Partial Glycosylation at Asparagine-2181 of the Second C-Type Domain of Human Factor V Modulates Assembly of the Prothrombinase Complex†

Suhng Wook Kim,‡ Thomas L. Ortel,‡ Mary Ann Quinn-Allen,‡ Lina Yoo,‡ Laura Worfolk,‡ Xin Zhai,§ Barry R. Lentz,§ and William H. Kane*,‡

Division of Hematology, Departments of Medicine and Pathology, Duke University Medical Center, Durham, North Carolina 27710, and Department of Biochemistry and Biophysics and The Program in Molecular and Cellular Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7260

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ABSTRACT: Thrombin-activated factor Va exists as two isoforms, factor Va_1 and factor Va_2, which differ in the size of their light chains and their affinity for biological membranes. The heterogeneity of the light chain remained following incubation of factor Va with *N*-glycanase. However, we found that the factor V C2 domain, which contains a single potential glycosylation site at Asn-2181, was partially glycosylated when expressed in COS cells. To confirm the structural basis for factor Va_1 and factor Va_2, we mutated Asn-2181 to glutamine (N2181Q) and expressed this mutant using a B domain deletion construct (rHFV des B) in COS cells. Thrombin activation of N2181Q released a light chain with mobility identical to that of factor Va_2 on SDS−PAGE. The functional properties of purified N2181Q were similar to those of factor Va_2 in prothrombinase assays carried out in the presence of limiting concentrations of phosphatidylserine. The binding of human factor Va_1 and factor Va_2 to 75:25 POPC/POPS vesicles was also investigated in equilibrium binding assays using proteins containing a fluorescein-labeled heavy chain. The affinity of human factor Va_2 binding to POPC/POPS vesicles was approximately 3-fold higher than that of factor Va_1. These results indicate that partial glycosylation of factor V at asparagine-2181 is the structural basis of the light chain doublet and that the presence of this oligosaccharide reduces the affinity of factor Va for biological membranes.

Thrombin-activated coagulation factor V is an essential component in the prothrombinase complex, which activates the zymogen prothrombin to thrombin. This complex consists of the enzyme factor Xa, the protein cofactor factor Va, calcium ions, and a phospholipid membrane surface (*1*, *2*). Human factor V circulates in plasma as a 330 kDa single-chain inactive precursor with the domain structure A1-A2-B-A3-C1-C2 (Figure 1) (*3*, *4*). Activation and inactivation of the cofactor are regulated by limited and specific proteolysis. Activation of factor V by thrombin or factor Xa results in the formation of an active heterodimer consisting of a heavy chain and a light chain held together by a tightly bound calcium ion. The heavy chain is derived from the amino-terminal region of factor V and has a molecular mass of 105 000 Da. The light chain is released from the carboxyl-terminal region and appears as a doublet with molecular masses of ∼71 and ∼74 kDa on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE)[1] (*5*−*11*).

In 1993, Rosing et al. (*12*) separated two forms of human factor Va, designated factor Va_1 and factor Va_2, that exclusively contained either the 74 or 71 kDa light chain, respectively. In the presence of limiting concentrations of membranes, the concentration of factor Va_1 required for half-maximal rates of prothrombin activation was approximately 10-fold higher than that of factor Va_2. Rosing et al. (*12*) concluded that the functional differences between factor Va_1 and factor Va_2 were due to differences in membrane binding properties since factor Va_1 bound to planar anionic phospholipid bilayers with a 45-fold lower affinity than factor Va_2. Subsequent studies by Hoekema et al. (*13*) have demonstrated that the overall procoagulant activity of factor Va_1 is at least 7 times higher than that of factor Va_2, in the presence of membranes containing 10% PS, since factor Va_1 is less susceptible to inactivation by APC. More recently, Koppaka et al. (*14*) characterized the membrane binding properties of bovine factor Va_1 and factor Va_2 and found only a 3−7-fold difference in the affinity of binding to anionic phospholipid vesicles. The difference between these two observations was hypothesized to be due either to differences between the bovine and human proteins or to

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* To whom correspondence should be addressed: Division of Hematology, Departments of Medicine and Pathology, DUMC-3656, Duke University Medical Center, Durham, NC 27710. Telephone: (919) 684-8952. Fax: (919) 681-6160. E-mail: william.kane@duke.edu.

‡ Duke University Medical Center.

§ University of North Carolina at Chapel Hill.

[1] Abbreviations: HFVa, plasma-derived human factor Va; rHFVa, recombinant human factor Va; rHFVa N2181Q, recombinant human factor Va with an Asn → Gln mutation at position 2181; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; APC, activated protein C; ELISA, enzyme-linked immunosorbent assay.

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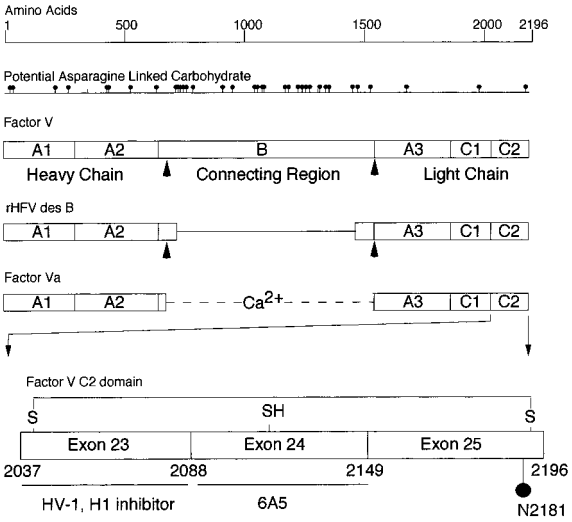

FIGURE 1: Structure of human factor V. The domain structures for factor V, rHFV des B, and thrombin-activated factor Va are depicted by the labeled boxes. The arrows indicate the locations of the thrombin cleavage sites at Arg-709 and Arg-1545. The thin line indicates deletion of amino acids 811−1491 in the rHFV des B construct. The locations of potential asparagine-linked glycosylation sites are denoted with the black circles at the top of the figure. An expanded schematic figure of the factor V C2 domain is shown below. Exons 23−25 of the factor V gene encode the C2 domain of factor V (*37*). The sequences within the C2 domain of factor V required for expression of epitopes for the factor V inhibitor H1 and the monoclonal antibodies HV-1 and 6A5 are shown below the diagram (*16*). The location of the glycosylation site at asparagine-2181 is indicated by the large labeled black circle.

differences in the physical state of the membrane lipids in the two binding assays. Taken together, these results indicate that the abilities of factor Va_1 and Va_2 to promote thrombin generation may differ significantly depending on the local concentrations of anionic phospholipid membranes, factor Xa, and APC.

The molecular basis for the observed heterogeneity of the factor Va light chain has not been elucidated. Previous data from our laboratory have strongly suggested that the observed heterogeneity originates from the carboxyl-terminal C2 domain of the factor Va light chain. We found that recombinant factor V releases a doublet light chain following activation with thrombin. However, deletion of the factor V C2 domain or replacement of the carboxyl terminal amino acids of the C2 domain (residues 2037−2196) with homologous factor VIII sequences resulted in a single light chain species (*15*, *16*). The presence of a potential asparagine-linked oligosaccharide attachment at asparagine-2181 suggested that the heterogeneity of the factor V light chain is due to alternative glycosylation at this site.

In this paper, we demonstrate that the molecular basis of the factor Va light chain doublet is alternative glycosylation at asparagine-2181. This information, together with recent progress in the elucidation of the structure and function of the C2 domain, provides important new insights into the molecular interactions regulating membrane-dependent assembly and inactivation of the prothrombinase complex.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes, vectors, cell culture media, and T4 DNA ligase were obtained from GIBCO/BRL (Gaithersburg, MD). Kits for DNA sequencing (Sequenase Version 2.0) and site-directed mutagenesis (T7-GEN In Vitro Mutagenesis) were from U.S. Biochemicals (Cleveland, OH). Human prothrombin, thrombin, and factor Xa were obtained from Haematologic Technologies Inc. (Essex Junction, VT). Molecular mass markers were obtained from Bio-Rad (Hercules, CA). 1-Palmitoyl-2-oleoylphosphatidylserine (POPS) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) were from Avanti Polar Lipids (Alabaster, AL). Thromboplastin-L was from Pacific Hemostasis (Huntersville, NC). All other reagents were from Sigma (St. Louis, MO).

Phospholipid Vesicle Preparation. Phospholipid vesicles were prepared as described previously (*17*) with slight modification. Appropriate amounts of POPC and POPS in a chloroform/methanol solution were pipetted with glass syringes (Hamilton, Reno, NV) into Corex tubes (Corning, Corning, NY). The chloroform was evaporated under a stream of nitrogen gas, and the dried phospholipids were resuspended in the appropriate buffer. The lipid suspension was then sonicated under a mild stream of nitrogen for 10 min in an ice bath. After sonication, the suspension was centrifuged at 72 000 rpm for 25 min at 15 °C in a Beckman TL100 centrifuge, yielding a homogeneous suspension of vesicles. Phospholipid concentrations were determined with the phosphate assay described by Gomori (*18*).

Construction and Expression of Factor V Mutants. The codon encoding asparagine-2181 in the factor V cDNA was mutated to encode glutamine using standard techniques (*19*). The mutagenic oligonucleotide was 5′-ATTCCTAAAA-CATGGCAACAAAGTATTGCACTT-3′. The mutation was confirmed by DNA sequencing and inserted into the deletion mutant lacking residues 811−1491 in the B domain (rHFV des B) (Figure 1) (*20*). Wild-type and N2181Q mutant constructs were subcloned into the expression vector pDX and expressed in COS-7 cells as previously described (*20*). The C2 domain of factor V was also expressed in COS cells using a pDX expression construct (*21*). Serum free conditioned medium (DMEM/F12) containing 5 mg/mL bovine serum albumin and 2.5 mM $CaCl_2$ was harvested 48−96 h after transfection and was stored at −70 °C until it was used. Factor V antigen was quantitated using a monoclonal antibody-based ELISA with monoclonal antibodies HV-1 and 6A5 being used for capture and detection, respectively (Figure 1) (*16*). Clotting assays for factor V procoagulant activity were performed as previously described (*21*).

Metabolic Labeling and Immunoprecipitation of Recombinant Proteins. Recombinant proteins expressed in COS cells were labeled with [^{35}S]methionine as previously described (*15*, *16*, *20*, *21*). Aliquots of conditioned medium containing labeled rHFV des B were activated with 3 units/mL thrombin for 5 min at 37 °C, followed by the addition of 50 μM PPACK. The rHFV des B and rHFVa were then immunoprecipitated using a polyclonal anti-factor V antibody and analyzed by SDS−PAGE and autoradiography (*20*). Recombinant factor Va was deglycosylated using the enzyme *N*-glycanase. Recombinant factor Va from 1 mL of conditioned medium was first immunoprecipitated and then resuspended in 170 μL of a buffer containing 0.5% SDS, 50 mM 2-mercaptoethanol, 0.1 mM PMSF, and 0.1 M Tris-HCl (pH 7.5). The protein was then denatured by heating at 100 °C for 5 min. Triton X-100 was added to a final concentration of 3.5%, and the sample was incubated with 0.3 unit of *N*-glycanase for 18 h at 37 °C, as recommended

by the manufacturer (Genzyme). The reaction was then stopped by boiling the sample in the presence of SDS and 2-mercaptoethanol. The samples were analyzed by SDS−PAGE and autoradiography. To investigate the glycosylation of the factor V C2 domain, we expressed rHFV C2 in the presence of tunicamycin. COS-7 cells expressing rHFV C2 in the presence or absence of 10 μg/mL tunicamycin were labeled overnight with [^{35}S]methionine. Recombinant proteins were then immunoprecipitated and analyzed by SDS−PAGE and autoradiography as previously described.

Purification. Human factor V was purified essentially as described by Kane (*22*) with slight modifications (*23*). Factor Va was prepared by incubating factor V (1.2 mg) for 30 min with thrombin (2.8 μg). After activation, 60 nM PPACK was added to inactivate the thrombin. The factor Va was then purified on a Mono S HR 5/5 ion-exchange column (Pharmacia, Piscataway, NJ) to separate factor Va_1 and factor Va_2 as described previously (*12*). To purify recombinant factor V, conditioned medium containing wild-type or mutant factor V was applied to a SP-Sepharose Fast-Flow FPLC column (26 mm × 10 cm) at a flow rate of 3 mL/min. The column was then washed with 1.0 L of a buffer containing 50 mM NH_4Cl, 5 mM $CaCl_2$, and 25 mM HEPES (pH 7.5). The recombinant proteins were eluted with a 250 mL linear salt gradient (50 to 1000 mM NH_4Cl) at a flow rate of 3 mL/min. Fractions containing factor V activity were pooled and concentrated using an Amicon concentrator with a PM-10 membrane and then diluted 5-fold in 50 mM NaCl, 10 mM $CaCl_2$, and 20 mM Tris-HCl (pH 7.4). The samples were then applied to a Mono Q HR 5/5 FPLC column equilibrated with the same buffer at a flow rate of 0.5 mL/min. Bound proteins were eluted using a linear salt gradient (50 to 1000 mM NaCl), and 0.5 mL fractions were collected and assayed for factor V activity with a clotting assay. Fractions containing >5 units/mL factor V were pooled and stored at −70 °C until further use. Purified recombinant factor V and factor Va were characterized by SDS−PAGE using 7.5% acrylamide gels as described previously (*24*).

Prothrombinase Assay. Factor Va procoagulant activity was determined by measuring the rate of thrombin generation catalyzed by prothrombinase in the reaction mixture containing 3 pM factor Xa, 1.4 μM prothrombin, 0.5 μM phospholipid vesicles, and varying concentrations of factor Va in 20 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 1 min, and then the reactions were quenched by adding 10 μL of 50 mM EDTA and 200 mM MOPS (pH 7.4). Thrombin activity was determined by incubating test samples with 50 μL of 0.5 mM S2238 (Chromogenix, Molndal, Sweden) and measuring the absorbance at 405 nm using a V_{max} kinetic microtiter reader (Molecular Devices, Menlo Park, CA). Thrombin concentrations were determined using a standard curve prepared with purified human thrombin.

Fluorescence Anisotropy Measurements. Purified factor Va_1 and factor Va_2 were dialyzed into 20 mM Tris-HCl (pH 7.4) and 1 mM EDTA, and the respective heavy and light chain subunits were isolated by anion exchange chromatography on a FPLC Mono-Q HR 5/5 column in the presence of 1 mM EDTA. The purified heavy chain of factor Va was then labeled with fluorescein−5-maleimide (Molecular Probes) as described by Krishnaswamy (*25*) with minor modifications (*14*, *17*). A 2.2 μL aliquot of a stock solution of fluorescein−5-maleimide (58.5 mM in *N*,*N*-dimethylformamide) was added to 200 μL of buffer [20 mM TES, 150 mM NaCl, and 5 mM $CaCl_2$ (pH 7.4)] and mixed well. Factor Va heavy chain (600 μL) in the same buffer (1.2 mg/mL) was treated with this probe solution, and the mixture was then incubated in the dark at 4 °C for 18 h with constant mixing. The modified protein was then dialyzed extensively in 20 mM TES, 150 mM NaCl, and 5 mM $CaCl_2$ (pH 7.4) at 4 °C and stored at −80 °C. The concentration of labeled factor Va heavy chain was determined using the Bradford assay (Bio-Rad), and the molar ratio of fluorescein to protein (0.88) was calculated using the molar absorptivity of protein-conjugated fluorescein at 492 nm (ϵ = 83 000 M^{-1} cm^{-1}; Molecular Probes). Factor Va_1 and factor Va_2 were reconstituted using a fluorescein-labeled heavy chain and unlabeled light chains in a molar ratio of 1.2:1 in a buffer composed of 20 mM TES, 150 mM NaCl, and 5 mM $CaCl_2$ (pH 7.4). The purity of the fluorescein-labeled factor Va heavy chain and light chain was established on the basis of SDS−PAGE. Activity of reconstituted factor Va was evaluated with the clotting assay. The binding of the two forms of factor Va to phospholipid vesicles was followed by monitoring the fluorescence anisotropy of the labeled proteins as increasing concentrations of phospholipid vesicles were added (*14*). Two data sets were fit using the SCoP package (Simulation Control Program; Simulation Resources, Berrien Spring, MI) as described previously (*14*).

RESULTS

N-Glycanase Treatment. To determine whether the heterogeneity of the factor Va light chain is due to glycosylation, we treated recombinant factor Va with *N*-glycanase, an enzyme that removes asparagine-linked glycans from protein substrates (*26*). Analysis of the digestion products by SDS−PAGE revealed that deglycosylation increased the mobility of the factor Va heavy chain, resulting in a doublet with an apparent molecular mass of 91−87 kDa (Figure 2). The mobility of the factor Va light chain also increased; however, the product appeared as a doublet with an apparent molecular mass of 69−67 kDa. On the basis of the amino acid sequence, the predicted molecular masses of the deglycosylated heavy and light chains are 80 and 71 kDa, respectively. Therefore, in this experiment, the heavy chain does not appear to be completely deglycosylated. In contrast, the larger component of the light chain doublet is close to the predicted size of the deglycosylated product. These results suggest that the heterogeneity of the light chain may be unrelated to oligosaccharide heterogeneity. Alternatively, the light chain may migrate anomalously and be resistant to complete deglycosylation by *N*-glycanase (*27*).

C2 Domain Expression in COS Cells. Since indirect evidence suggested that the heterogeneity of the factor Va light chain was due to post-translational modifications at the carboxyl terminus of the light chain, we expressed the C2 domain in COS cells and analyzed the recombinant protein by immunoprecipitation and SDS−PAGE (Figure 3). The recombinant C2 domain appeared as a 21−18 kDa doublet. Tunicamycin inhibits the lipid-linked pathway for asparagine-linked glycosylation by blocking the first step in the synthesis of the oligosaccharide−lipid intermediate (*28*). When rHFV C2 was expressed in the presence of tunicamycin, only the 18 kDa component was observed, suggesting that the 21−

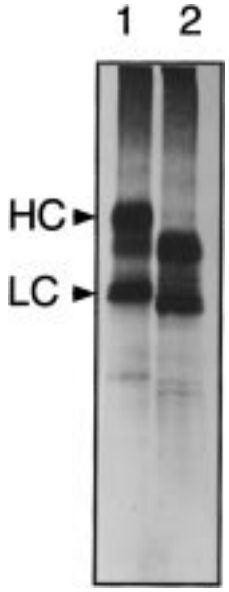

FIGURE 2: Treatment of recombinant factor Va with *N*-glycanase. COS-7 cells were transfected with an expression vector encoding B domain-deleted factor V (rHFV des B) and then labeled with [^{35}S]methionine. Recombinant factor V was immunoprecipitated from conditioned medium using a rabbit polyclonal anti-human factor V antibody and activated with thrombin as described in Experimental Procedures. Factor Va was then incubated in the presence or absence of 1750 milliunits/mL *N*-glycanase for 18 h at 37 °C. The recombinant factor Va was then analyzed by SDS−PAGE on 5 to 12% acrylamide gradient gels: lane 1, recombinant factor Va; and lane 2, recombinant factor Va treated with *N*-glycanase.

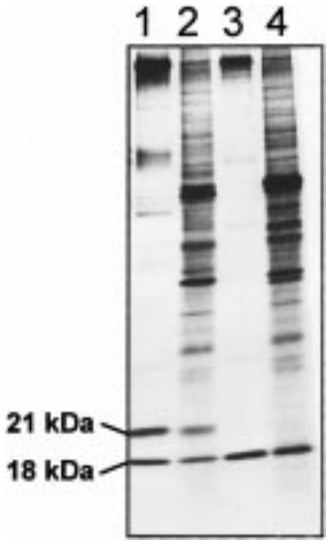

FIGURE 3: Expression of rHFV C2 in COS cells. COS-7 cells were transfected with an expression vector encoding the C2 domain of factor V (rHFV C2) and then labeled with [^{35}S]methionine in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 10 μg/mL tunicamycin as described in Experimental Procedures. Recombinant proteins in the conditioned medium (lanes 1 and 3) or cell lysates (lanes 2 and 4) were then immunoprecipitated using a rabbit polyclonal anti-human factor V antibody and analyzed by SDS−PAGE on 8 to 12% acrylamide gradient gels.

18 kDa doublet observed in the absence of tunicamycin is due to partial glycosylation of the C2 domain.

Site-Directed Mutagenesis. We had previously determined that the structural basis of the light chain doublet was located within the C2 domain, and using recombinant factor V−factor VIII chimeras, we further localized the structural basis

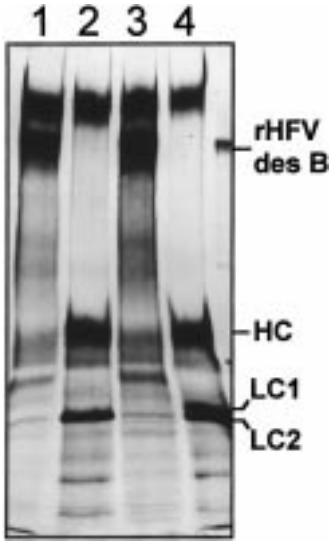

FIGURE 4: Expression of rHFV des B N2181Q. COS-7 cells were transfected with an expression vector encoding rHFV des B N2181Q (lanes 1 and 2) or rHFV des B (lanes 3 and 4) and then labeled with [^{35}S]methionine as described in Experimental Procedures. Conditioned medium was incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 2 nM thrombin for 2 min at 37 °C, and the recombinant proteins were then immunoprecipitated using a rabbit polyclonal anti-human factor V antibody. Recombinant proteins were analyzed by SDS−PAGE on 5 to 12% acrylamide gradient gels. The migration of single-chain rHFV des B, the factor V heavy chain (HC), the factor Va_1 light chain (LC1), and the factor Va_2 light chain (LC2) is depicted. The 270 kDa band present in all four lanes is nonspecifically immunoprecipitated (*20*).

of the doublet to the 50 carboxyl-terminal amino acids of the domain. Since N-linked glycosylation usually occurs at asparagine residues in Asn-X-Ser/Thr consensus sequences where X is any amino acid except Pro, this region contains a single potential N-linked glycosylation site at Asn-2181 which is not conserved in the factor VIII C2 domain. Therefore, site-directed mutagenesis was utilized to mutate this amino acid to a glutamine (N2181Q), and the resultant mutant was expressed in a B domain deletion construct (rHFV des B) in COS cells. Immunoprecipitation of a thrombin-activated [^{35}S]methionine metabolically labeled N2181Q mutant revealed that the light chain was a single band of ~71 kDa, similar to the lower band of the light chain doublet from rHFVa (Figure 4).

Isolation and Characterization of Recombinant Factor Va N2181Q. Factor V B domain deletion mutants (rHFV des B) containing the wild-type sequence or the N2181Q mutation were expressed in COS-7 cells as described in Experimental Procedures. The N2181Q mutation did not affect the level of recombinant factor V secreted into the conditioned medium, and typically, 1 L of pooled conditioned medium was used for isolation of the recombinant proteins. We next used ion exchange chromatography on a Mono S column to isolate factor Va_1 and factor Va_2 from thrombin-activated HFVa, rHFVa, and the N2181Q mutant. Figure 5 shows the Mono S elution pattern of $HFVa_1$ and $HFVa_2$, $rHFVa_1$ and $rHFVa_2$, and rHFVa N2181Q. When developed with a linear gradient of NH_4Cl (50 to 1000 mM), factor Va activity was eluted from the column in two well-separated protein peaks at 450 and 750 mM NH_4Cl for plasma-derived factor Va and at 600 and 800 mM NH_4Cl for recombinant wild-type factor Va. These experiments demonstrate that plasma-derived factor Va and recombinant factor Va have

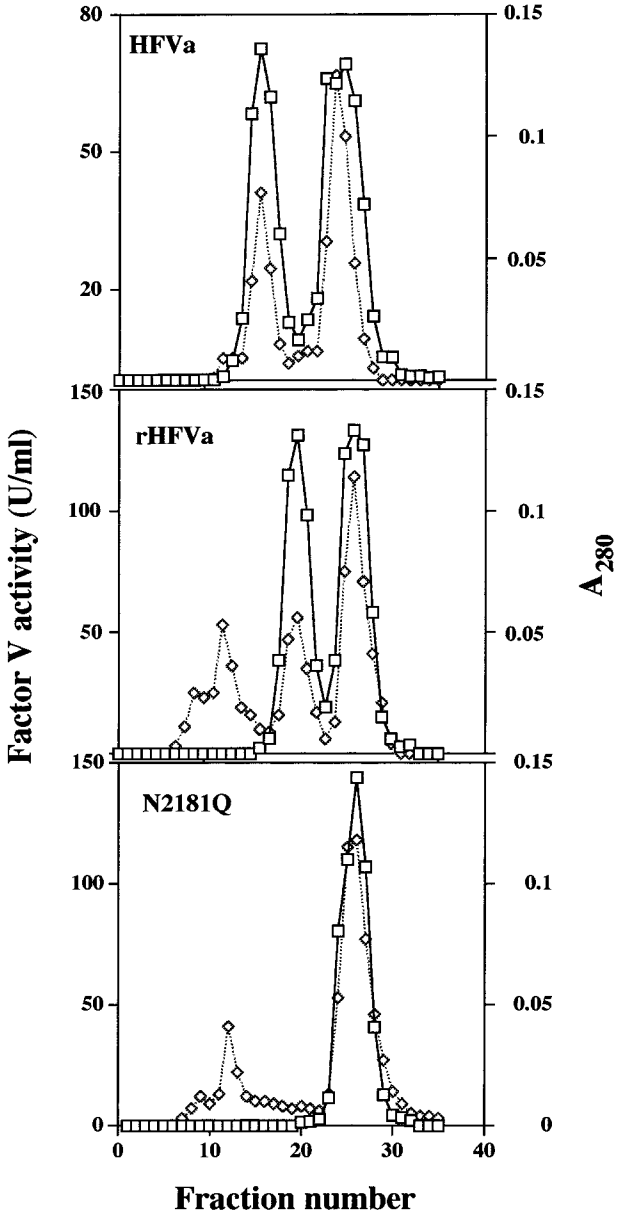

FIGURE 5: Chromatography of factor Va on a Mono S column. Factor Va was purified by FPLC using a Mono S column as described in Experimental Procedures. Factor Va was eluted using a 15 mL NH_4Cl gradient (from 0.05 to 1.0 M NH_4Cl). Fractions (0.5 mL) were collected, and the absorbance at 280 nM (◇) and the factor V clotting activity (□) were determined.

slightly different cationic properties, most likely due to differences in post-translational modification. SDS−PAGE analysis showed that the first peak consisted of factor Va molecules with a light chain with an M_r of 74 kDa and that the factor Va that eluted in the second peak contained the light chain with an M_r of 71 kDa for both plasma-derived factor Va and recombinant wild-type factor Va (Figure 6). In contrast, when thrombin-activated N2181Q was fractionated by chromatography on a Mono S column, a single protein peak eluted at 800 mM NH_4Cl. Analysis of this peak by SDS−PAGE revealed a single 71 kDa light chain with mobility identical to that of factor Va_2.

Comparison of Cofactor Activities. Since factor Va_1 and factor Va_2 express different cofactor activities at limiting concentrations of factor Xa and phospatidylserine, we compared the cofactor activities of plasma-derived and recombinant factor Va_1 and factor Va_2 to that of the

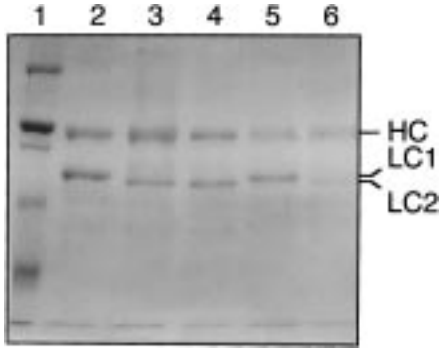

FIGURE 6: SDS−PAGE analysis of purified factor Va_1, factor Va_2, and rHFVa N2181Q. Factors Va_1 and Va_2 were purified using a FPLC Mono S column and analyzed by SDS−PAGE using a 7.5% acrylamide gel followed by Coomassie staining: lane 1, molecular mass markers (Bio-Rad, SDS−PAGE standards, high range; 200, 116.3, 97.4, 66.2, and 45 kDa); lane 2, $HFVa_1$; lane 3, $HFVa_2$; lane 4, rHFVa N2181Q; lane 5, $rHFVa_1$; and lane 6, $rHFVa_2$. The mobilities of the heavy chain (HC) and light chain (LC) are shown on the right side of the figure.

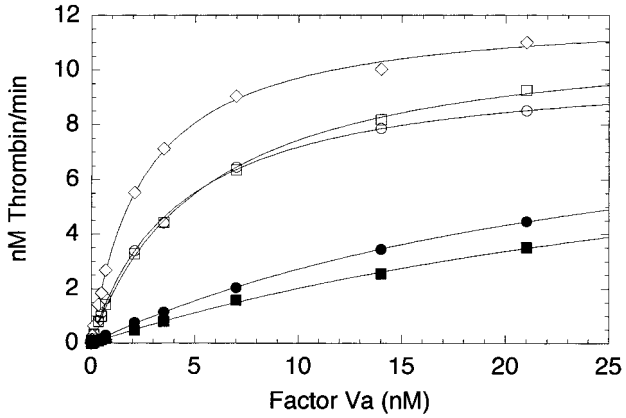

FIGURE 7: Cofactor activity of factor Va N2181Q. Rates of thrombin generation were determined in a reaction mixture containing 20 mM Tris (pH 7.4), 0.15 M NaCl, 2.7 mM KCl, 10 mg/mL BSA, 3 mM $CaCl_2$, 3 pM factor Xa, 0.5 μM phospholipid vesicles (POPC/POPS, 95:5 molar ratio), 1.4 μM prothrombin, and amounts of $HFVa_1$ (●), $HFVa_2$ (○), $rHFVa_1$ (■), $rHFVa_2$ (□), or rHFVa N2181Q (◇). The curves were derived by fitting data into the Michaelis−Menten equation using the Kaleidagraph software package.

Table 1: Kinetic Analysis of Factor Va Cofactor Activity

	0.5 μM PC/PS (95:5)		0.5 μM PC/PS (75:25)	
	$K_{1/2Va}$ (nM)	V_{max} (nmol of IIa/min)	$K_{1/2Va}$ (nM)	V_{max} (nmol of IIa/min)
$HFVa_1$	29.1	10.6	0.67	12.3
$HFVa_2$	4.26	10.2	0.33	12.0
$rHFVa_1$	41.1	10.3	1.61	13.4
$rHFVa_2$	5.49	11.5	0.40	12.7
rHFVa N2181Q	2.54	12.2	0.22	11.9

thrombin-activated N2181Q mutant. Half-maximal rates of prothrombin activation in the presence of 3 pM factor Xa and 0.5 μM POPC/POPS (95:5 molar ratio) were observed at 41.1 nM $rHFVa_1$, 5.49 nM $rHFVa_2$, 29.1 nM factor Va_1, 4.26 nM factor Va_2, and 2.54 nM rHFVa N2181Q (Figure 7 and Table 1). The differences between the two forms of factor Va were reduced when the experiment was performed using phospholipid vesicles containing a higher mole percentage of phosphatidylserine (0.5 μM POPC/POPS, 75:25 molar ratio) (Table 1). In both cases, the cofactor activities

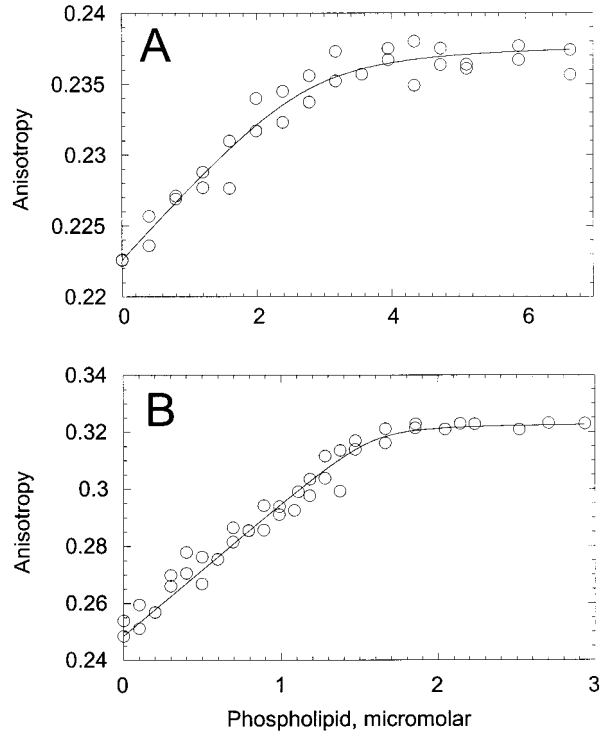

FIGURE 8: Titration of fluorescein-labeled factor Va with phospholipid vesicles. Effect of increasing concentrations of POPC/POPS (75:25) on the fluorescence anisotropy of fluorescein-labeled factor Va_1 (A) and Va_2 (B). The initial concentration of Va was 0.1 μM in 20 mM TES, 150 mM NaCl, and 2 mM $CaCl_2$ (pH 7.4). The solid lines represent simulations using SCoP to fit globally. The binding parameters are as follows: K_d for Va_1 = 5.1 nM and the stoichiometry for Va_1 is 27 phospholipids per site, and K_d for Va_2 = 1.6 nM and the stoichiometry for Va_2 is 17.3 phospholipids per site.

of plasma-derived factor Va_1 were similar to that of $rHFVa_1$ and the cofactor activity of factor Va_2 was similar to that of $rHFVa_2$ and rHFVa N2181Q. The maximum rates of prothrombin activation were similar when saturating concentrations of factor Va_1, factor Va_2, or rHFVa N2181Q were used (data not shown).

Membrane Binding Properties. We have characterized the binding of human factor Va_1 and factor Va_2 to phospholipid vesicles in equilibrium binding experiments using the fluorescence anisotropy of the fluorescein-labeled protein (see Experimental Procedures). Titration curves obtained using fixed concentrations of fluorescein−Va and varying concentrations of POPC/POPS (75:25) are shown in Figure 8. An increase in anisotropy values was seen in both cases, and saturable behavior was evident at high vesicle concentrations. Since we had two sets of data for each form of factor Va, the values of the dissociation constant and stoichiometry were obtained for the phospholipid vesicles by global analysis of these data sets using SCoP. The global analysis using SCoP yielded a K_d of 5.1 nM and a stoichiometry of 27 phospholipids per site for factor Va_1, and a K_d of 1.6 nM and a stoichiometry of 17.3 phospholipids per site for factor Va_2. These data show that the binding of human factor Va_2 to POPC/POPS (75:25) vesicles was about 3-fold tighter than that of factor Va_1 at 2 mM $CaCl_2$ and that factor Va_2 occupied slightly less area on the membrane surface. The membrane binding properties of rHFVa N2181Q were not characterized in this study since this mutant appeared to be functionally similar to factor Va_2.

DISCUSSION

Partial N-linked glycosylation is frequently observed in many eukaryotic proteins (*29*). Partial glycosylation results in the synthesis of heterogeneous glycoprotein variants, which differ from one another in the number and position of N-linked oligosaccharides. In some cases, differences in glycosylation have no apparent effect on the functional behavior of the protein (*30*, *31*). However, in other cases, this may offer an advantage, since individual glycoforms may have unique physical or biological properties (*32*, *33*).

Activation of purified human factor V by thrombin results in release of a 105 kDa heavy chain and two forms of the light chain ($M_r \sim$ 74 and 71 kDa). This light chain doublet has been reported previously several times, in both human and bovine factor Va (*6*, *9*−*11*, *22*). This heterogeneity of the light chain domain was also reported to be an intrinsic property of both plasma and platelet factor Va (*12*). The structural basis for the light chain doublet in factor Va had been hypothesized to be either a proteolytic event at the carboxyl terminus of the light chain or the synthesis of two different species of factor Va (*11*).

The results presented in this paper clearly demonstrate that the two isoforms of factor Va result from partial glycosylation at asparagine-2181. First, we expressed the factor V C2 domain in COS cells since we had previously localized the structural basis of the light chain doublet to this domain and demonstrated that this domain plays an important role in binding of factor V to phosphatidylserine (*15*, *16*, *21*). The factor V C2 domain is expressed as a 21−18 kDa doublet, indicating that two species of the C2 domain are synthesized. When the factor V C2 domain is expressed in the presence of tunicamycin, only the 18 kDa species is observed which indicates that the 21 kDa species results from partial glycosylation of the C2 domain. Second, we have previously demonstrated that the thrombin-generated recombinant factor Va light chain also appeared as a doublet on SDS−PAGE (*20*). We found that the heterogeneity of the recombinant factor Va light chain remained following treatment with N-glycanase, an enzyme that removes asparagine-linked oligosaccharide chains. Resistance of the light chain to complete deglycosylation could explain these results. We therefore used site-directed mutagenesis to mutate the potential glycosylation site at asparagine-2181. As expected, the N2181Q mutant released only the 71 kDa light chain following activation with thrombin. Third, we purified factor Va_1 and factor Va_2 from plasma-derived factor Va, from recombinant factor Va, and from the N2181Q mutant. The characteristic elution pattern on Mono S chromatography and SDS−PAGE analysis clearly demonstrates that the N2181Q mutant contains a single light chain with mobility similar to that of factor Va_2. Fourth, the N2181Q mutant was functionally indistinguishable from wild-type factor Va_2 in its ability to enhance the factor Xa-catalyzed activation of prothrombin in the presence of limiting concentrations of anionic phospholipid vesicles and factor Xa. We have previously demonstrated that the properties of recombinant factor Va were similar to those of the plasma-derived protein based on procoagulant activity (*20*) and APC inactivation.[2] In the

[2] C. J. Wei et al. (1999), manuscript in preparation.
[3] S. Macedo-Ribeiro et al. (1999), manuscript submitted for publication.
[4] S. W. Kim et al. (1999), manuscript submitted for publication.

study presented here, we found that recombinant factor Va_1 and factor Va_2 elute at slightly higher salt concentrations during Mono S chromatography, compared to the corresponding plasma-derived proteins. This suggests differences in post-translational modification. However, the cofactor activities and migration of the heavy and light chains on SDS−PAGE are virtually identical for the recombinant and plasma-derived proteins.

Although the potential N-linked glycosylation site at asparagine-2181 is conserved in human (*34*), bovine (*35*), and murine (*36*) factor V, there are significant species differences between the reported membrane binding affinities of factor Va_1 and factor Va_2. In 1993, Rosing et al. (*12*) reported that the two forms of human factor Va differed 45-fold in their affinity for planar phospholipid bilayers containing DOPS/DOPC (20:80). In contrast, Koppaka et al. (*14*) recently reported that bovine factors Va_1 and Va_2 differed only 3−7-fold in their affinity for small unilamellar phospholipid vesicles containing 25% phosphatidylserine. Furthermore, evidence suggesting that bovine factor Va_1 and factor Va_2 may also differ to some extent in their interactions with factor Xa was presented (*14*). In this paper, we find that human factor Va_2 binds to small unilamellar vesicles containing POPC/POPS (75:25) with approximately 3-fold higher affinity than human factor Va_1, similar to data reported by Koppaka et al. (*14*). The observed differences in binding affinity are consistent with the 2-fold difference in factor Va concentration required for half-maximal rates of prothrombin activation in the presence of phospholipid vesicles containing 25% phosphatidylserine. The quantitative differences between our results and those reported by Rosing et al. (*12*) are likely due to differences in the physical state of the phospholipid vesicles used here and by Koppaka et al. (*14*) compared to the planar phospholipid bilayers used in the work of Rosing et al. (*12*). The functional differences between factor Va_1 and factor Va_2 are enhanced as the concentration of phosphatidylserine in the membrane decreases and as the concentration of factor Xa becomes limiting (Table 1) (*12*−*14*). Since the exposure of phosphatidylserine is thought to be a limiting factor during blood coagulation, the alternative glycosylation of the factor Va light chain at asparagine-2181 may modulate both assembly and inactivation of the prothrombinase complex in vivo (*12*, *13*). On the basis of the recently solved crystal structures of the nonglycosylated human factor V C2 domain, we can now locate asparagine-2181 in the structure.[3] Interestingly, asparagine-2181 is located in close proximity to the putative phosphatidylserine-binding site.[4] Therefore, since asparagine-linked oligosaccharide chains can be considered bulky hydrophilic structures, it is possible that glycosylation of asparagine-2181 directly interferes with the interaction of factor Va with phosphatidylserine. Alternatively, the presence of this oligosaccharide chain could have effects on the conformation of the C2 domain that might lead to impaired membrane binding. Finally, the possibility still exists that the oligosaccharide chain alters the interaction between factor Va and factor Xa on a membrane surface.

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