Identification of Functionally Important Amino Acid Residues within the C2-Domain of Human Factor V Using Alanine-Scanning Mutagenesis†

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Received September 27, 1999; Revised Manuscript Received December 9, 1999

ABSTRACT: We have previously determined that the C2-domain of human factor V (residues 2037−2196) is required for expression of cofactor activity and binding to phosphatidylserine (PS)-containing membranes. Naturally occurring factor V inhibitors and a monoclonal antibody (HV-1) recognized epitopes in the amino terminus of the C2-domain (residues 2037−2087) and blocked PS binding. We have now investigated the function of individual amino acids within the C2-domain using charge to alanine mutagenesis. Charged residues located within the C2-domain were changed to alanine in clusters of 1−3 mutations per construct. In addition, mutants W2063A, W2064A, (W2063, W2064)A, and L2116A were constructed as well. The resultant 30 mutants were expressed in COS cells using a B-domain deleted factor V construct (rHFV des B). All mutants were expressed efficiently based on the polyclonal antibody ELISA. The charged residues, Arg^{2074}, Asp^{2098}, Arg^{2171}, Arg^{2174}, and Glu^{2189} are required for maintaining the structural integrity of the C2-domain of factor V. Four of these residues (Arg^{2074}, Asp^{2098}, Arg^{2171}, and Arg^{2174}) correspond to positions in the factor VIII C-type domains that have been identified as point mutations in patients with hemophilia A. The epitope for the inhibitory monoclonal antibody HV-1 has been localized to Lys^{2060} through Glu^{2069} in the factor V C2-domain. The epitope for the inhibitory monoclonal antibody 6A5 is composed of amino acids His^{2128} through Lys^{2137}. The PS-binding site in the factor V C2-domain includes amino acid residues Trp^{2063} and Trp^{2064}. This site overlaps with the epitope for monoclonal antibody HV-1. These factor V C2-domain mutants should provide valuable tools for further defining the molecular interactions responsible for factor V binding to phospholipid membranes.

Thrombin-activated coagulation factor V is an essential nonenzymatic cofactor of the prothrombinase complex, accelerating the activation of prothrombin by factor Xa in the presence of calcium and a phospholipid membrane surface (*1*, *2*). It is synthesized as a single chain polypeptide of approximately 330 kDa with the domain structure A1-A2-B-A3-C1-C2 (*3*−*5*). The A1- and A2-domains form the heavy chain and the A3-, C1-, and C2-domains form the light chain after activation by thrombin (*6*, *7*) or factor Xa (*8*). The B-domain is a connecting domain and is not essential for procoagulant activity.

Factor V is similar structurally and functionally to factor VIII, which is an essential component of the X-ase complex, consisting of factor IXa, factor VIIIa, calcium ions, and a phospholipid surface (*1*). Both cofactors have three A-type domains, which share ∼30% amino acid identity with each other and with the triplicated A-type domains in the copper-binding plasma protein ceruloplasmin (*9*, *10*). On the basis of the crystal structure of ceruloplasmin (*11*), the three-dimensional structures of three A-domains of factors V and VIII have been predicted (*12*, *13*). In addition to the three A-type domains, factor V and factor VIII possess two C-type domains at the carboxyl-terminal end of the light chain (*1*). These tandem modules are ∼150 amino acids in length and belong to the major eukaryotic subfamily of discoidin domains (*14*).

The C2-domain of factor V consists of residues Gly^{2037} through Tyr^{2196}. The exact biological roles of the C-domains in factor V are not well-characterized. However, we have previously determined that the C2-domain of factor V is required for expression of cofactor activity and binding to phosphatidylserine (PS)[1] (*15*). Naturally occurring factor V inhibitors and a monoclonal antibody (HV-1) recognized epitopes in the amino terminus (residues 2037−2087) of the C2-domain and blocked PS binding (*16*, *17*). The monoclonal antibody 6A5 bound to an epitope in the central region (residues 2088−2148) of the C2-domain. This antibody

† This work was supported by grants HL43106 and HL54939 from the National Institutes of Health.

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[1] The abbreviations used are: PS, phosphatidylserine; ELISA, enzyme-linked immunosorbent assay; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine; rHFV des B, the wild-type recombinant factor V mutant, which lacks amino acids 811−1491 of the B-domain; rHFV des B/C2, the recombinant factor V mutant lacking residues 811−1492 of the B-domain and 2037−2196 of the C2-domain.

10.1021/bi992256r CCC: $19.00
Published on Web 02/22/2000

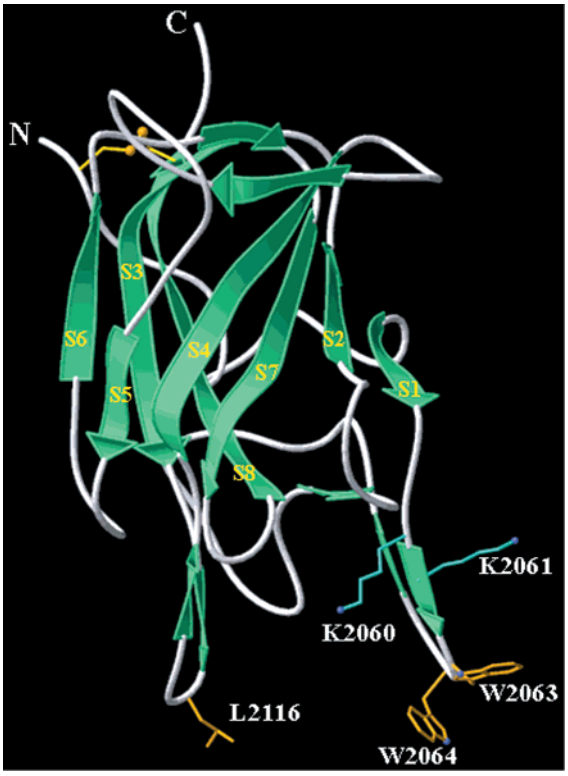

FIGURE 1: Structure of the factor V C2-domain. A ribbon plot is shown highlighting the beta structure of the factor V C2-domain and the exposed loops at the bottom of the molecule (*20*). The individual beta strands are numbered in yellow. The amino (N) and carboxyl (C) termini are labeled in white. The disulfide bond formed by Cys^{2038} and Cys^{2193} is depicted by the yellow balls and sticks. Selected side chains at the bottom of the barrel are shown explicitly.

inhibited procoagulant activity, but it did not block the binding of factor V to PS (*17*). Molecular models of the C2-domain of factor V were reported based on the crystal structure of the N-terminal domain of galactose oxidase, which has a low sequence homology with C-domains of factors V and VIII (*18*, *19*). On the basis of these models, two tryptophan residues, Trp^{2063} and Trp^{2064}, were proposed to be involved in PS binding (*18*, *19*). Recently, the three-dimensional structure of the C2-domain of factor V has been solved in two independent crystal forms, at a maximal resolution of 1.8 Å (Figure 1) (*20*). In this study, we have investigated the function of individual amino acids within the C2-domain, using charge to alanine mutagenesis. We identified several amino acid residues essential for structural integrity and regions responsible for binding of monoclonal antibodies, inhibitors, and PS. Our studies demonstrate that tryptophan residues 2063 and 2064 are required for optimal factor Va binding to PS, supporting the model for membrane binding suggested by the two C2-domain crystal structures (*20*).

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes, vectors, cell culture media, and T4 DNA ligase were obtained from GIBCO/BRL (Gaithersburg, MD). Human prothrombin, thrombin, and factor Xa were obtained from Haematologic Technologies Inc. (Essex Junction, VT). Molecular weight markers were obtained from Bio-Rad (Hercules, CA). 1-Palmitoyl-2-oleoyl-phosphatidylserine (POPS), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), brain L-α-phosphatidylcholine, and brain L-α-phosphatidylserine were from Avanti Polar Lipids (Alabaster, AL). All other reagents were from Sigma (St. Louis, MO).

Site-directed Mutagenesis and Expression. To facilitate mutagenesis of the factor V C2-domain, an XbaI site was created at nucleotide 6241 of the factor V cDNA (*3*, *4*). The XbaI−SalI fragment containing the factor V C2-domain was subcloned into pBluescript SK to generate a cassette vector. Mutagenesis was performed within the cassette vector using the QuikChange site-directed mutagenesis kit (Stratagene). The mutations were confirmed by DNA sequencing and inserted into a vector designed for expression of a deletion mutant lacking residues 811 through 1491 of the B-domain (rHFV des B) (*21*). Mutant proteins were expressed in COS-7 cells as previously described (*21*) with minor modifications. In the present study, COS cells were transfected using LipofectAMINE (Life Technologies, Gaithersburg, MD). Serum free conditioned medium (DMEM/F12), containing 5 mg/mL bovine serum albumin and 2.5 mM $CaCl_2$, was harvested 48 h following transfection and stored at −70 °C until use. Clotting assays for factor V procoagulant activity were performed as previously described (*21*).

Factor V ELISA and Epitope Mapping of Antibodies. Factor V antigen was quantitated by ELISA using monoclonal antibody AHV-5146 (Haemotologic Technologies Inc.) for capture and biotinylated affinity purified polyclonal rabbit anti-human factor V antibody for detection (*17*). Standard curves were constructed from purified wild-type factor V (rHFV des B). Epitope mapping of the murine monoclonal antibodies HV-1 and 6A5 was performed as previously described (*17*).

Prothrombinase Assay. The activity of factor V mutants was determined by measuring rates of thrombin generation in a prothrombinase assay as previously described (*21*). Conditioned media containing rHFV des B mutants were activated with 2 nM thrombin for 5 min at 37 °C and diluted immediately for assay. Cofactor activity was determined in the presence of saturating concentrations of factor Xa and phospholipid vesicles in reaction mixtures containing 5 nM factor Xa, 1.4 μM prothrombin, 5 μM phospholipid vesicles (POPC/POPS, 75/25, mol/mol), factor Va, 20 mM Tris, pH 7.4, 150 mM NaCl, 2.7 mM KCl, 10 mg/mL BSA, and 3 mM $CaCl_2$. Cofactor activity was also determined in the presence of limiting concentration of phospholipid vesicles in similar reaction mixtures containing 0.5 μM phospholipid vesicles (POPC/POPS, 95/5, mol/mol).

Phospholipid Binding Assay. The binding of factor V mutants to phospholipid was investigated using a solid-phase ELISA, as previously described (*17*). Detection was performed using the biotinylated affinity purified polyclonal rabbit anti-human factor V antibody.

RESULTS

Mutagenesis and Expression. We have previously shown that binding of factor V to immobilized phosphatidylserine requires the presence of the C2-domain, and that the amino-terminal portion of the domain is required for binding. To investigate the functional roles of charged amino acid residues in membrane binding, 22 mutants of factor V were generated where one to three neighboring charged amino

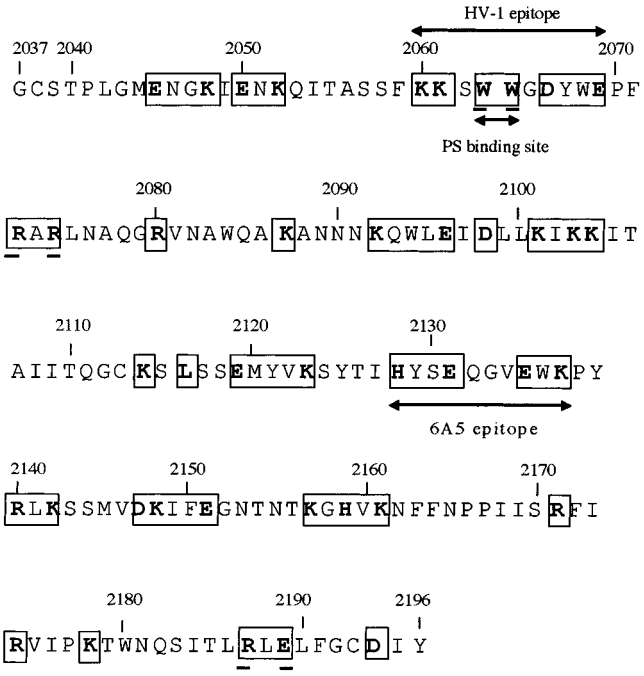

FIGURE 2: Amino acid residues of factor V C2-domain targeted for charged-to-alanine scanning mutagenesis. Segments that contain mutations in each variant protein are boxed and the mutated residues are shown in bold. The positions of six single residue mutant constructs, W2063A, W2064A, R2072A, R2074A, R2187A, and E2189A are underlined. The binding sites for PS, antibodies HV-1, and 6A5 localized in this study are also indicated (solid arrows).

acids (Asp, Glu, Lys, Arg, and His) of C2-domain were replaced with alanine. While these experiments were in progress, we were successful in solving the structure of the C2-domain in two independent crystal forms. Analysis of these structures suggested that tryptophans 2063−2064 and leucine 2116 might play a role in membrane binding. Therefore, mutants W2063A, W2064A, (W2063, W2064)A, and L2116A were also constructed (Figure 2). All these mutants were expressed in COS cells using a B-domain deleted factor V construct (rHFV des B), and serum-free media were analyzed for the activity and expression of mutant factor V. The concentration of recombinant factor V in conditioned media was estimated using a polyclonal antibody factor V ELISA. The average concentration for factor V mutants ranged from 0.9 to 5.5 μg/mL, which was similar to the average concentration of 2.1 μg/mL observed for rHFV des B. The potential effects of mutations on the secretion of recombinant factor V was not further investigated. The specific activities of mutants (R2072, R2074)A, D2098A, (K2101, K2103, K2104)A, R2171A, and (R2187, E2189)A were less than 11% of wild-type. The specific activities of mutants (W2063, W2064)A, (K2157, H2159, K2161)A, and R2174A were also significantly lower than wild-type (Figure 3). We further analyzed mutants (R2072, R2074)A and (R2187, E2189)A by generating individual charge-to-alanine mutants (R2072A, R2074A, R2187A and E2189A). While the specific activities of mutants R2072A and R2187A were similar to wild-type, the specific activities of mutants R2074A and E2189A were less than 15% of wild-type. These results indicate that the R2074A and E2189A mutations were responsible for the decreased activity of mutants (R2072, R2074)A and (R2187, E2189)A.

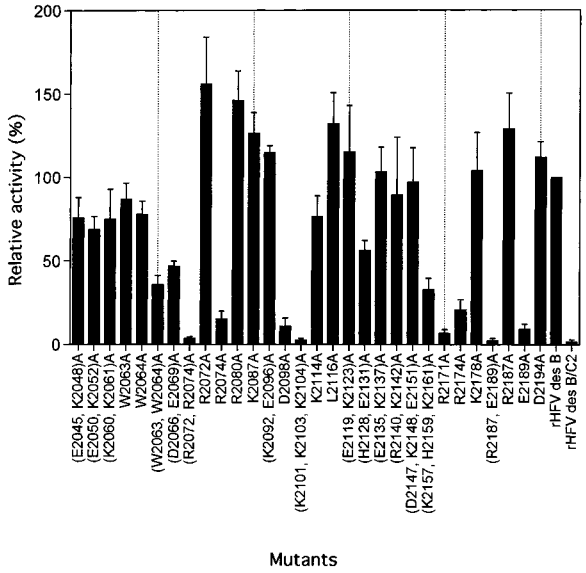

FIGURE 3: Specific activity of factor V C2-domain mutants. The specific activity of factor V C2-domain mutants (see Figure 2) relative to wild-type protein (rHFV des B) was determined by clotting assay. Protein concentration was estimated by ELISA using a rabbit polyclonal anti-factor V antibody and purified wild-type factor V for construction of a standard curve. Mutant rHFV des B/C2 was used as a control. Bars are expressed as a mean ± SD of three independent transfections.

Epitope Mapping. We next used the factor V C2-domain mutants together with the crystal structure of the C2-domain to localize the nonoverlapping epitopes for two monoclonal antibodies, HV-1 and 6A5. This information was then used to identify mutants that were improperly folded. Monoclonal antibody HV-1 bound poorly to the mutants (K2060, K2061)A, (W2063, W2064)A, (D2066, E2069)A, (R2072, R2074)A, R2074A, D2098A, (K2101, K2103, K2104)A, R2171A, R2174A, (R2187, E2189)A, and E2189A (Figure 4). Monoclonal antibody 6A5 bound poorly to the mutants (R2072, R2074)A, R2074A, (K2092, E2096)A, D2098A, (K2101, K2103, K2104)A, (H2128, E2131)A, (E2135, K2137)A, R2171A, R2174A, (R2187, E2189)A, and E2189A (Figure 5). Both antibodies bound poorly to the mutants (R2072, R2074)A, R2074A, D2098A, (K2101, K2103, K2104)A, R2171A, R2174A, (R2187, E2189)A, and E2189A suggesting that these mutants were incorrectly folded. Western blot analysis using polyclonal anti-factor V antibodies demonstrated single chain mutant proteins, confirming that observed loss of activity was not secondary to proteolysis (data not shown).

Mutants (K2060, K2061)A, (W2063, W2064)A, and (D2066, E2069)A bound poorly only to HV-1. These amino acid residues, which are exposed in both crystal forms of the isolated C2-domain, appear to contribute to the epitope for HV-1. Mutants (K2092, E2096)A, (H2128, E2131)A, and (E2135, K2137)A bound poorly to only 6A5, suggesting that these exposed side chains contribute to the epitope for 6A5.

Phosphatidylserine Binding Assay. Each of the mutants was then tested for its ability to bind immobilized PS. We have used a solid-phase phospholipid binding ELISA to screen our mutants for their ability to bind to PS. We previously found through the use of this assay that phospholipid binding is specific for PS and that factor V does not bind to the plate if the plate is coated with phospha-

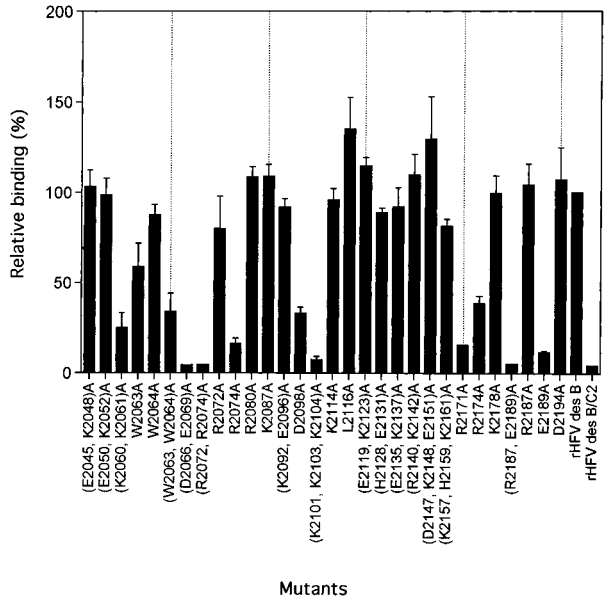

FIGURE 4: Binding of factor V C2-domain mutants to monoclonal antibody HV-1. Microtiter plate wells were coated with antibody HV-1 and incubated overnight at 4 °C. The wells were then blocked and incubated with conditioned media containing the factor V mutants. Captured factor V antigen was detected with biotinylated rabbit anti-human factor V. Binding of factor V mutants to HV-1 was determined at a fixed concentration of factor V (1 μg/mL) and normalized to the value obtained for wild-type factor V. Bars are expressed as a mean ± SD of three independent transfections.

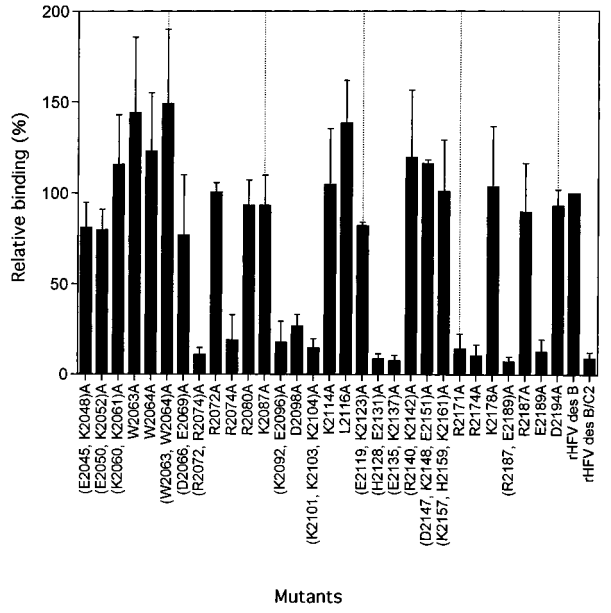

FIGURE 5: Binding of factor V C2-domain mutants to monoclonal antibody 6A5. Microtiter plate wells were coated with rabbit anti-human factor V and incubated overnight at 4 °C. The wells were then blocked and incubated with conditioned media containing the factor V mutants. Captured factor V antigen was detected with biotinylated monoclonal antibody 6A5. Binding of factor V mutants to 6A5 was determined at a fixed concentration of factor V (1 μg/mL) and normalized to the value obtained for wild-type factor V. Bars are expressed as a mean ± SD of three independent transfections.

tidylcholine or if the factor V mutant that lacks the C2-domain (rHFV des B/C2) is used (*15*, *17*). When we tested rHFV des B/C2 as a control, the relative binding was less than 13% of wild-type. The relative binding of all

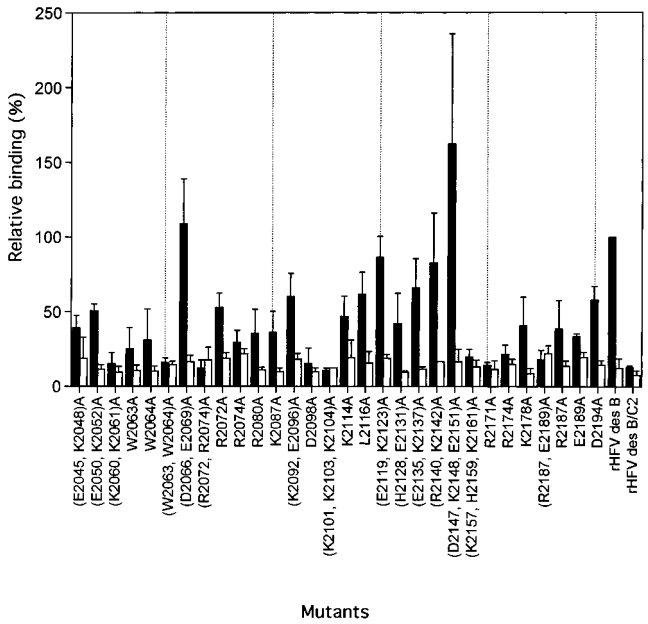

FIGURE 6: Binding of factor V C2-domain mutants to phosphatidylserine and phosphatidylcholine. Microtiter plate wells were coated with 3 μg/mL brain L-α-phosphatidylserine (black bars) and 3 μg/mL brain L-α-phosphatidylcholine (white bars) and blocked. The wells were then incubated with conditioned media containing mutants, wild-type (rHFV des B), or C2-domain deletion mutant (rHFV des B/C2). Binding was detected with the same polyclonal rabbit anti-factor V antibody used to estimate the protein concentrations. Binding of factor V mutants to phosphatidylserine or phosphatidylcholine was determined at a fixed concentration of factor V (0.3 μg/mL) and normalized to the value obtained for wild-type factor V. Bars are expressed as a mean ± SD of three independent transfections.

mutants, except (D2066, E2069)A and (D2147, K2148, E2151)A, was decreased. The relative binding of mutants (K2060, K2061)A, (W2063, W2064)A, (R2072, R2074)A, D2098A, (K2101, K2103, K2104)A, (K2157, H2159, K2161)A, R2171A, R2174A, and (R2187, E2189)A to PS was significantly decreased (less than 22% of wild-type) (Figure 6). The modifications in mutants (R2072, R2074)A, D2098A, (K2101, K2103, K2104)A, R2171A, R2174A, and (R2187, E2189)A that appear to disrupt C2-folding result in severely decreased PS binding. The modifications in mutants (K2092, E2096)A, (H2128, E2131)A, and (E2135, K2137)A that prevent expression of the 6A5 epitope partially decrease PS binding. Mutations that prevent expression of the HV-1 epitope result in severely decreased PS binding ((K2060, K2061)A and (W2063, W2064)A), except for mutant (D2066, E2069)A in which binding to PS is normal or modestly increased. Mutant (K2157, H2159, K2161)A, which is not part of HV-1 epitope, has severely decreased PS binding. Since mutants (R2072, R2074)A, D2098A, (K2101, K2103, K2104)A, R2171A, R2174A, and (R2187, E2189)A appear to be incorrectly folded, we conclude that the modified amino acid residues in mutants (K2060, K2061)A and (W2063, W2064)A contribute to the interaction of PS with the factor V C2-domain.

Prothrombinase Assay at Saturating and Limiting Membrane Concentrations. To confirm that the modified amino acid residues in mutants (K2060, K2061)A and (W2063, W2064)A are involved in binding to PS, the activity of factor V mutants was determined by measuring rates of thrombin

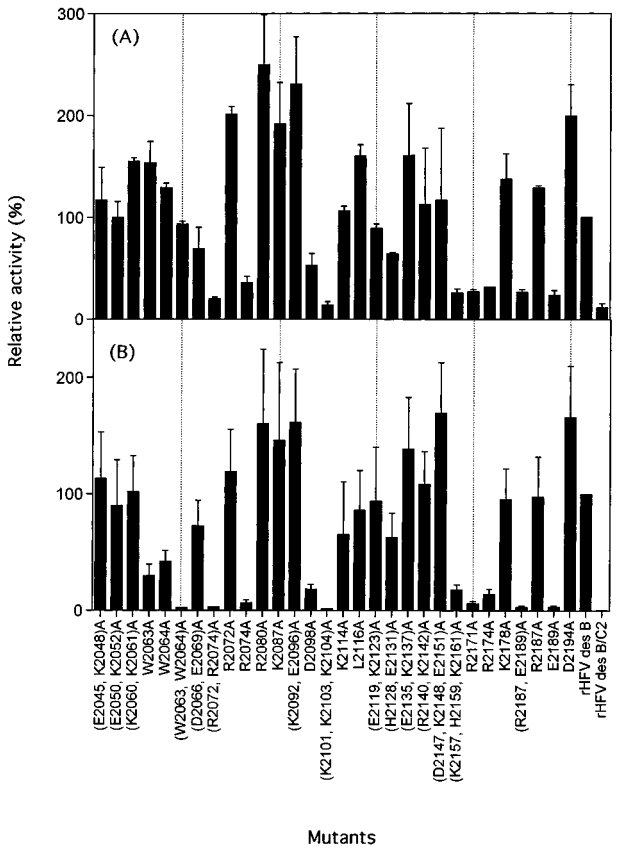

FIGURE 7: Prothrombinase assay at saturating and limiting membrane concentrations. (A) The rate of thrombin generation in a prothrombinase assay was determined in the presence of saturating concentrations of factor Xa and phospholipid vesicles in reaction mixtures containing 5 nM of factor Xa, 1.4 μM prothrombin, 5 μM phospholipid vesicles (POPC/POPS, 75/25, mol/mol), factor Va, 20 mM Tris, pH 7.4, 150 mM NaCl, 2.7 mM KCl, 10 mg/mL BSA, and 3 mM $CaCl_2$. (B) Cofactor activity was also determined in the presence of limiting concentrations of phospholipid vesicles in similar reaction mixtures containing 0.5 μM phospholipid vesicles (POPC/POPS, 95/5, mol/mol).

generation in a prothrombinase assay at saturating and limiting concentrations of phospholipid vesicles (Figure 7). Modifications in the mutants (R2072, R2074)A, D2098A, (K2101, K2103, K2104)A, R2171A, R2174A, (R2187, E2189)A, and E2189A that appear to disrupt folding of the C2-domain result in severely decreased activities at both saturating and limiting membrane concentrations. The activities of mutant (K2157, H2159, K2161)A were also decreased at both saturating and limiting membrane concentrations. The most striking activity differences between saturating and limiting membrane concentrations were observed with mutants W2063A, W2064A, and (W2063, W2064)A. The relative activities of mutants W2063A, W2064A, and (W2063, W2064)A were 153.6, 129.2, and 93.3% of wild-type at saturating membrane concentrations. However, when we measured the prothrombinase activity of mutants W2063A, W2064A, and (W2063, W2064)A at limiting lipid concentrations (0.5 μM POPC/POPS (95/5, mol/mol)), the relative activities of these mutants were 29.5, 41.8, and 2.5% of wild-type, respectively, indicating that amino acid residues Trp^{2063} and Trp^{2064} contribute to the interaction of the factor V C2-domain with PS.

DISCUSSION

Single site mutations of amino acid residues located at binding interfaces typically results in a 2−10-fold decrease in binding affinity (*22*). In this study, we expressed a series of factor V mutants in which clusters of charged residues within the C2-domain were replaced with alanine in order to investigate structure−function relationships (*22*). We used site-directed mutagenesis to alter 39 charged residues in the C2-domain of factor V to alanine. Alanine was used as a substitute, because it is uncharged and has the smallest amino acid side chain, except glycine, which is not preferred, because it can alter the main chain conformation of the protein. Six mutants, (R2072, R2074)A, D2098A, (K2101, K2103, K2104)A, R2171A, R2174A, and (R2187, E2189)A showed significant decreases in cofactor activity, binding to monoclonal antibodies and binding to PS. Since interactions between an antibody and antigen are dependent on shape complementarity of the interacting surfaces as well as direct contact with the epitope site, disruption of either the epitope site or of the antigen's structural conformation can lead to loss of antibody binding (*23*, *24*). The charged residues modified in these mutants seem to be required for maintaining the structural integrity of the factor V C2-domain. The polar side chains of buried Arg^{2074} and Glu^{2189} play an important role in stabilizing the N-terminal loop (Gly^{2043}–Lys^{2052}) via strong hydrogen bonding interactions with main chain atoms of residues Gly^{2043}, Met^{2044}, Glu^{2045}, and Ile^{2049}. Curiously, replacement of exposed charged residues within this loop (see Figures 1 and 8) does not seem to impair functionality of the protein, indicating that the proper three-dimensional conformation of this loop is of paramount importance for correct folding. The exposed residues Asp^{2098} and Arg^{2171} are conserved in all discoidin domains of coagulation factors sequenced to date. Formation of a salt bridge between the carboxylate of Asp^{2098} and the guanidinium group of Arg^{2171} seems to be very important in stabilizing the three-dimensional structure of discoidin domains. This salt bridge is also conserved in the discoidin domain of a fungal galactose oxidase (*20*, *25*). The side chain of Arg^{2174} is positioned between the indol moieties of residues Trp^{2094} and Trp^{2136} (also strictly conserved in coagulation factors) and is further stabilized by a salt bridge to Glu^{2096}.

The importance of amino acid residues Arg^{2074}, Asp^{2098}, Arg^{2171}, and Arg^{2174} in factor V is further supported by the fact that point mutations of corresponding amino acid residues in factor VIII cause hemophilia A. Residue Asp^{2098} is conserved at Asp^{2074} in the C1-domain of factor VIII, whereas residues Arg^{2074}, Arg^{2171}, and Arg^{2174} are conserved at Arg^{2209}, Arg^{2304}, and Arg^{2307} in its C2-domain. Mutations D2074G, R2209Q (R2209G), R2304H (R2304C), and R2307Q (R2307L) have been reported to cause mild to severe hemophilia A (*26*). Recently, Pipe and Kaufman demonstrated that the factor VIII cofactor activity and von Willebrand factor binding of R2307Q mutant factor VIII was only mildly reduced compared to wild-type, whereas secretion and stability of the mutant protein was significantly impaired (*27*). These investigators also found that R2307L mutant factor VIII was not secreted in detectable amounts. They proposed that defective secretion and intracellular degradation of factor VIII was the molecular mechanism for hemophilia A in patients expressing these mutations. How-

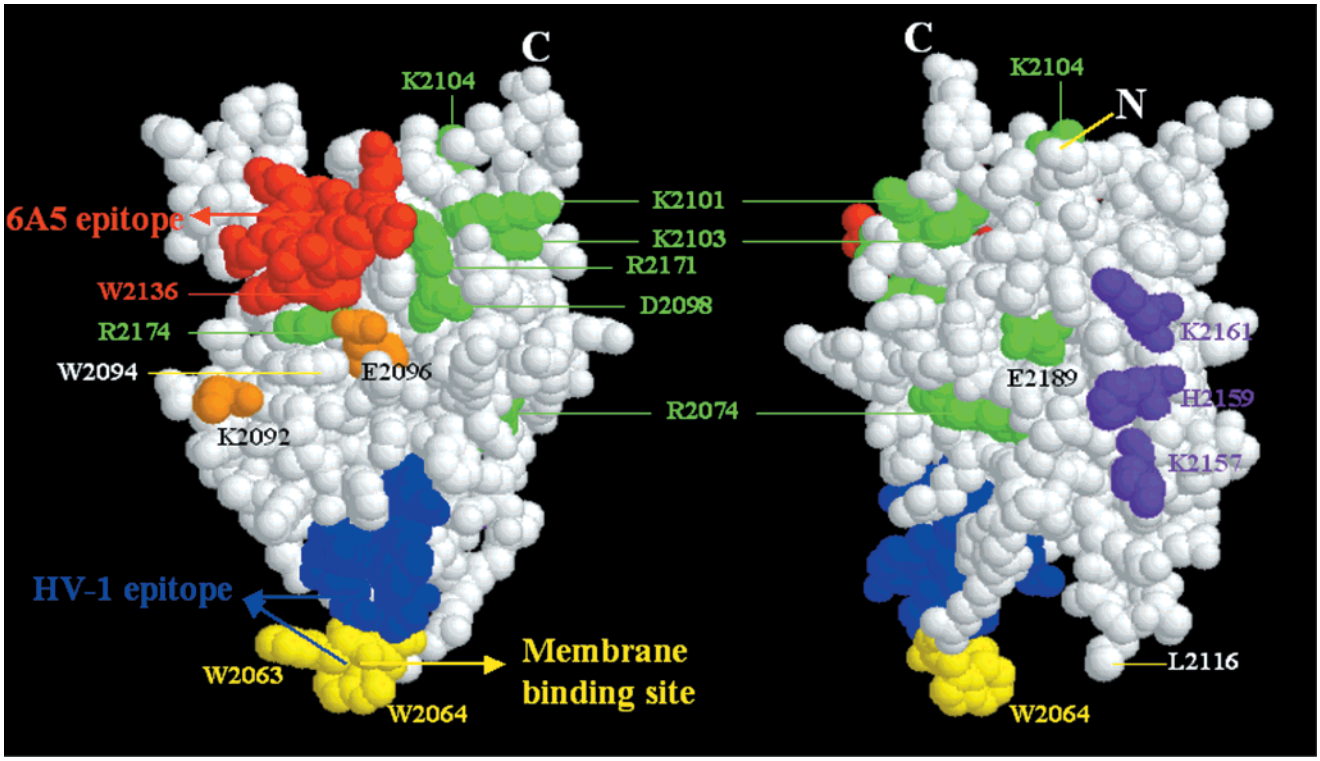

FIGURE 8: Space-filling representation of the C2-domain of factor V. The epitope for monoclonal antibody 6A5 is colored red and the epitope for monoclonal antibody HV-1 is colored blue. The amino acid residues involved in phosphatidyserine binding are colored yellow and these residues are also involved in the binding to monoclonal antibody HV-1. Functionally important residues are colored either green (defect in cofactor activity, PS binding, and monoclonal antibodies binding), purple (defect in cofactor activity and PS binding) or orange (defect in monoclonal antibody 6A5 binding). The molecule on the right is rotated approximately 90° about the *z*-axis compared to the molecule on the left. The figures were generated using the RasMol software package.

ever, in the present study, we found that the R2174A mutant factor V was expressed at a level similar to the wild-type protein, although specific activity was less than 21% of normal. The different effects of the R2174A mutation in factor V and the R2307Q and R2307L mutations in factor VIII appear to be due to the nature of the substituted amino acid side chain. Supporting this view, hemophilia A patients expressing the R2307L mutation have a more severe bleeding disorder than patients expressing the R2307Q mutation (*27*).

Mutation of Lys^{2101}, Lys^{2103}, and Lys^{2104} also appears to disrupt the folding of the factor V C2-domain. The aliphatic parts of these amino acid side chains (especially the rather buried Lys^{2104}) make several important van der Waals contacts with a number of neighboring side chains (e.g., Thr^{2106}, Pro^{2166}, and Ile^{2169}) and partially protect the disulfide bridge Cys^{2038}—Cys^{2193}. Residues Lys^{2103} and Lys^{2104} are replaced by hydrophobic residues in all C1-domains of coagulation factors V and VIII. These observations suggest that replacement of these side chains in the C2-domain by alanine might disturb proper protein folding. Alternatively, it is possible that the impaired activity and PS binding of this mutant could be explained by involvement of these amino acid side chains in C1−C2 interactions.

Several explanations can be advanced for the loss of activity of mutant (K2157, H2159, K2161)A. First, it is possible that Lys^{2157} might be involved in electrostatic interactions with the phosphate group of a bound PS molecule. Second, His^{2159} participates in a network of hydrogen bonds connecting major structural elements. However, it seems more likely that the almost complete loss of activity derives from grossly impaired inter-domain interactions. Location of this region opposite to the putative extended membrane-binding site suggests that it constitutes a potential assembly site for the A3-domain. Supporting this view, both C2 monomers are linked through their beta-strands Val^{2160}—Phe^{2164} (S6) in the dimeric crystal form (*20*), and the neighboring strand (S5) in galactose oxidase is responsible for interactions with the catalytic domain (*28*).

The C2-domain of factor V contains epitopes for inhibitory autoantibodies and monoclonal antibodies. We previously showed that the spontaneously arising factor V inhibitor H1, which was associated with fatal hemorrhagic outcome (*29*), bound to the N-terminal region of the C2-domain and blocked binding of factor V to PS (*16*, *17*). The murine monoclonal antibody HV-1 and inhibitor H1 were found to bind to the same structural region in factor V, since HV-1 competes with the H1 inhibitor for binding to factor V and HV-1 also blocked binding of factor V to PS. The antibodies HV-1 and H1 rapidly neutralized the procoagulant activity of factor Va. In the present study, the epitope for the inhibitory monoclonal antibody HV-1 has been localized to Lys^{2060} through Glu^{2069} in the factor V C2-domain. This region was also localized to the PS-binding site in the crystal structure, which is consistent with the fact that monoclonal antibody HV-1 and inhibitor H1 blocked the binding of factor V to PS. These residues are part of a prominent loop, which adopts two extremely different conformations in the solved crystal structures. Solvent exposed, highly flexible regions are usually associated with enhanced antigenicity (*30*). The model for the factor V C2-domain depicted in Figure 8 shows the "open" crystal form. The flatter conformation of this loop in the "closed" crystal form is more typical of antibody

binding sites (*20*) (not shown). However, proof of the precise loop conformation recognized by HV-1 and H1 will require further experiments.

We have previously demonstrated that the monoclonal antibody 6A5 binds to an epitope in the central region of the factor V C2-domain that requires the presence of residues Ala^{2088}−Lys^{2148}. In the present study, we show that this epitope is composed of amino acids in two discontinuous segments of the C2-domain, Lys^{2092} through Glu^{2096} and His^{2128} through Lys^{2137}. On the basis of the crystal structure, the loop containing His^{2128} through Lys^{2137} between strands S4 and S5 seems to be the major epitope for monoclonal antibody 6A5 (*20*). Since Glu^{2096} interacts with the side chain of Arg^{2174} and the proper location of the Trp^{2136}−Arg^{2174}−Trp^{2094} sandwich is important for the conformation of the His^{2128}−Lys^{2137} loop, the mutation of Glu^{2096} might disrupt the His^{2128}−Lys^{2137} loop, and therefore, the epitope for 6A5 through a domino effect. Mutants within the 6A5 epitope retained >50% procoagulant activity (Figures 3 and 7), although slightly decreased binding to immobilized PS was observed for mutant (H2128, E2131)A, using the ELISA assay (Figure 6). We have previously demonstrated that 6A5 inhibits prothrombinase activity, but does not block the interaction of factor Va with PS. These data, together with the factor V C2-domain structure suggest that the amino acid residues comprising the 6A5 epitope are not directly involved in binding to factor Xa or PS, but that this epitope located near the factor Xa binding site in the factor Va light chain. Confirmation of this hypothesis will require characterization of purified factor Va mutants with more quantitative binding assays. The mutants described in this study should also prove valuable in characterizing the epitopes for other C2-domain antibodies and factor V inhibitors (*31*).

Finally, we have demonstrated that the PS-binding site in the factor V C2-domain includes amino acid residues Trp^{2063} and Trp^{2064}. First, mutation of these residues blocked the binding of factor V to immobilized PS. Second, prothrombinase assays in the presence of membranes containing limiting concentrations of PS confirmed that these residues are essential in the factor V-membrane interactions under these conditions. These findings support the model for the interaction of the factor V C2-domain with PS membranes that we have proposed based on the C2-domain crystal structures (*20*). This model predicts that tryptophan residues 2063 and 2064 may be inserted into the phospholipid bilayer during membrane recognition and binding. This membrane-binding site appears to be identical to the epitope for monoclonal antibody HV-1, which blocks factor V binding to PS (*17*). Interestingly, the association of the anticoagulant protein Annexin V with phospholipid membranes also involves direct interactions between a mobile tryptophan residue and the membrane bilayer (*32*). Substitution of alanine for tryptophan 185 in Annexin V results in a modest decrease in membrane-binding affinity, since at least five additional amino acid side chains also contribute to the binding interaction (*32*). We had also hypothesized that Leu^{2116}, which is located at the bottom of the second putative membrane-binding spike, also penetrated the lipid bilayer and contributed to membrane recognition and binding. We did not detect any functional defect associated with the L2116A mutation. It is possible that the major functional role of Leu^{2116} resides in the maintenance of the "closed" conformation of the C2-domain, by virtue of its van der Waals interactions with Trp^{2064}. The ϵ-amino group of Lys^{2061} was predicted to interact with the negative charge of the phosphate group of PS. Consistent with this model, mutant (K2060,2061)A did not bind to PS in the ELISA plate binding assay. However, mutant (K2060,2061)A expressed normal procoagulant activity even in the presence of limiting concentrations of phospholipid membranes. The precise roles of these two amino acid residues in PS binding will, therefore, require further clarification.

Previous studies have suggested that a membrane-binding peptide from the homologous factor VIII C2-domain forms an amphipathic alpha helical structure in the presence of SDS micelles (*33*) or dodecylphosphocholine micelles (*34*). This peptide corresponding to amino acid residues 2303−2324 in factor VIII and 2170−2191 in factor V. On the basis of the crystal structures for the C2-domains of factors V (*20*) and VIII (*35*), this sequence is not likely to adopt a helical structure in the absence of detergents, since it forms the S7 and S8 strands of the highly conserved antiparallel beta structure. Substititution of alanine for several of the charged residues within this region in factor V (Arg^{2171}, Arg^{2174}, and Glu^{2189}) leads to misfolding of the C2-domain an loss of PS binding and procoagulant activity (Figures 3, 4, and 6). In contrast, mutation of Lys^{2178} or Arg^{2187} does not appear to have a major effect on procoagulant activity. Interestingly, the loop between the S7 and S8 strands in factor V contains Asn^{2181}, which is partially glycosylated in the native protein, giving rise to the glycoforms factor Va_1 and factor Va_2 (*36*). This glycosylation site is located near the "bottom" of the factor V C2-structure (*20*). Factor Va_1, which is glycosylated at this site, has up to an 80-fold lower affinity for phosphatidylserine-containing membranes compared to factor Va_2 (*36*−*38*). Taken together, these data suggests that the loop between strands S7 and S8 may contribute either directly or indirectly to the PS-binding site in the factor V C2-domain.

The present study has localized several functionally important epitopes within the factor V C2-domain structure. Two tryptophan residues at the apex of one of three variable loops located on the "bottom" of the C2-domain are involved in binding to PS membranes. High-resolution mapping of these variable loops, combined mutations of putative interactive side chains, and detailed characterization of selected factor V mutants should help to further define the precise molecular basis for this important interaction.

ACKNOWLEDGMENT

We wish to thank Dr. Jane Richardson for assistance in the preparation of Figure 1. We also wish to thank Drs. Deborah Lewis and Thomas Ortel for critically reviewing this manuscript.

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BI992256R