrombinase complex. Two solvent-exposed hydrophobic residues located in the C2 domain, Trp^{2063} and Trp^{2064}, have been proposed to contribute to factor Va membrane interactions by insertion into the hydrophobic membrane bilayer. However, the prothrombinase activity of rHFVa W(2063, 2064)A was found to be significantly impaired only at low concentrations of PS (5 mol %). In this study, we find that 10-fold higher concentrations of mutant factor Va are required for half-maximal prothrombinase activity on membranes containing 25% PS. The ability of the mutant factor Va to interact with factor Xa on a membrane was also impaired since 4-fold higher concentrations of factor Xa were required for half-maximal prothrombinase activity. The interaction of factor Va with 25% PS membranes was also characterized using fluorescence energy transfer and surface plasmon resonance. We found that the affinity of mutant factor Va for membranes containing 25% PS was reduced at least 400-fold with a $K_d > 10^{-7}$ M. The binding of mutant factor Va to 25% PS membranes was markedly enhanced in the presence of factor Xa, indicating stabilization of the factor Va−factor Xa−membrane complex. Our findings indicate that Trp^{2063} and Trp^{2064} play a critical role in the high-affinity binding of factor Va to PS membranes. It remains to be determined whether occupancy of this PS binding site in factor Va is also required for high-affinity binding to factor Xa.

Coagulation factor Va binds to negatively charged phospholipid membranes and serves as a protein cofactor for the activation of prothrombin by the prothrombinase complex. The prothrombinase complex consists of the enzyme factor Xa, the protein cofactor factor Va, calcium ions, and a phospholipid membrane surface (*1*, *2*). Formation of the prothrombinase complex requires the presence of a procoagulant membrane surface. Activated platelets, platelet microparticles, and damaged vascular cells provide this surface in vivo (*3*). Factor Va binds to phospholipid vesicles containing 25% phosphatidylserine (PS)[1] and 75% phosphatidylcholine with a K_d of approximately 10^{-9} M (*3*−*6*). The binding of factor Va to phospholipid membranes does not require calcium ions (*4*, *7*−*9*), and appears to involve both hydrophobic (*10*, *11*) and electrostatic interactions (*8*, *12*, *13*). Formation of the prothrombinase complex proceeds via the initial formation of separate factor Va−membrane and factor Xa−membrane complexes (*14*). This is followed by rapid association of factor Xa and factor Va on the membrane surface to form the prothrombinase complex (*14*−*16*).

Factor V is synthesized as a single chain pro-cofactor with a domain structure A1-A2-B-A3-C1-C2. Activation of factor V by thrombin results in an active cofactor consisting of an amino-terminal heavy chain (A1-A2) and a carboxyl terminal light chain (A3-C1-C2). The light chain mediates the binding of factor Va to phospholipid membranes (*7*−*9*). Membrane binding sites have been localized within the A3 (*4*, *17*, *18*), C1 (*19*), and C2 (*20*−*23*) domains of factor Va. The C2 domain of factor V (residues 2037−2196) is required for the binding of factor V to PS (*20*−*23*). Deletion of the C2 domain prevents the binding of factor V to PS (*20*). Monoclonal antibodies and factor V inhibitors that bind to the amino terminus of the C2 domain block factor V binding to PS and inhibit procoagulant activity (*22*, *23*). The isolated factor V C2 domain binds to membranes containing PS as well as to soluble phosphatidylserine (C6PS) (*21*).

Structural studies of the factor V C2 domain (*24*, *25*) and recent alanine scanning mutagenesis studies (*26*, *27*) provide

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[1] Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; D-PE (dansyl-phosphatidyl-ethanolamine), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol-amine-*N*-(5-(dimethylamino)-1-naphthalenesulfonyl); PC, hen egg L-α-phosphatidylcholine; PS, brain L-α-phosphatidylserine; rHFVa, recombinant factor Va; factor Va_1, the form of factor Va with a 74-kDa light chain due to glycosylation at Asn^{2181}; factor Va_2, the form of factor Va with a 71-kDa light chain due to the absence of glycosylation at Asn^{2181}; ELISA, enzyme-linked immunosorbent assay; FRET, fluorescence resonance energy transfer; SPR, surface plasmon resonance; RU, resonance unit.

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further evidence that the C2 domain interacts with the membrane. The three-dimensional structure of the C2 domain was solved in monomeric and dimeric crystal forms (*25*). The factor V C2 domain exhibits a distorted jelly roll β-barrel motif, consisting of eight major antiparallel strands arranged in two β-sheets of five and three strands packed against one another. Three adjacent loops (Ser^{2058}−Trp^{2068}, Asn^{2076}−Asn^{2082}, and Gly^{2112}−Tyr^{2121}) protrude like spikes from the bottom of the β-barrel. At the apex of spike-1, the indole moieties of two consecutive tryptophan residues (Trp^{2063}/Trp^{2064}) are fully exposed to solvent. We have demonstrated that monoclonal antibody HV-1 and the inhibitory antibody H1, which map to spike-1, interfere with binding to immobilized PS and abolish procoagulant activity (*26*, *28*). Substitution of alanine residues for Trp^{2063}/Trp^{2064} in spike-1 also blocks binding of factor V to immobilized PS in an ELISA binding assay (*26*). Although the ability of this mutant to promote thrombin generation was markedly impaired on phospholipid membranes containing <10 mol % PS, thrombin generation was not significantly reduced on membranes containing >20 mol % PS (*26*, *27*).

The apparent discrepancy between the normal functional activity of this mutant on 25% PS membranes and its inability to bind to immobilized PS suggested that the PS binding ELISA might not accurately reflect binding of factor Va to phospholipid membranes and/or that Trp^{2063}/Trp^{2064} may play only a minor role in membrane binding. We have now characterized the prothrombinase complex assembly in the presence of mutant factor Va and investigated factor Va binding to phospholipid membranes using both fluorescence resonance energy transfer (FRET) and surface plasmon resonance (SPR). In this study, we demonstrate that (i) Alanine substitutions for Trp^{2063}/Trp^{2064} decrease factor Va membrane binding affinity by at least 400-fold (estimated $K_d > 10^{-7}$ M). (ii) Alanine substitutions for Trp^{2063}/Trp^{2064} have no effect on maximal rates of thrombin generation on 25% PS membranes in the presence of saturating concentrations of factor Va or factor Xa. (iii) The mutant factor Va has a 4-fold reduced affinity for factor Xa on 25% PS membranes. (iv) Factor Xa promotes the binding of mutant factor Va to 25% PS membranes.

MATERIALS AND METHODS

Materials. Human prothrombin, human thrombin, human factor Xa, and bovine factor Va were obtained from Haematologic Technologies Inc. (Essex Junction, VT). Molecular weight markers were obtained from Bio-Rad (Hercules, CA). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), hen egg L-α-phosphatidylcholine (PC), brain L-α-phosphatidylserine (PS), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(5-(dimethylamino)-1-naphthalenesulfonyl) (D-PE) were from Avanti Polar Lipids (Alabaster, AL). Hi-5 cells, cell culture media, and Lipofectamine were from Invitrogen (Carlsbad, CA). All other reagents were from Sigma (St. Louis, MO).

Recombinant Proteins. Native and mutant recombinant factor Va was expressed in COS cells using a B-domain deleted construct and purified as previously described (*6*). The concentration of purified $rHFVa_2$ was estimated by measuring the absorbance at 280 nm using an extinction coefficient ($E^{1\%}_{280}$) of 1.74 (*4*). Native and mutant recombinant factor V C2 domain constructs were expressed in Hi-5 cells; recombinant proteins were isolated using established methods (*21*). The concentration of purified $rHFVa_2$-C2 was determined by measuring the absorbance at 280 nm using an extinction coefficient of 2.85 that was calculated based on the amino acid composition of $rHFVa_2$-C2 (*21*, *29*). Alternative glycosylation of factor V at Asn^{2181} results in two glycoforms, $rHFVa_1$ and $rHFVa_2$, that can be separated by chromatography on a Mono S column. The recombinant proteins utilized in the present study, $rHFVa_2$ and $rHFVa_2$-C2, are not glycosylated at Asn^{2181}.

Deglycosylation of Recombinant Factor Va. To further investigate heterogeneity in the factor Va heavy chain, native and mutant factor Va (7 μg) were deglycosylated using recombinant *N*-glycanase F (Glyko, Novato, CA) according to the manufacturer's instructions.

Circular Dichroism Spectroscopy. Native and mutant rHFV-C2 was dialyzed into 100 mM NaF, 20 mM K_2HPO_4 pH 7.4 and concentrated using a Centricon 3 Filter (Millipore, Watertown, MA). Circular dichroism (CD) experiments were carried out at 23 °C using an Applied Photophysics PiStar-180 Circular Dichroism spectrometer with a 1-mm quartz cuvette. The CD spectra of 7.6 μM rHFV-C2 and 4.6 μM rHFV-C2 W(2063, 2064)A were recorded between 190 and 260 nm using 0.2-nm steps and a 4-nm bandwidth. The mean molar ellipticity was calculated using the equation: $[\theta] = \theta/10nCl$, where θ is the measured ellipticity in millidegrees, C is the molar concentration of rHFV-C2, n is the number of amino acid residues, and l is the path length in centimeters. The predicted secondary structure of native and mutant rHFV-C2 was estimated using the CDPro software suite and a reference set of 43 proteins of known secondary structure (http://lamar.colostate.edu/~sreeram/CDPro/) (*30*).

Phospholipid Vesicle Preparation. Phospholipids in chloroform solution were pipetted into Corex tubes (Corning, Corning, NY) using glass syringes (Hamilton, Reno, NV). The solvent was evaporated under a stream of nitrogen gas; and the dried phospholipids were resuspended in 150 mM NaCl, 20 mM Tris pH 7.4. The lipid suspension was then sonicated using a Braun model 2000 probe sonicator on low output (0.25% duty cycle, 15 W) for 30 min under a stream of nitrogen in an ice-bath. After sonication, the suspension was centrifuged at 72 000 rpm for 25 min at 15 °C in a Beckman TL 100 centrifuge to obtain a homogeneous suspension of vesicles. Phospholipid concentration was determined by inorganic phosphate assay (*31*). The size of the phospholipid vesicles was determined using a DynaPro-LSR Dynamic Light Scattering instrument (Lakewood, NJ). Phospholipid vesicles prepared using this method had an average diameter of 24−30 nm.

Prothrombinase Assay. The kinetics of prothrombin activation were investigated by measuring the rate of thrombin generation catalyzed by prothrombinase in the reaction mixture containing factor Xa, 1.4 μM prothrombin, phospholipid vesicles, and factor Va in 25 mM Tris, pH 7.4, 0.15 M NaCl, 2.7 mM KCl, 10 mg/mL BSA, and 3 mM $CaCl_2$. The reaction mixtures were incubated at 37 °C for one minute and then quenched by adding 10 μL of 50 mM EDTA, 200 mM MOPS, pH 7.4. The samples were then diluted and incubated with 50 μL of 0.5 mM S2238 (Chromogenix,

Molndal, Sweden) at 22 °C. The absorbance at 405 nm (mOD/min) was monitored for 3 min using a Vmax kinetic microtiter plate reader (Molecular Devices, Menlo Park, CA). Thrombin concentrations were determined using a standard curve prepared with purified human thrombin (0−15 nM). Control experiments confirmed that rates of thrombin generation by prothrombinase were linear under these conditions. Prothrombin activation in the presence of native and mutant recombinant factor Va was investigated in reaction mixtures containing either 0.2 nM factor Xa and 0−40 nM factor Va or 0.2 nM factor Va and 0−10 nM factor Xa. The effect of membrane PS content on prothrombin activation was investigated in reaction mixtures containing 1 nM factor Va, 0.2 nM factor Xa, and 5 μM PC/PS phospholipid vesicles containing 0−40% PS. Kinetic parameters for prothrombin activation were analyzed by nonlinear regression performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego CA, www.graphpad.com). Rates of thrombin generation were plotted against the concentration of the variable protein and values for $V_{Va\rightarrow\infty}$, $K_{1/2\ Va}$, $V_{Xa\rightarrow\infty}$, and $K_{1/2\ Xa}$ were obtained by fitting the data to a hyperbola.

Fluorescence Resonance Energy Transfer Studies. The binding of factor Va to phospholipid vesicles was determined using membranes labeled with 2.5 mol % dansyl-phosphatidylethanolamine as described by Gilbert el al. (*32*). Fluorescence measurements were made using an SLM-8100 fluorescence spectrophotometer assembled in the "L" configuration and equipped with MC-200 monochromators and Glanz-Thompson polarizers in the excitation and emission beams. To avoid variations in polarization-dependent transmission of the monochromators, the excitation polarization was set at 0° while the emission polarizer was set at 54.7°. Fluorophores in factor Va were excited at 280 nm (4-nm band-pass) and energy transfer to dansyl groups in the phospholipid membrane was detected by measuring emission at 545 nm (16-nm band-pass). Schott UG-11 and KV450 filters were used in the excitation and emission paths, respectively, to reduce light scattering artifacts. All measurements were made in a buffer containing 150 mM NaCl, 20 mM Tris pH 7.4 at 25 °C. Samples were mixed by carefully pipetting up and down after addition of protein and allowed to equilibrate for 15 s before beginning measurements. Fluorescence measurements were integrated over 5−10 s, and serial readings were obtained to confirm that equilibrium had been established. The final values represent the mean of 3−6 repeated readings. The volume added during each titration was less than 5% of the initial sample volume. Control experiments confirmed that photobleaching of dansyl groups and protein fluorophores was <0.25% per minute of irradiation at 280 nm. Data were analyzed using a simple bimolecular model, in which each factor Va binding site is independent and comprised of a discrete number of phospholipid monomers (*4*, *32*). The relationship between the observed fluorescence and the dissociation constant, K_d is described by the equations (*32*):

$$F_c = \frac{F}{F_b} - 1$$

$$F_c = F_\infty \cdot \frac{K_d + [V_t] + [PL_t/n] - \sqrt{(K_d + [V_t] + [PL_t/n])^2 - 4[V_t][PL_t/n]}}{2[PL_t/n]}$$

where F_c is the corrected fluorescence, F is the observed fluorescence, F_b is the fluorescence of the vesicles in the absence of added factor Va, F_∞ is the corrected fluorescence signal associated with saturation of all factor Va binding sites, V_t is the total factor Va concentration, PL_t is the total phospholipid concentration, and n is the number of phospholipid monomers per factor Va binding site. Values K_d were determined by fitting the data to the above equation using the Marquardt algorithm and GraphPad Prism version 4.00 for Windows. Fitted data were evaluated by visual inspection of residuals, standard errors for binding parameters, and independence of best fit parameters on initial parameter estimates.

Surface Plasmon Resonance Studies. Surface plasmon resonance binding experiments were carried out using a BIAcore 3000 instrument (BIAcore AB, Uppsala, Sweden). All experiments were carried out at 25 °C. The surface of a Pioneer Sensor Chip L1 was first preconditioned by injecting 20 μL of 40 mM octyl glucososide for 2 min. The sensor chip surfaces were then coated with either POPC/POPS (75:25 mol/mol) or POPC. Approximately 30 μL of a solution containing 0.15 mM (115 μg/mL) vesicles, 150 mM NaCl, and 20 mM Tris, pH 7.4 was injected at a flow rate of 5 μL/min, which produced a signal of approximately 5000 resonance units (RU). Nonspecific binding sites on the coated flow cells were then blocked by injecting 250 μL of 0.1 mg/mL BSA at a flow rate of 5 μL/min. After blocking, repeat injections of BSA resulted in a minimal signal increase (<20 RU). These experiments confirmed that the vesicles remained intact during the binding and regeneration steps. Binding experiments were carried out using a running buffer containing 150 mM NaCl, 5 mM $CaCl_2$, 20 mM HEPES pH 7.4, and a control flow cell coated with POPC vesicles. The sensor chip was allowed to equilibrate until the baseline drift was less than 0.5 RU/min. Kinetic experiments were performed using flow rates of 15 μL/min. The association and dissociation phases were monitored for 300 s. To regenerate the sensor chip, complete dissociation of bound protein was achieved using 10 or 20 μL of 10 mM NaOH. Kinetic experiments were performed using five or more concentrations of each protein. The phospholipid vesicles were then removed from the sensor chip using 20 μL of 40 mM octyl glucoside. The sensor chip was recoated with fresh vesicles prior to the next experiment. Data were analyzed using BIAevaluation version 3.03 software (Biacore Inc., Piscataway, NJ). For each experiment, the signal from the control surface (POPC) was subtracted to correct for refractive index changes due to changes in buffer composition. Derivative plots were evaluated for mass transport effects.

Statistics Analysis. Statistical analyses were performed using Student's t test.

RESULTS

Purification and Characterization. To further define the role of Trp^{2063}/Trp^{2064} in factor Va binding to phospholipid membranes and assembly of the prothrombinase complex, we purified the mutant rHFVa W(2063, 2064)A. The mutant

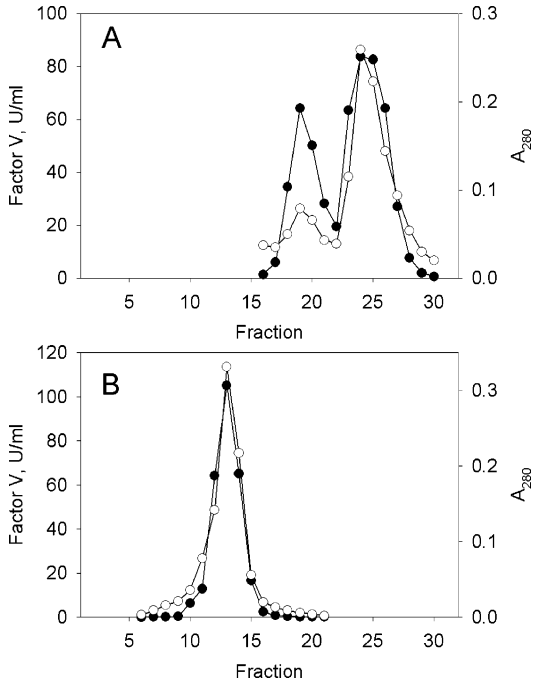

FIGURE 1: FPLC chromatography of rHFVa (A) and rHFVa W(2063,2064)A (B) on Mono S. Factor Va was purified by FPLC chromatography using a Mono S HR 5/5 column as previously described (*6*). Factor Va was eluted at a flow rate of 0.5 mL/min using a 15-mL NH_4Cl gradient (0.05−1.0 M NH_4Cl) at 4 °C. Fractions (0.5 mL) were collected and absorbance at 280 nm (open circles) and factor V clotting activity (closed circles) were determined.

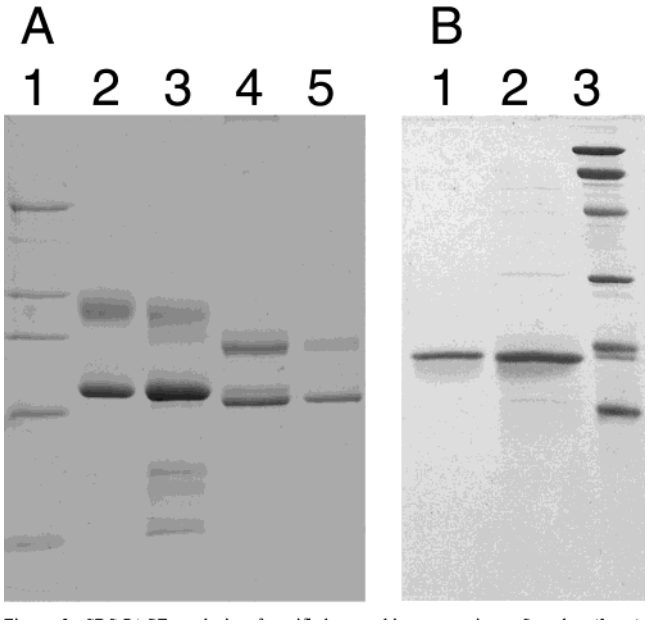

FIGURE 2: SDS−PAGE analysis of purified recombinant proteins. Samples (3 μg) were electrophoresed on 7.5% (A) or 15% (B) acrylamide gels and detected by Coomassie Blue staining. (A) Recombinant factor Va. Molecular weight markers: 200, 116, 97, 66, 45 kDa (lane 1), $rHFVa_2$ (lane 2), $rHFVa_2$ W(2063,2064)A (lane 3), $rHFVa_2$ treated with *N*-glycanase (lane 4), $rHFVa_2$ W(2063,-2064)A, treated with *N*-glycanase (lane 5). (B) Recombinant factor V C2 domain. $rHFV_2$-C2 (lane 1), $rHFV_2$-C2 W(2063,2064)A, (lane 2), molecular weight markers: 97, 66, 45, 31, 21.5, 14 kDa (lane 3).

factor Va was expressed in COS-7 cells and purified by FPLC chromatography as previously described (*6*). Chromatography of native rHFVa on Mono S results in elution of two activity peaks corresponding to factor Va_1 (fractions 16−21) and factor Va_2 (fractions 22−27) (Figure 1A). Approximately 31 ± 3.7% of the total factor Va activity eluted with factor Va_1, whereas 68.9 ± 3.7% eluted with factor Va_2 (mean ± SEM of six experiments). In contrast, the mutant eluted as a single peak (fraction 13) following chromatography on Mono S (Figure 1B). Fractions from the leading edge of the activity peak contained mostly $rHFVa_1$ W(2063, 2064)A, whereas the fractions from the trailing edge contained $rHFVa_2$ W(2063, 2064)A (data not shown). Although trace contaminants were detected in the $rHFVa_2$ W(2063, 2064)A preparations (Figure 2A), the concentrations of native and mutant protein preparations estimated by absorbance at 280 nm were identical to those obtained by ELISA using monoclonal antibodies AHV5146 (heavy chain) and 6A5 (light chain) (*26*). The mutant factor Va heavy chain migrates slightly faster than the heavy chain of the wild-type protein. This difference was noted in three independent protein preparations and appears to be due to differences in heavy chain glycosylation since the native and mutant heavy chains have identical mobilities following deglycosylation using *N*-glycanase (Figure 2A).

Native and mutant $rHFV_2$-C2 were also purified as previously described (Figure 2B) (*21*). Circular dichroism (CD) was utilized to further assess the effects of the W(2063, 2064)A mutation on folding of the factor V C2 domain. The spectral data were analyzed using the CDPro suite of programs and a reference set of 43 proteins of known secondary structure (*30*). Each of the three programs within the suite, SELCON3, CONTINLL, and CDSTR, yielded similar results. The predicted secondary structure for $rHFV_2$-C2 was 0−8% helix, 32−39% beta, 21−27% turn, and 31−40% unordered. The predicted secondary structure for rHFV-C2 W(2063, 2064)A was 2−10% helix, 30−34% beta, 25−27% turn, and 32−42% unordered. These results indicate that the W(2063, 2064)A mutation does not lead to major changes in the backbone secondary structure of the C2 domain. This does not eliminate the possibility that these amino acid substitutions may result in slight local changes in secondary structure.

Prothrombinase Activity. The ability of native and mutant factor Va to promote thrombin generation was investigated in prothrombinase assays to detect defects in both the formation and the activity of the prothrombinase complex. At saturating concentrations of factor Va, both native and mutant proteins expressed similar cofactor activities (turnover number ∼5000−6000 min^{-1}) in the presence of 0.2 nM factor Xa and 5 μM 75:25 PC/PS vesicles (Figure 3, Table 1). Approximately 10-fold higher concentrations of W(2063, 2064)A were required for half-maximal rates of prothrombin activation compared to rHFVa ($p < 0.05$). These results indicate that the ability of the mutant to form the prothrombinase complex is impaired but that once formed the mutant and native complexes have equal cofactor activities. To further investigate this difference, we next studied assembly of the prothrombinase complex in the presence of 5 μM 95:5 PC/PS vesicles. The maximal rates of thrombin formation and concentration of rHFVa required for half-maximal thrombin formation were similar to that observed in the presence of 5 μM 75:25 PC/PS vesicles (Figure 3, Table 1). In contrast, the ability of W(2063, 2064)A to promote

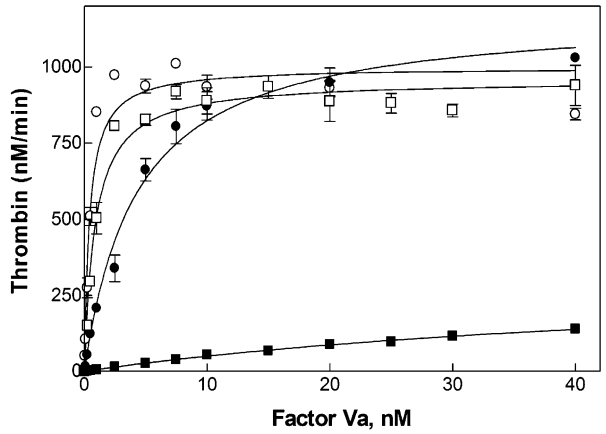

FIGURE 3: Prothrombinase activity as a function of factor Va concentration. Rates of thrombin generation were determined in reaction mixtures containing 25 mM Tris, pH 7.4, 150 mM NaCl, 2.7 mM KCl, 10 mg/mL BSA, 3 mM $CaCl_2$, 0.2 nM factor Xa, 1.4 μM prothrombin, factor Va and 5 μM PC/PS phospholipid vesicles. PC/PS, 95:5, mol/mol (squares), PC/PS, 75:25, mol/mol (circles), rHFVa (open symbols), rHFVa W(2063,2064)A (closed symbols).

assembly of the prothrombinase complex in the presence of limiting concentrations of PS membranes is dramatically reduced ($p < 0.05$).

We also performed experiments utilizing a fixed concentration of factor Va (0.2 nM) and variable concentrations of factor Xa (Figure 4, Table 1). In the presence of 5 μM 75:25 PC/PS vesicles the maximal rates of thrombin formation were again similar for both native and mutant proteins. Approximately 4-fold higher concentrations of factor Xa were required for half-maximal thrombin generation with W(2063, 2064)A compared to rHFVa ($p < 0.05$). In the presence of 0.2 nM rHFVa, approximately 8-fold higher concentrations of factor Xa were required for half-maximal thrombin generation on 5% PS membranes compared to membranes containing 25% PS ($p < 0.05$). No thrombin generation was detected on membranes containing 5% PS in the presence of 0.2 nM W(2063, 2064)A.

We also investigated the ability of factor Va to promote prothrombin activation on 5 μM phospholipid vesicles containing varying amounts of PS (Figure 5). Increasing the concentration of PS in the membrane resulted in similar rates of thrombin generation in the presence of both wild type and mutant factor Va. Half-maximal rates of thrombin generation occurred at approximately 6.1% PS in the presence of rHFVa. However, at PS concentrations below 10% little or no thrombin generation was observed in the presence of mutant W(2063, 2064)A and 19.4% PS was required for half-maximal rates of thrombin generation ($p < 0.05$). These results confirm that Trp^{2063}/Trp^{2064} are required for assembly of the prothrombinase complex on membranes containing low concentrations of PS.

Fluorescence Resonance Energy Transfer Membrane Binding Studies. We next investigated the binding of rHFVa and mutant W(2063, 2064)A to phospholipid vesicles in solution using the membrane binding assay previously described by Gilbert et al. (*32*). In this assay, the binding of factor Va to the phospholipid membrane is detected by monitoring energy transfer (FRET) between excited tryptophan residues in factor Va and dansyl-phosphatidylethanolamine (D-PE) in the phospholipid membrane. The factor Va C2 domain contains seven tryptophan residues; several of these residues are located in close proximity to the putative membrane binding site (*25*) including Trp^{2064} (0 Å), Trp^{2063} (~5 Å), Trp^{2068} (~12 Å), and Trp^{2084} (~20.4 Å). In preliminary experiments, we found that bovine factor Va bound to 3 μM 72.5:25:2.5 PC/PS/D-PE vesicles with a K_d of 0.84 ± 0.10 nM (mean ± SEM of eight experiments, data not shown). Similar results were obtained when the concentration of phospholipid vesicles was decreased to 0.3 or 0.6 μM. The affinity of rHFVa for 72.5:25:2.5 PC/PS/D-PE vesicles was approximately 2.5-fold higher than observed for bovine factor Va (Figure 6A) with a K_d of 0.34 ± 0.06 nM (mean ± SEM of eight experiments, $p < 0.05$). The observed affinity of human factor Va for 25% PS membranes is in general agreement with the values of 0.53 and 1.6 nM reported previously (*5*, *6*). The affinity of rHFVa for phospholipid vesicles containing 5% PS was reduced approximately 8.5-fold compared to 25% PS vesicles (Figure 6A) with a K_d of 2.87 ± 0.31 nM (mean ± SEM of two experiments, $p < 0.05$). In contrast to rHFVa, no binding was detected between W(2063, 2064)A and 25% PS membranes (Figure 6A) even at protein concentrations as high as 57 nM (not shown). The energy transfer associated with binding of W(2063, 2064)A to the phospholipid membrane should be decreased by 50% due to mutation of two of the four tryptophan residues located in close proximity to the membrane binding site (see discussion). On the basis of manual fitting of the data, we estimate that the affinity of W(2063, 2064)A for 25% PS membranes must be reduced over 400-fold with a $K_d > 100$ nM (Figure 6A).

Since the alanine substitutions for Trp^{2063}/Trp^{2064} appeared to have indirect effects on glycosylation of the factor Va heavy chain, we also characterized the interaction of the isolated factor V C2 domain with phospholipid vesicles. We found that rHFV C2 bound to 3 μM 72.5:25:2.5 PC/PS/D-PE vesicles approximately 21-fold more weakly than rHFVa (Figure 6B) with a K_d of approximately 7.1 ± 0.9 nM (mean ± SEM of eight experiments, $p < 0.05$). This value is in good agreement with the binding affinity recently reported by Majumder et al. (*33*) obtained by measuring changes in intrinsic fluorescence, but significantly higher than initially reported by Srivastava et al. (*21*). No binding of rHFVa C2 W(2063, 2064)A to phospholipid vesicles was detected at protein concentrations up to 150 nM (Figure 6B). Because the alanine substitutions for Trp^{2063}/Trp^{2064} could decrease the energy transfer associated with membrane binding, we also performed a competition experiment using rHFVa C2 W(2063, 2064)A (Figure 6C). This experiment demonstrated that rHFVa C2 W(2063, 2064)A did not compete with the 93 nM rHFVa C2 domain for binding to phospholipid vesicles at concentrations as high as 700 nM confirming that the membrane binding affinity of the mutant protein must be reduced at least 20-fold.

Surface Plasmon Resonance Studies. To monitor the association and dissociation of factor Va binding to phospholipid membranes, we established an assay for factor Va binding to immobilized phospholipid vesicles using surface plasmon resonance. In these experiments, a solution containing phospholipid vesicles was injected over the hydrophobic surface of an L1 Sensor Chip to create a membrane coated surface. Factor Va binding to the membrane was monitored by surface plasmon resonance using a BIAcore 3000 biosensor. In control experiments, no binding of factor Va was

Table 1: Kinetic Analysis of Prothrombinase[a]

protein	membrane	turnover no. (min^{-1})	$K_{1/2\ Va}$ (nM)	turnover no. (min^{-1})	$K_{1/2\ Xa}$ (nM)
rHFVa	75:25 PC/PS	4997 ± 126	0.47 ± 0.06	2491 ± 76	0.67 ± 0.09
	95:5 PC/PS	4789 ± 94	0.88 ± 0.10	1702 ± 74	5.2 ± 0.2
rHFVa W(2063,2064)A	75:25 PC/PS	5895 ± 182	4.3 ± 0.4	2669 ± 148	2.6 ± 1.0
	95:5 PC/PS	[b]	[b]	[b]	[b]

[a] Kinetic parameters derived from data shown in Figures 3 and 4. Rates of thrombin generation are expressed as the turnover number for the prothrombinase complex. Error values represent standard error of the fit for data averaged from three independent experiments. [b] Rate of thrombin generation was too low to calculate kinetic parameters.

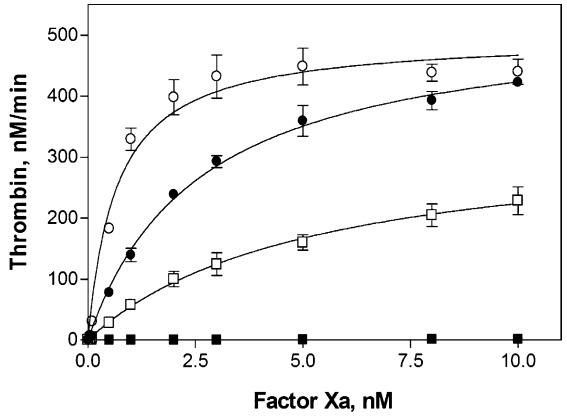

FIGURE 4: Prothrombinase activity as a function of factor Xa concentration. Rates of thrombin generation were determined in a reaction mixture containing 25 mM Tris, pH 7.4, 150 mM NaCl, 2.7 mM KCl, 10 mg/mL BSA, 3 mM $CaCl_2$, 0.2 nM factor Va, 1.4 μM prothrombin, factor Xa and 5 μM PC/PS phospholipid vesicles. PC/PS, 95:5, mol/mol (squares), PC/PS, 75:25, mol/mol (circles), rHFVa (open symbols), rHFVa W(2063,2064)A (closed symbols).

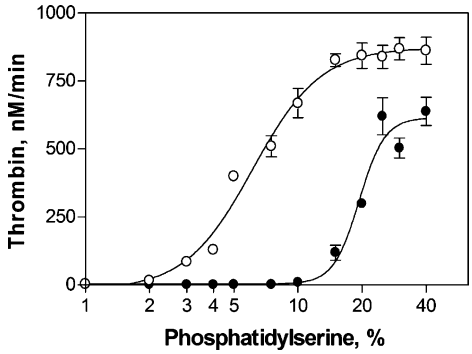

FIGURE 5: Prothrombinase activity as a function of membrane PS content. Rates of thrombin generation were determined in a reaction mixture containing 25 mM Tris, pH 7.4, 150 mM NaCl, 2.7 mM KCl, 10 mg/mL BSA, 3 mM $CaCl_2$, 0.2 nM factor Xa, 1.4 μM prothrombin, 1 nM factor Va and 5 μM phospholipid vesicles with varying mole percentages of PS. rHFVa (open circles), rHFVa W(2063,2064)A (closed circles).

observed when the L1 Sensor Chip was coated with 100% PC phospholipid membranes (not shown). In contrast, high-affinity binding of factor Va was observed when the L1 Sensor Chip was coated with POPC/POPS (75:25) phospholipid membranes (Figure 7A). Analysis of these data indicated that it could not be fit to a simple 1:1 Langmuir binding model. The shape of binding isotherms varied significantly as a function of flow rate (5−75 μL/min) indicating a mass transport effect. Attempts to eliminate mass transport by increasing the flow rate or decreasing the immobilization density of phospholipid vesicles were not successful, precluding quantitative analysis of association and dissociation rate constants. We next investigated the interaction of 5 nM W(2063, 2064)A with immobilized phospholipid vesicles in the presence or absence of 5 nM factor Xa. There was no detectable binding of mutant W(2063, 2064)A to immobilized membranes (Figure 7B) even at concentrations as high as 50 nM (not shown). Binding of 5 nM factor Xa alone to immobilized phospholipid vesicles was also not detected due to the relatively low affinity of factor Xa binding. However, binding of W(2063, 2064)A to immobilized membranes was detected in the presence of factor Xa, indicating that high-affinity interaction between membrane bound factor Xa and factor Va stabilized the membrane bound complex. In contrast, 1 μM prothrombin did not promote binding of W(2063, 2064)A to immobilized membranes (data not shown). These findings confirm that W(2063, 2064)A has a dramatically reduced affinity for phospholipid membranes and provide a mechanistic explanation for the ability of W(2063, 2064)A to promote assembly of the prothrombinase complex on 25% PS membranes.

DISCUSSION

The recently solved crystal structures of the C2 domains of factor V (*25*) and factor VIII (*34*) have suggested models for the interaction of these cofactors with phospholipid membranes that involve insertion of solvent-exposed hydrophobic amino acid side chains into the hydrophobic membrane core and interactions of positively charged amino acid side chains with PS. Consistent with this model, our initial characterization of a series of factor V mutants containing alanine substitutions in the C2 domain indicated that mutation of Trp^{2063} and/or Trp^{2064} resulted in decreased binding to immobilized PS by ELISA assay and reduced cofactor activity in the presence of membranes containing 5% PS (*26*). Nicolaes et al. (*27*) have also reported that rHFVa W(2063, 2064)A expresses limited cofactor activity in the presence of membranes containing <10% PS. However, the relative importance of Trp^{2063}/Trp^{2064} in membrane binding has not been completely defined since rHFVa W(2063, 2064)A appears to express normal cofactor activity in the presence of 25% PS membranes and the interaction of this mutant protein with phospholipid membranes has not been characterized (*26*, *27*).

In this study, the binding of rHFVa W(2063, 2064)A to phospholipid vesicles has been investigated using a well-characterized fluorescence energy transfer assay (*32*). In this assay, binding of factor Va is monitored by energy transferred from excited fluorophores in factor Va to dansyl groups in the phospholipid membrane. In addition to Trp^{2063}/Trp^{2064}, two other tryptophan residues, Trp^{2068} and Trp^{2084} are located within 21 Å of the putative membrane-binding site in the factor V C2 domain. Because the Förster distance

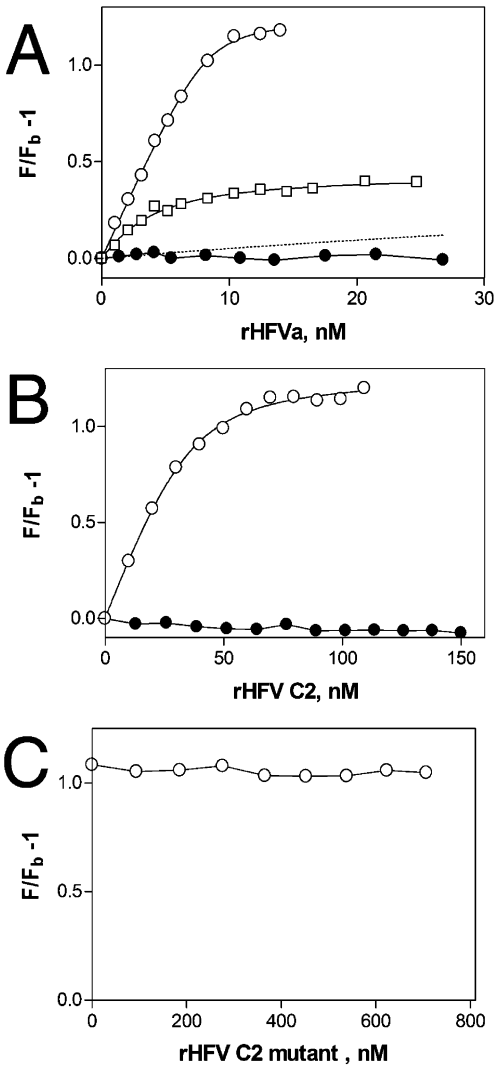

FIGURE 6: Binding of factor Va and factor V C2 domain to phospholipid vesicles. (A) Native and mutant factor Va binding to phospholipid vesicles. Reaction mixtures contained 150 mM NaCl, 25 mM Tris pH 7.4 and either 0.6 μM 72.5:25:2.5 PC/PS/D-PE (open circles), or 0.6 μM 92.5:5:2.5 PC/PS/D-PE (open squares) or 3.0 μM 72.5:25:2.5 PC/PS/D-PE (closed circles). Fluorescence measurements (F) were made as described under experimental procedures after incremental addition of rHFVa (open symbols) or rHFVa W(2063,2064)A (closed circles) at 25 °C. The lines for rHFVa indicate the best fit for the data corresponding to a K_d of 0.33 ± 0.14 nM and n of 73 ± 2 phospholipid monomers per molecule of rHFVa in the presence of 25% PS vesicles and a K_d of 2.6 ± 1.2 nM and n of 256 ± 184 phospholipid monomers per molecule of rHFVa in the presence of 5% PS vesicles. The dotted line depicts the predicted factor Va binding curve characterized by a K_d of 100 nM and maximum fluorescence, $F/F_b - 1$ of 0.64 (see text). (B) Binding of native and mutant rHFV C2 domain to phospholipid vesicles. Reaction mixtures contained 3 μM 72.5:25:2.5 PC/PS/D-PE, 150 mM NaCl and 20 mM Tris pH 7.4. Fluorescence measurements (F) were made as described under experimental procedures after incremental addition of rHFV C2 (open circles) or rHFV C2 W(2063,2064)A (closed circles) at 25 °C. The line for rHFV C2 indicates the best fit for the data corresponding to a K_d of 5.5 ± 1.7 nM and n of 86 ± 7 phospholipid monomers per molecule of rHFV C2. (C) Binding of rHFV C2 domain to phospholipid vesicles in the presence of mutant rHFV C2 domain. Reaction mixtures contained 93 nM rHFV C2, 3 μM 72.5:25:2.5 PC/PS/D-PE, 150 mM NaCl and 20 mM Tris pH 7.4. Fluorescence measurements (F) were made as described under the experimental procedures after incremental addition of rHFV C2 W(2063,2064)A (open circles) at 25 °C.

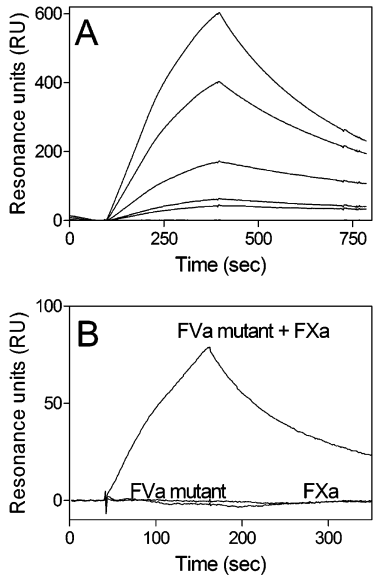

FIGURE 7: Binding of native and mutant factor Va to immobilized phospholipid membranes. (A) Sensorgram depicting binding of native factor Va to immobilized POPC/POPS (75:25) membranes. POPC/POPS (75:25) vesicles (~5000 RU) were immobilized on the surface of a BIAcore L1 sensor chip as described in the experimental procedures. Association and dissociation kinetic data were obtained at 0, 0.5, 1, 3, 5, and 10 nM (from bottom to top) of rHFVa. Association and dissociation times were 300 s. Experiments were performed in a buffer containing 150 mM NaCl, 5 mM $CaCl_2$, 20 mM HEPES pH 7.4, at a flow rate of 15 μL/min and at 25 °C. After each association/dissociation cycle, the surface of the sensor chip was regenerated using 10 mM NaOH. (B) Binding of mutant factor Va and factor Xa to immobilized phospholipid membranes. Experimental conditions were the same as those in panel A except that the flow rate was 60 μL/min and the association and dissociation times were 120 and 180 s, respectively. Samples injected were 5 nM rHFVa W(2063,2064)A, 5 nM factor Xa and 5 nM rHFVa W(2063,2064)A plus 5 nM factor Xa.

(R_0) for the Trp-dansyl pair is 21−24 Å (*35*), Trp^{2068} and Trp^{2084} should transfer energy when rHFVa W(2063, 2064)A binds to the membrane surface. We were not able to detect binding of rHFVa W(2063, 2064)A to 25% PS membranes at protein concentrations up to 57 nM. Analysis of these binding data indicate that the binding affinity of rHFVa W(2063, 2064)A for 25% PS membranes must be reduced over 400-fold with a K_d > 100 nM (Figure 6A). This suggests that the contribution of Trp^{2063}/Trp^{2064} to the free energy of membrane binding is at least 3.6 kcal/mol. The dramatic effect of these alanine substitutions on membrane binding was confirmed in competition experiments using a factor V C2 domain construct (Figure 6C) and in surface plasmon resonance experiments using immobilized phospholipid vesicles (Figure 7B). The membrane binding affinity of individual amino acids has been investigated using model peptides and electron paramagnetic resonance (*36*). These studies indicate that the free energy associated with insertion of the indole side chain of tryptophan into a membrane bilayer is 2.26 ± 0.15 kcal/mol. It remains to be determined whether the side chains of Trp^{2063}/Trp^{2064} are inserted into the membrane bilayer during membrane binding or whether the alanine substitutions at these positions could also have some affects on the orientation or conformation of the putative membrane binding spike-1. Substantial effects on the folding of the factor V C2 domain are unlikely, since we have previously demonstrated that mutations predicted

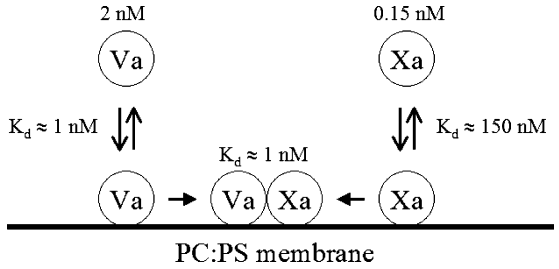

FIGURE 8: Reaction steps required for prothrombinase assembly on 25% PS membranes. Prothrombinase assembly proceeds after initial formation of separate factor Va-PC/PS and factor Xa-PC/PS complexes (*14*). Physiologic concentrations of factor Va are near the K_d for formation of factor Va/PC/PS. In contrast, the maximum concentrations of factor Xa generated during blood coagulation are at least 100-fold lower than the K_d for formation of factor Xa-PC/PS (*40*). The PC/PS bound proteins react rapidly to form the prothrombinase complex with an estimated $K_d \sim 1$ nM (*14*). Adapted from Krishnaswamy et al. (*14*).

to disrupt folding of the factor V C2 domain result in a complete loss of cofactor activity and reactivity with the monoclonal antibody 6A5 (*26*). The factor V C2 domain is responsible for 11.1 kcal/mol of the 12.9 kcal/mol associated with binding of factor Va to phospholipid membranes. Although the present results confirm that Trp^{2063}/Trp^{2064} make a significant contribution to the free energy associated with membrane binding, it seems likely that other interactions involving the A3 (*4*, *17*, *18*), C1 (*19*), and C2 (*20*−*23*) domains of factor Va also make significant contributions to membrane binding affinity.

We have developed a surface plasmon resonance (SPR)-based membrane binding assay for factor Va using phospholipid vesicles immobilized on an L1 sensor chip. Similar assays have been utilized successfully for characterization of a number of peripheral membrane binding proteins (*37*, *38*) including the homologous clotting protein, factor VIII (*39*). Quantitative analysis of association and dissociation rates requires that the binding data be free of mass transport effects, since such data may lead to erroneous conclusions even when corrections for mass transport effects are incorporated into the data analysis (*38*). It is likely that our inability to eliminate mass transport in factor Va membrane binding experiments is due to the extremely fast association constant for membrane binding which has been estimated to be 5.7×10^7 M^{-1} s^{-1} (*14*) near the upper limit for detection by the BIAcore 3000 biosensor. Nevertheless, the SPR membrane binding experiments have confirmed the markedly reduced membrane binding affinity of rHFVa W(2063, 2064)A compared to the native protein. Furthermore, these experiments demonstrated that factor Xa enhances the binding of rHFVa W(2063, 2064)A to the membrane surface.

The membrane binding properties and cofactor activity of rHFVa W(2063, 2064)A provide important insights into the role of Trp^{2063}/Trp^{2064} in assembly of the prothrombinase complex. At physiological concentrations of factor Xa (~0.15 nM), very little factor Xa is bound to 25% PS membranes in the absence of factor Va since factor Xa binds to membranes with a relatively low binding affinity of 150 nM (Figure 8) (*14*, *40*). In contrast, the binding of factor Va to phospholipid membranes is readily detected since the physiological concentration of factor Va (2 nM) is close to the K_d for membrane binding (*4*, *40*). Trp^{2063}/Trp^{2064} are critical for the binding of factor Va to phospholipid membranes since the K_d for rHFVa W(2063, 2064)A is substantially higher than physiological concentrations of factor Va. However, in the presence of factor Xa, the factor Va−factor Xa−membrane complex is stabilized by the high-affinity interaction between membrane bound factor Va and factor Xa which leads to formation of the prothrombinase complex in the absence of interactions between Trp^{2063}/Trp^{2064} and the membrane bilayer. The ability of factor Va to promote assembly of the prothrombinase complex on phospholipid membranes containing <10% PS is highly dependent on interactions between Trp^{2063}/Trp^{2064} and the membrane bilayer. This may be solely a consequence of the decreased affinity of factor Va (Figure 6A) and factor Xa for membranes containing low concentrations of PS (*41*). Alternatively, PS binding to the membrane binding site localized to the factor Va C2 domain may also regulate the interaction of factor Va with factor Xa as suggested by the data in Figure 4.

Normally, little or no PS is expressed on the outer leaflet of cellular membranes (*42*, *43*). During platelet activation, the extracellular membrane PS content increases to between 5 and 11% depending on the strength of the platelet activator (*44*). Our findings indicate that Trp^{2063} and Trp^{2064} play a critical role in the expression of procoagulant activity at concentrations of membrane PS similar to that seen on the surface of resting or activated platelets. Further characterization of the interaction of rHFVa W(2063, 2064)A with platelets from factor V-deficient patients may provide additional insights into the regulation of the prothrombinase complex by cellular membranes in vivo. Furthermore, characterization of prothrombinase complex assembly in the presence of rHFVa W(2063, 2064)A and soluble PS should also allow us to define any possible role for PS binding to factor V C2 domain in regulating the interaction of factor Va with factor Xa to form a productive prothrombinase complex (*21*, *45*).

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