Partial Glycosylation of Asn^{2181} in Human Factor V as a Cause of Molecular and Functional Heterogeneity. Modulation of Glycosylation Efficiency by Mutagenesis of the Consensus Sequence for N-Linked Glycosylation†

Gerry A. F. Nicolaes,* Bruno O. Villoutreix, and Björn Dahlbäck

Wallenberg Laboratory, Department of Clinical Chemistry, Malmö University Hospital, Lund University, Malmö, Sweden

Received May 20, 1999; Revised Manuscript Received August 16, 1999

ABSTRACT: Coagulation factor V (FV) circulates in two forms, FV1 and FV2, having slightly different molecular masses and phospholipid-binding properties. The aim was to determine whether this heterogeneity is due to the degree of glycosylation of Asn^{2181}. FVa1 and FVa2 were isolated and digested with endoglycosidase PNGase F. As judged by Western blotting, the FVa2 light chain contained two N-linked carbohydrates, whereas FVa1 contained three. Wild-type FV and three mutants, Asn^{2181}Gln, Ser^{2183}Thr, and Ser^{2183}Ala, were expressed in COS1 cells, activated by thrombin, and analyzed by Western blotting. Wild-type FVa contained the 71 kDa−74 kDa doublet, whereas the Asn^{2181}Gln and Ser^{2183}Ala mutants contained only the 71 kDa light chain. In contrast, the Ser^{2183}Thr mutant gave a 74 kDa light chain. This demonstrated that the third position in the Asn-X-Ser/Thr consensus affects glycosylation efficiency, Thr being associated with a higher degree of glycosylation than Ser. The Ser^{2183}Thr mutant FVa was functionally indistinguishable from plasma-purified FVa1, whereas Asn^{2181}Gln and Ser^{2183}Ala mutants behaved like FVa2. Thus, the carbohydrate at Asn^{2181} impaired the interaction between FVa and the phospholipid membrane, an interpretation consistent with a structural analysis of a three-dimensional model of the C2 domain and the position of a proposed phospholipid-binding site. In conclusion, we show that the FV1−FV2 heterogeneity is caused by differential glycosylation of Asn^{2181} related to the presence of a Ser rather than a Thr at the third position in the consensus sequence of glycosylation.

Blood coagulation factor V (FV)[1] is a 330 kDa single-chain plasma glycoprotein that circulates in human blood at a concentration of 21 nM (*1*). Its domain organization, A1−A2−B−A3−C1−C2, is similar to that of factor VIII (FVIII), and the two proteins share ∼40% amino acid sequence identity in their A and C domains (*2*). Upon activation of FV via limited proteolysis mediated by either thrombin, meizothrombin, or factor Xa (*3*−*8*), the active procoagulant cofactor, factor Va (FVa), is formed (Figure 1). FVa is a heterodimeric molecule composed of a heavy chain (A1−A2) noncovalently linked to a light chain (A3−C1−C2), the chain association being dependent on tightly bound calcium (*9, 10*). In the prothrombinase complex, i.e., factor Xa and FVa assembled on a phospholipid surface in the presence of calcium ions, FVa enhances the rate of prothrombin activation by more than 3 orders of magnitude (*11, 12*). Intact FV has only limited cofactor activity in prothrombin activation (*11*). It was recently found that FV not only works as a procoagulant protein but also plays a role as an anticoagulant molecule in the protein C anticoagulant pathway. There, together with protein S, it functions as a synergistic cofactor of activated protein C (APC) in the proteolytic inactivation of coagulation FVIIIa (*13, 14*).

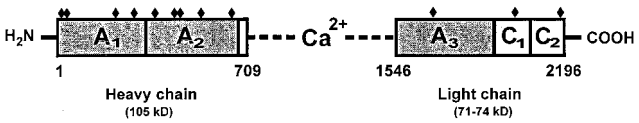

FIGURE 1: Schematic representation of the human FVa structure. The A and C domains are illustrated with their amino acid residue boundaries given. Diamonds denote the 12 potential N-glycosylation sites (◆). Nine of these sites are located in the heavy chain and three in the light chain, at positions Asn^{1675}, Asn^{1982}, and Asn^{2181}.

FV in human plasma and platelets is present in two forms, FV1 and FV2, having slightly different molecular masses and charges (*15*). The FV1 form is slightly bigger than FV2, and the FV1:FV2 ratio in blood is approximately 30:70. The heterogeneity has been found to reside in the light chain, which on SDS−polyacrylamide gel electrophoresis (SDS−PAGE) migrates as a closely spaced 71 kDa−74 kDa doublet. The two FVa forms, FVa1 (74 kDa light chain) and FVa2 (71 kDa light chain), can be separated by cationic exchange chromatography, suggesting the molecular mass

† This work was supported by a grant from the Niels Stensen Stichting, Amsterdam, The Netherlands (G.A.F.N.) and by grants from the Swedish Medical Research Council (Projects 07143 and 11793), the Louis Jeantet Foundation of Medicine, a Senior Investigator award from the Strategic funds, the Alfred Österlund Trust, the Albert Påhlsson Trust; Ax:son Johnsons Trust, research funds (general and cancer funds) from the University Hospital in Malmö, and the Swedish National Network for Cardiovascular Research.

* To whom correspondence should be addressed: The Wallenberg Laboratory, Department of Clinical Chemistry, Malmö University Hospital, Lund University, S-205 02 Malmö, Sweden. Fax: +46 40 337044. E-mail: Gerry.Nicolaes@klkemi.mas.lu.se.

[1] Abbreviations: BSA, bovine serum albumin; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; FV, coagulation factor V; FVa, activated FV; Gal, galactose; GlcNAc, *N*-acetylglucosamine; KIU, kallikrein inhibitor unit; Man, mannose; PMSF, phenylmethanesulfonyl fluoride; PS, phosphatidylserine; S2238, D-Phe-(pipecolyl)-Arg-pNA; SDS, sodium dodecyl sulfate; SDS−PAGE, SDS−polyacrylamide gel electrophoresis.

10.1021/bi991165r CCC: $18.00
Published on Web 09/23/1999

difference between the two is associated with a surface charge difference. Ortel and colleagues, using recombinant FV variants, demonstrated that the FVa light chain heterogeneity resides in the C2 domain (*16*, *17*). In a meeting abstract from 1994, the same group hypothesized that the molecular mass heterogeneity was due to glycosylation differences (*18*). A single potential site for N-linked glycosylation is present in the C2 domain at Asn^{2181}, whereas two additional sites are present in the FVa light chain, at Asn^{1675} and Asn^{1982} (Figure 1).

The two forms of FV are functionally distinct, which is related to different phospholipid binding properties (*15*, *19*, *20*). FVa1 binds negatively charged phospholipid membranes less efficiently than FVa2, causing FVa1 to have a lower procoagulant potency than FVa2. This is particularly pronounced at low phospholipid concentrations, or a low percentage of phosphatidylserine (PS) in the membrane. In contrast, at high phospholipid levels and/or with a high PS content, little difference in phospholipid binding and procoagulant activity is observed. The two forms of FV also differ in their abilities to express anticoagulant APC cofactor activity, the FV1 form being less efficient than FV2. This indicates the anticoagulant activity of FV depends on the ability of FV to interact with phospholipid membranes.

FV is synthesized in the liver, and before secretion, the molecule undergoes several functionally important posttranslational modifications, including sulfation (*21*, *22*), phosphorylation (*23*), and glycosylation (*24*, *25*). FV is highly glycosylated, carrying both N- and O-linked carbohydrate side chains, the total carbohydrate content being estimated to account for 13−25% of the molecular mass (*25*). The majority (25) of the 37 potential N-linked glycosylation sites are located in the B domain, the remaining being distributed between the heavy chain (nine) and the light chain (three) (*2*, *26)*. During glycosylation of Asn, a dolichol-linked high-mannose oligosaccharide is transferred to an Asn residue, which is part of a tripeptide consensus sequence for N-linked glycosylation, Asn-X-Ser/Thr. In the consensus sequence, X can be any amino acid except Pro (*27*, *28*). The third amino acid in the consensus sequence has in a few cases been shown to influence the efficiency of the N-linked glycosylation process, Thr being associated with a higher degree of glycosylation than Ser (*29*).

To investigate whether the FV heterogeneity is caused by partial glycosylation of Asn^{2181}, the consensus sequence for glycosylation was modified by site-directed mutagenesis and the mutant FV variants were expressed in eukaryotic cells. We found that replacement of Ser^{2183} with a Thr increased the efficiency of glycosylation, whereas a Ser^{2183} to Ala mutation resulted in the loss of glycosylation. Functional analysis demonstrated that the carbohydrate side chain at Asn^{2181} impairs the interaction between FV and the phospholipid membrane. This is consistent with a structural analysis of a theoretical model of the C2 domain of FV and the position of a proposed binding site for the phospholipid.

MATERIALS AND METHODS

Materials. Restriction enzymes, aprotinin, leupeptin, pepstatin A, and PWO polymerase were purchased from Boehringer Mannheim. DNA ligation kit version 2 was from Takara Shuzo Co. Serum free cell culture media, Optimem Glutamax, were from Gibco BRL. The double-stranded DNA sequencing kit was from Perkin-Elmer. Bovine serum albumin, Hepes, ovalbumin, benzamidine, and Tris-HCl were obtained from Sigma Chemical Co. The phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids. Chromogenix supplied the chromogenic substrate S2238. Macrosep 100K concentrating cells were purchased from Pall Filtron.

Equipment for the gel electrophoretic analyses and Western blotting were from Bio-Rad Laboratories (Richmond, CA). FPLC equipment and column materials used for protein purification were purchased from Pharmacia (Uppsala, Sweden). Prestained molecular mass standards were from New England Biolabs. A rabbit polyclonal anti-human FVa light chain serum (8138) has been described previously (*30*). Alkaline phosphatase-conjugated pig anti-rabbit IgG was obtained from Dakopatts.

Proteins. Human α-thrombin, FXa, and prothrombin were purchased from Enzyme Research Laboratories. Purified plasma-derived FVa was prepared as previously described (*31*). The FVa1 and FVa2 forms were isolated by cationic chromatography as described previously (*15*). As judged by SDS−PAGE, they were homogeneous and contained either the 74 kDa (FVa1) or the 71 kDa (FVa2) light chain. FVa was brought into 25 mM Hepes (pH 7.5), 150 mM NaCl, and 5 mM $CaCl_2$ by buffer change on a PD-10 column (Pharmacia). Endoglycosidase H (EC 3.2.1.96) was purchased from Boehringer Mannheim. The enzyme PNGase (EC 3.5.1.52), purified as described previously (*32*), was a kind gift of J. Stenflo.

The FV antigen levels were determined by ELISA, as described previously (*8*). Molar thrombin and factor Xa concentrations were determined by active site titration with *p*-NPGB (*33*, *34*). Functional determinations of FV and FVa concentrations were performed using the FVa assay, as described below.

Mutagenesis. Three FV mutants, Ser^{2183} Ala, Ser^{2183}Thr, and Asn^{2181}Gln, were generated with the QuikChange site-directed mutagenesis kit (Stratagene) using the expression vector PMT2-V (containing a full-length cDNA of human FV) as a template (*35*). For each mutant, two complementary oligonucleotides containing the desired mutation were employed, the sequences of the three sense oligos being 5′-CTAAAACATGGAATCAA**GC**TATTACACTTCG-3′ (Ser^{2183}Ala), 5′-CTAAAACATGGAATCAAA**C**TATTACACTTCG-3′ (Ser^{2183}Thr), and 5′-CCTAAAACATGG**C**A**G**CAAAGTATTACAC-3′ (Asn^{2181}Gln). A 3.2 kb restriction fragment was isolated for each mutant using *Sna*BI and *Nhe*I (cleaves at unique sites), and the mutant fragments were used to replace the corresponding wild-type fragment in the PMT2-V vector. The presence of only the desired mutations in each of the three 3.2 kb fragments was confirmed by DNA sequencing.

Transient Expression of Recombinant FV. Recombinant FV was transiently expressed in COS1 cells as described previously (*35*, *36*). The serum-free medium was collected 72 h after transfection, and the expression levels were determined using both ELISA and the FVa assay that is described below. Before the phospholipid binding characteristics of the FV mutants were probed, they were concentrated using a Macrosep 100K concentrating cell and brought

into the appropriate buffer using a PD-10 column. Cell extracts were prepared using a Triton X-100 lysis buffer, as described previously (*37*). As a control, the PMT2 vector containing no FV cDNA was used to transfect cells. No FVa was detected in this mock medium, as judged by the ELISA and functional activity assay.

Preparation of Phospholipid Vesicles. Small unilamellar phospholipid vesicles were prepared by mixing the appropriate quantities of phospholipids dissolved in $CHCl_3/CH_3OH$ (9:1 v:v) in a glass tube, after which they were dried under a mild flow of nitrogen gas. The phospholipids were suspended in 25 mM Hepes and 150 mM NaCl (pH 7.5) and vigorously vortexed for 2 min. The phospholipid suspension was then sonicated for 10 min at 0 °C using an MSE Mark II 150 W ultrasonic disintegrator, with an 8 μm peak-to-peak amplitude. Phospholipid concentrations were determined by phosphate analysis as described previously (*38*).

SDS−Polyacrylamide Gel Electrophoresis and Western Blot Analysis. The purity and molecular size of the proteins were analyzed by SDS−PAGE under reducing conditions according to the method of Laemmli (*39*). Visualization of proteins was carried out after transfer to a PVDF membrane and subsequent incubation with a polyclonal rabbit antibody directed against the light chain of FVa and then an alkaline phosphatase-conjugated pig anti-rabbit IgG as described previously (*40*).

Enzymatic Deglycosylation of Plasma-Purified and Recombinant FVa. To FVa (20 μg/mL) were added 100 mM β-mercaptoethanol and 0.5% SDS (final concentrations in the premixture), and the mixture was incubated at 100 °C for 5 min. Deglycosylation buffer containing 100 mM Tris-HCl (pH 8.0), 2 mM PMSF, 10 mM 1,10-phenanthroline, 1.6% Nonidet P-40, 12.5 μg/mL benzamidine, 12.5 KIU/mL aprotinin, 1.25 μg/mL leupeptin, and 1.25 μg/mL pepstatin A was added (final concentrations). The total reaction volume was 160 μL, containing 14 μg/mL FVa. Deglycosylation was initiated by the addition of 0.3 μg of purified endoglycosidase PNGase F, having a specific activity of 25 units/μg of protein. The reaction mixture was incubated at 37 °C, and aliquots were drawn at intervals and mixed with SDS−PAGE sample preparation buffer [10% glycerol, 2% SDS, 2% β-mercaptoethanol, 100 mM Tris-HCl (pH 6.8), and 0.02% bromophenol blue (final concentrations)]. Heating the sample to 100 °C for 5 min inhibited PNGase activity.

FVa Assay and Functional Assessment of Phospholipid Binding of FVa. The procoagulant activity of FVa was measured as described previously (*41*). In brief, the rate of factor Xa-catalyzed prothrombin activation was measured at saturating concentrations of factor Xa (5 nM) and prothrombin (1 μM) and a limiting amount of FVa (between 1 and 35 pM). The buffer was 25 mM Hepes, 150 mM NaCl, and 2 mM $CaCl_2$ (pH 7.5) containing 0.5 mg/mL ovalbumin, and the temperature was 37 °C. The reaction mixture contained a saturating concentration of phospholipid vesicles (40 μM DOPS/DOPC, 10:90 molar ratio), unless otherwise stated. The generated thrombin was quantified using the chromogenic substrate S2238. FVa concentrations were determined from the rate of thrombin generation using a turnover number of 6000 mol of prothrombin activated per minute per mole of the factor Xa−Va complex (*41*). FV was quantified by the same method after prior activation with 5 nM thrombin. Control experiments showed thrombin activated recombinant FV to be stable for at least 45 min at 37 °C, with a <5% loss of functional activity (data not shown).

On the basis of the functional FVa assay, a quick method for detecting changes in lipid binding properties of mutant FV, as compared to those of wild-type FV, was created. In this assay, prothrombin activation was performed in the presence of (A) 5 μM DOPS/DOPC (5:95 molar ratio) or (B) 50 μM DOPS/DOPC (20:80 molar ratio). FVa variants having reduced phospholipid-binding ability were less efficient in supporting thrombin generation in the presence of 5 μM DOPS/DOPC (5:95 molar ratio) than in the presence of 50 μM DOPS/DOPC (20:80 molar ratio). The ratio of thrombin generated under condition A to that generated under condition B reflected the membrane binding ability of the FVa, with a low ratio suggesting poor phospholipid binding.

RESULTS

FVa1 and FVa2 Differ in the Number of Carbohydrate Side Chains, As Judged by Endoglycosidase Digestion. Isolated FVa1 and FVa2 were subjected to endoglycosidase treatment using PNGase F and analyzed by Western blotting using antibodies against the light chain, to determine the number of attached carbohydrate side chains (Figure 2). The mobility of both FVa1 and FVa2 light chains shifted slightly to lower molecular mass positions during the 48 h incubation, consistent with the liberation of carbohydrate side chains. The pattern observed for FVa1 suggested removal of three carbohydrate side chains, whereas FVa2 only lost two chains. The loss of the first carbohydrate side chain from the FVa1 light chain yielded a species with a molecular mass similar to that of the untreated FVa2 light chain. At the end of the incubation, the FVa1 and FVa2 light chains exhibited similar electrophoretic mobilities, both migrating as ~67 kDa proteins. It proved to be difficult to remove all the N-linked carbohydrate side chains from the FVa light chain, even though we varied the enzyme concentration between 10 and 1000% of the concentration used in the experiment illustrated in Figure 2 and extended the incubation time up to 5 days. The longer incubation times yielded nonspecific degradation, which interfered with the interpretation of the Western blots (observed in the 48 h incubation depicted in Figure 2). Similar experiments were also performed using 15 mIU of Endo H. Even after overnight incubation, the electrophoretic mobility of the FVa light chain did not change (data not shown), suggesting that it contained no high-mannose oligosaccharides.

Expression and Characterization of Recombinant FV Mutants. To elucidate whether the partial glycosylation of the FVa light chain was related to Ser^{2183} in the consensus sequence for N-glycosylation, it was mutated to either Thr or Ala. In addition, Asn^{2181} was mutated to Gln to determine whether the carbohydrate side chain at position 2181 affected the FXa cofactor activity of FVa. The three mutants (Ser^{2183}Thr, Ser^{2183}Ala, and Asn^{2181}Gln) and wild-type FV were transiently expressed in COS1 cells, the conditioned media being collected after 72 h. The expression levels of the four recombinant proteins varied between 100 and 200 ng/mL (Table 1), as estimated by an ELISA. The Ser^{2183}Thr mutant consistently expressed less efficiently than the other FV forms. The specific activities, i.e., the antigen:activity ratio, were similar for all FV variants.

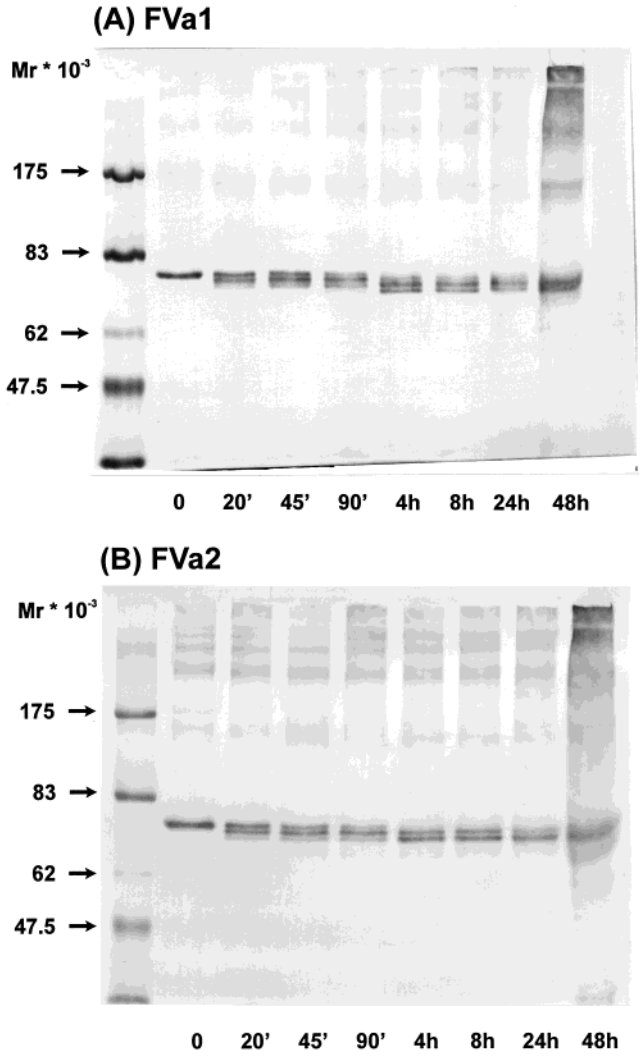

FIGURE 2: Removal of N-linked carbohydrate from plasma-purified human FVa1 and FVa2. FVa1 and FVa2 were incubated with PNGase F (see Materials and Methods) at 37 °C and analyzed by Western blotting after 7.5% SDS−PAGE, using a rabbit polyclonal antibody against the human FVa light chain. The various time points at which samples were drawn from the reaction mixtures for FVa1 (A) and FVa2 (B) are denoted. Each lane contained approximately 50 ng of FVa antigen. The molecular masses of the prestained markers are indicated.

Table 1: Expression Levels and Functional Analysis of Recombinant FV[a]

construct	antigen (ng/mL)	FVa activity (nM)	antigen:activity ratio	antigen in lysate (ng/mL)
wild-type FV	202 ± 16	0.31 ± 0.04	1.95 ± 0.3	54 ± 17
Ser^{2183}Ala	190 ± 16	0.36 ± 0.10	1.62 ± 0.3	45 ± 15
Ser^{2183}Thr	101 ± 18	0.17 ± 0.01	1.82 ± 0.4	16 ± 6
Asn^{2181}Gln	173 ± 7	0.32 ± 0.03	1.62 ± 0.1	40 ± 16

[a] FV concentrations were determined by an ELISA as described previously (*8*). FVa activities were determined using the chromogenic FVa assay as described. As a parameter of specificity, the ratio of FV antigen to activity was calculated, assuming 1 ng/mL ∼ 3.03 pM FV. The amount of FV antigen, present in 2 mL cell lysates obtained after harvesting conditioned media from culture dishes, was determined via the ELISA method. Data are the averages ± the standard error of the mean of eight different expressions.

The four recombinant FV variants were activated by thrombin and analyzed by Western blotting using an anti-

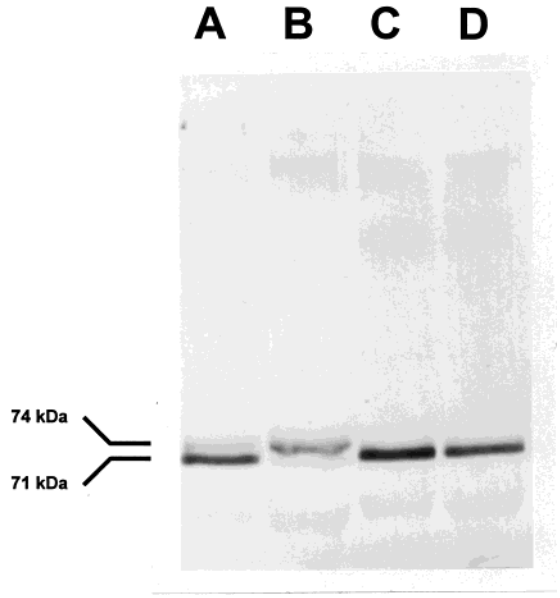

FIGURE 3: Western blotting of thrombin-activated wild-type and mutant FV. Concentrated conditioned media from COS1 cells (approximately 50 μg/mL FV) were incubated with thrombin (5 nM) for 20 min at 37 °C and then analyzed by Western blotting (7.5% SDS−PAGE under reducing conditions) using a rabbit anti-human FVa light chain antibody. The lane contents were as follows: (A) wild-type FVa, (B) the Ser^{2183}Thr FV mutant, (C) the Ser^{2183}Ala FV mutant, and (D) the Asn^{2181}Gln FV mutant. Estimated molecular masses are indicated.

serum specific for the FVa light chain (Figure 3). The wild-type FVa light chain appeared mainly as a 71 kDa species, even though a weak band was detected at the slightly higher molecular mass position expected for the FVa1 form (74 kDa). This suggested that the majority of wild-type recombinant FV is of the FV2 type. After activation of the Ser^{2183}Ala and Asn^{2181}Gln mutants, their light chains migrated as the FVa2 light chain, which proved that the FVa1−FVa2 light chain heterogeneity is due to partial glycosylation. This was further illustrated by the results obtained with the Ser^{2183}Thr mutant, which yielded a light chain migrating to the position expected for the FVa1 light chain species. Thus, the replacement of Ser with a Thr at position 2183 resulted in more efficient glycosylation of Asn^{2181}. Since Ser^{2183}Ala and Asn^{2181}Gln mutants had similar electrophoretic mobilities, it was concluded that the Ser^{2183}Ala mutant was not glycosylated at position Asn^{2181}. Deglycosylation of the four recombinant FV variants yielded FVa light chains with similar molecular masses (data not shown). It was consistently observed that deglycosylation of recombinant FV was complete at concentrations of PNGase F that were much lower than those required for plasma-purified FVa.

The C2 domain of FV is important for membrane binding (*16*), and plasma-derived FVa1 has in prothrombinase-based assays been shown to interact less efficiently than FVa2 with phospholipid containing low phosphatidylserine concentrations (*15*). Similar behavior was reproduced with the different FVa mutants, the Ser^{2183}Thr mutant being significantly less efficient than the other recombinant FV molecules ($p < 0.05$ using the Student's t test) under suboptimal phospholipid conditions, 5 μM DOPS/DOPC (5:95 molar ratio) versus 50 μM DOPS/DOPC (20:80 molar ratio) (Figure 4). This indicated a loss of membrane binding capacity for the

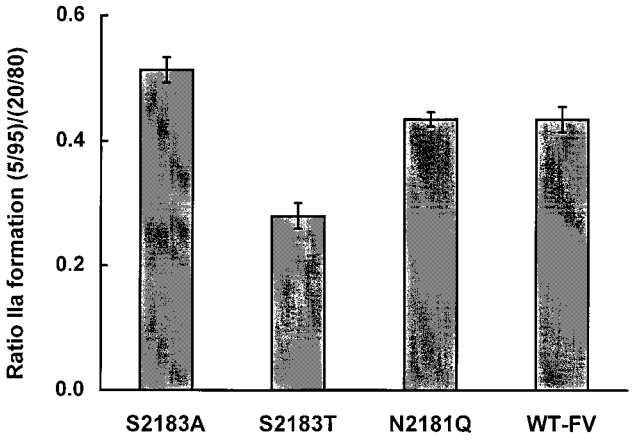

FIGURE 4: FXa cofactor activity of mutant FV under different phospholipid conditions. Rates of prothrombin activation were determined by addition of limiting amounts of thrombin-activated recombinant FV (170 ng) to reaction mixtures containing saturating amounts of factor Xa (5 nM) and prothrombin (1 μM). The buffer was 25 mM Hepes and 150 mM NaCl (pH 7.5) containing 2 mM $CaCl_2$ and 0.5 mg/mL ovalbumin. The ratios between the rates of prothrombin activation at 5 μM DOPS/DOPC (5:95 molar ratio) phospholipid and 50 μM DOPS/DOPC (20:80 molar ratio) are given. Data represent the average $\pm$ the standard error ($n = 4$).

Ser^{2183}Thr mutant, as compared to the other recombinant FV molecules.

Separation of Recombinant FVa1 and FVa2 by Ion Exchange Chromatography. The FVa1 and FVa2 forms have different charges, giving them distinct behavior on cationic ionic exchange chromatography (*15*). The separation of the two forms on a Resource S column using FPLC allowed an accurate quantification of the ratio between the two forms in the various recombinant FV preparations. FVa1 eluted from the column at ~330 mM NH_4Cl, whereas FVa2 was recovered at ~500 mM NH_4Cl. Western blotting confirmed the presence of the 74 and 71 kDa light chain forms in the first and second peak, respectively (Figure 5). The results suggested the presence of a carbohydrate at Asn^{2181} for neutralizing positive charges on the C2 domain of FV and hence influencing the chromatographic behavior of FVa. The distribution of the FVa activity in the two peaks eluting from the FPLC column was used as a means of estimating the relative degree of glycosylation of Asn^{2181} (Figure 5).

Glycosylation of Asn^{2181} Affects the Procoagulant Activity of FVa. The effects of the phospholipid composition and concentration for expression of procoagulant activity of the recombinant FV mutants were investigated using a prothrombinase-based assay system containing purified proteins (Figure 6). At low concentrations of phospholipid vesicles containing <15 mol % phosphatidylserine, FVa1 forms were considerably less efficient than the FVa2 forms in prothrombin activation. In the presence of vesicles containing higher mole percentages of phosphatidylserine, FVa1 and FVa2 were almost equally active in supporting prothrombinase activity. The recombinant FVa1 and FVa2 behaved just like their respective plasma-derived form. Not only the mole percentage of phosphatidylserine but also the concentration of phospholipid was found to be important. Thus, increasing concentrations of phospholipid vesicles containing 5 mol % phosphatidylserine resulted in an increased level of prothrombin activation, and above 10−15 μM, only small differences between the FVa forms were observed. From this,

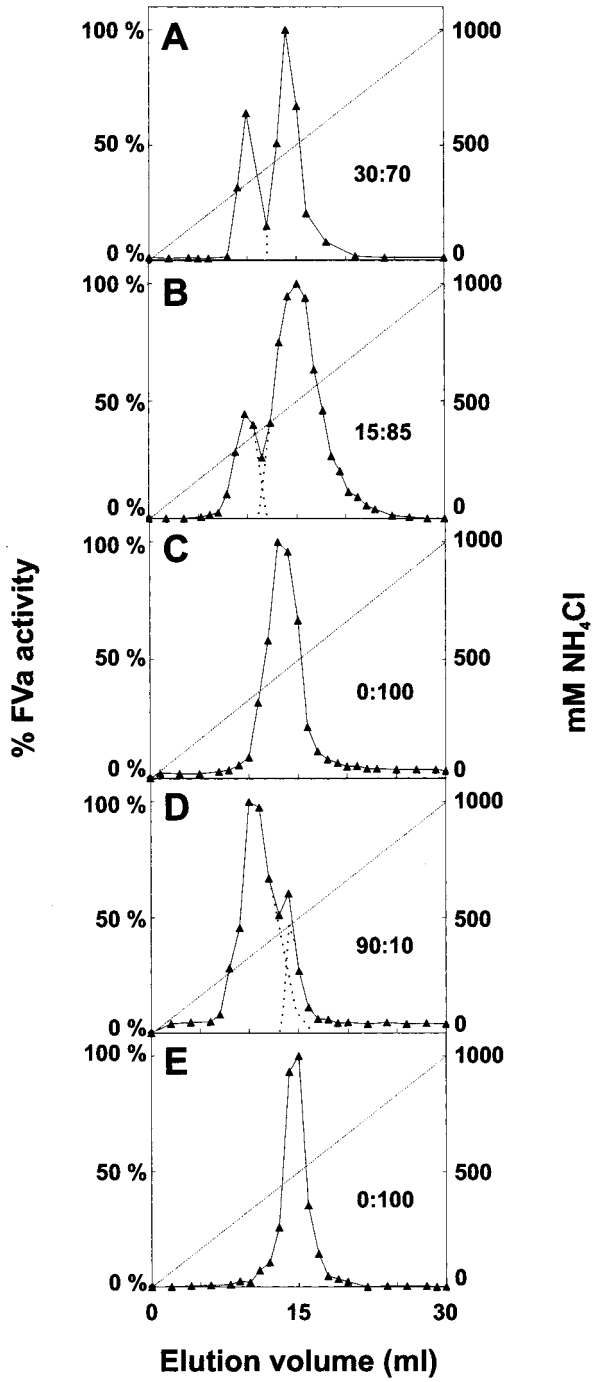

FIGURE 5: Separation of recombinant FVa1 and FVa2 forms by FPLC. Recombinant FV was, after concentration on Macrosep-100, activated by 5 nM thrombin at 37 °C in 25 mM Hepes and 150 mM NaCl (pH 7.5) containing 5 mM $CaCl_2$ and 1 mg/mL BSA. The activation process was monitored by a prothrombin-based FVa assay, and when full activation was achieved, the incubation was allowed to continue for an additional 15 min to ensure complete FV activation. Thrombin was inhibited by the addition of PPACK (5 nM), and the samples were loaded on a 1 mL Resource S column. The column was washed with buffer A, 25 mM Hepes (pH 6.5) containing 5 mM $CaCl_2$, until the baseline absorbency was reached. The FVa forms were eluted with a linear gradient of NH_4Cl (0 to 1 M). Collected fractions were screened for FVa activity as described in Materials and Methods: (A) plasma-purified FVa, (B) recombinant wild-type FVa, (C) the Ser^{2183}Ala mutant, (D) the Ser^{2183}Thr FV mutant, and (E) the Asn^{2181}Gln FV mutant. The distribution of the FVa activity in the two peaks is indicated.

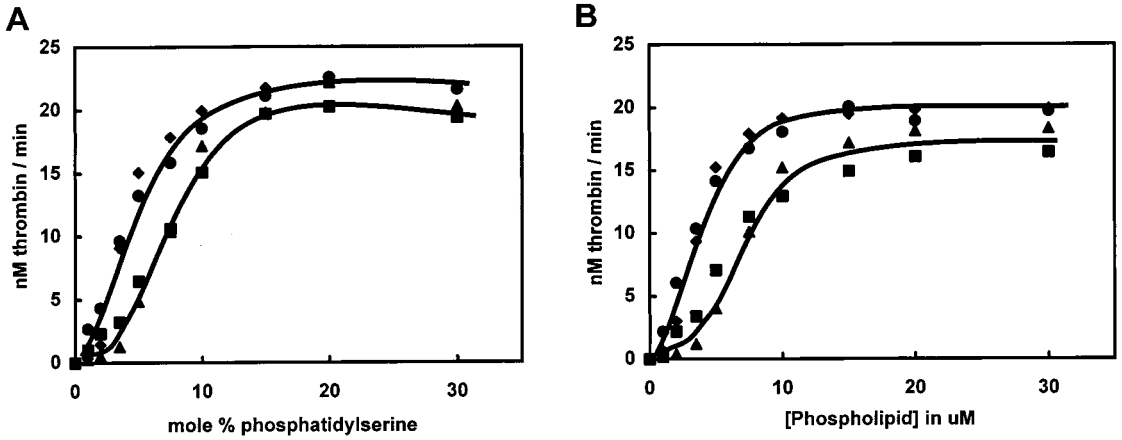

FIGURE 6: FXa cofactor activity of FVa mutants related to phospholipid composition and concentration. FVa (1.5 nM) was incubated with 2.5 pM factor Xa and phospholipid vesicles at 37 °C in 25 mM Hepes, 150 mM NaCl, and 3 mM $CaCl_2$ (pH 7.5) containing 5 mg/mL BSA. The phospholipid vesicles were (A) 5 μM DOPS/DOPC containing varying mole percents of phosphatidylserine or (B) increasing concentrations of 5:95 (molar ratio) DOPS/DOPC vesicles. After 5 min, prothrombin activation was started by the addition of prothrombin to a final concentration of 1 μM, and 3 and 6 min later, the amount of thrombin was quantified. Quantitation of the generated thrombin was performed for the 3 and 6 min samples in duplicate. The data are averages of three different experiments with variation per data point being <5% of its value: (◆) plasma-derived FVa2, (■) the $Ser^{2183}Thr$ mutant, (▲) plasma-derived FVa1, and (●) the $Asn^{2181}Gln$ mutant.

it can be concluded that the functional differences between FVa1 and FVa2 are due to the presence or absence of the carbohydrate side chain at Asn^{2181}.

DISCUSSION

The FVa light chain heterogeneity is an intrinsic property of the C2 domain (*15*, *16*). The aim of the investigation described here was to determine whether partial glycosylation of the single N-glycosylation site in the C2 domain, at Asn^{2181}, was the cause of the heterogeneity. Endoglycosidase treatment suggested that the light chains of FVa1 and FVa2 contain three and two carbohydrate side chains, respectively. Thus, FVa1 contains N-linked carbohydrates at all three potential glycosylation sites, at Asn^{1675}, Asn^{1982}, and Asn^{2181}, whereas FVa2 presumably contains carbohydrates only at Asn^{1675} and Asn^{1982}. Despite extensive digestion under denaturing conditions, the deglycosylation of plasma-derived FVa1 and FVa2 was incomplete, suggesting the accessibility of the carbohydrate side chains for the PNGase F to be limited. This agrees with an observation made by Fernandez et al. (*25*) that treatment of FV with *N*-glycanase under nondenaturing conditions did not change the electrophoretic mobility of the FV light chain. In contrast, we found deglycosylation of recombinant FV to be highly efficient, indicating that the oligosaccharide structures of recombinant FV (derived from COS1 cells) may be somewhat different from those of plasma FV (derived from hepatocytes). Although COS1 cells have no record of abnormal glycosylation and have proved to be valuable for studies of N-linked glycosylation of recombinant proteins (*42*, *43*), it is likely that small differences in the fine structure of the carbohydrate between recombinant and plasma-derived FV exist, which may affect the susceptibility to PNGase F. Another less likely possibility is that the reduced susceptibility of plasma-derived FV is the result of carbohydrate modification during purification, or during exposure of FV to plasma in vivo (*24*).

Our results demonstrated that wild-type FV, and the two mutants $Ser^{2183}Thr$ and $Ser^{2183}Ala$, differ in their degrees of glycosylation. The efficiency of glycosylation at Asn^{2181} related to which amino acid residue was present in the third position in the consensus sequence for N-glycosylation, with the following order: Ala < Ser < Thr. This observation agrees with the consensus sequence-assisted catalysis mechanism for oligosaccharyl transferase, proposed by Bause and Legler (*44*). According to this mechanism, the residue at the third position plays an active role in the catalysis of oligosaccharide transfer to the Asn residue by formation of a charge relay system with the Asn saccharide receptor and the dolicholpyrophosphate-bound oligosaccharide, which facilitates the saccharide transfer. Partial glycosylation at Asn^{2181} due to the Ser in position 2183 thus explains the heterogeneity of FV, despite the presence of only one gene for FV.

Expression levels of wild-type FV and two of the mutants, $Ser^{2183}Ala$ and $Asn^{2181}Gln$, were similar, whereas the level of expression of $Ser^{2183}Thr$ was consistently lower. Although this suggests that the carbohydrate at Asn^{2181} impairs the secretion of FV, additional studies, including metabolic pulse labeling of FV, are required to address this question properly. Complete blockage of the N-linked glycosylation by tunicamycin has been shown to impair the overall secretion of recombinant FV, using CHO T9 cells (*45*).

The interaction between FVa and phospholipid membranes was probed indirectly via a prothrombinase-based assay system. Plasma-derived and recombinant FVa2 ($Asn^{2181}Gln$ and $Ser^{2183}Ala$ mutants), compared to FVa1 ($Ser^{2183}Thr$ and plasma-derived), demonstrated higher procoagulant cofactor activity in the presence of low concentrations of phospholipid containing less than 15 mol % phosphatidylserine. This suggests that the polar carbohydrate attached to Asn^{2181} interferes with binding of FVa1 to the phospholipid membrane surface. In this context, it is interesting to note that modification of the carbohydrate side chains in FV has been found to affect the procoagulant activity of FV. Thus, the procoagulant activity increased slightly when recombinant FV was expressed in the presence of DMJ, an inhibitor of a 1,2-mannosidase (causing inhibition of N-linked oligosaccharide processing) (*45*). Increased FV activity was also observed after neuramidase treatment (*24*). The possible

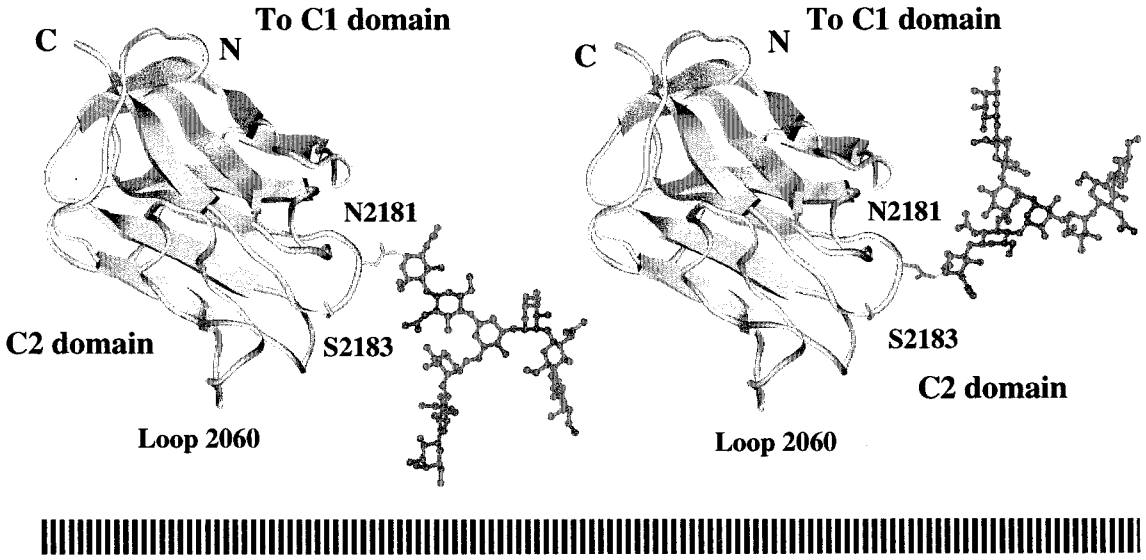

FIGURE 7: Molecular model of the C2 domain including the carbohydrate side chain. The ribbon diagram of the C2 module is drawn (left and right) with the same orientation and with two possible orientations for the Asn^{2181} side chain to which a common carbohydrate motif (GlcNAc-GlcNAc-Man-[Man-GlcNAcGal]$_2$) has been grafted. The loop 2060 most likely contacts the phospholipid surface directly. The exact location of the glycan cannot be predicted at present. The glycan could be slightly closer to the protein surface, and/or the module could be tilted slightly differently with respect to the membrane plane. In all cases, it is clear that the size of a common carbohydrate motif is such that it could interfere with membrane binding.

interference with the FVa−membrane interaction by a carbohydrate side chain attached to Asn^{2181} was theoretically tested by structural analysis of a molecular model of the C2 domain (*46*). In this model, Asn^{2181} is located in a solvent-exposed loop where a carbohydrate side chain can easily be accommodated. The Asn^{2181} residue was found to be nearby another solvent-exposed loop containing several Trp and positively charged amino acids, which was proposed to constitute part of the phospholipid binding site in the C2 domain (Figure 7). The C2 domain is known to interact with the membrane (*17*), and electron microscopy demonstrated that the A1−A3 domains are distant from the membrane (*47*, *48*). A likely possibility is that the C1 and C2 domains adopt a rod-like structure with the membrane binding site being located on the opposite side of the N-terminus of the C2 domain (*46*, *49*). Collectively, these data are compatible with the idea that the charged and hydrophobic/aromatic loop residue 2060 of FV is directly involved in the interaction with the phospholipids. It is known that protein−membrane interactions often involve both hydrophobic effects and other thermodynamic forces, such as electrostatic interactions. Moreover, structural (*50*) and statistical analyses (*51*) of membrane proteins indicate that aromatic residues are preferentially found at the membrane−solvent interface.

The results presented here are compatible with a localization of Asn^{2181} close to the membrane (Figure 7). The structure of the core of N-linked oligosaccharides is remarkably conserved between different glycoproteins (*52*, *53*). Thus, by grafting a commonly found N-linked carbohydrate core motif (GlcNAc-GlcNAc-Man-[Man-GlcNAcGal]$_2$) to Asn^{2181} onto the C2 model, we can further interpret our biochemical data. Despite the fact that the exact orientation of this N-linked glycan is not yet known, we propose an approximate position for the membrane surface relative to the C2 module (Figure 7). Regardless of the exact position of the glycan, it is expected to be very close to the membrane and may therefore interfere with membrane binding.

The C2 domain is highly conserved among at least three species (human, bovine, and murine), exhibiting a striking degree of identity of 87.4% between human and mouse and 89.9% between human and bovine FV, and no insertions or deletions are observed among the three species. Even more noteworthy is the 100% degree of identity among the three species in the region between amino acid residues 2161 and 2194, which includes the glycosylation site (numbering as in human FV). The bovine FVa light chain is known to be heterogeneous just like the human FVa light chains. The high degree of conservation between species indicates that the C-terminal region of the FVa light chain is important for proper FV function. Moreover, it appears that the light chain heterogeneity is also highly conserved, and therefore, it is apparently functionally important. The FVa2 form is likely to be more procoagulant at low phospholipid concentrations. However, it is difficult to conclude which are the overall functional consequences of the glycosylation because the two forms of FV and FVa differ in their sensitivities toward APC and have different potencies as anticoagulant APC cofactors (*19*, *20*). The availability of well-defined recombinant FV mutants mimicking the FV1 and FV2 forms will allow a detailed elucidation of pro- and anticoagulant properties of the two FV forms.

In conclusion, we demonstrate that the FV heterogeneity is caused by partial glycosylation at Asn^{2181}, which is due to the presence of a Ser rather than a Thr at the third position in the consensus sequence for N-linked glycosylation. After replacement of the Ser with a Thr, an increased level of glycosylation at Asn^{2181} was obtained. A three-dimensional model for the C2 domain was used to interpret the modulation of FVa activity and membrane binding ability by the carbohydrate side chain in the C2 domain.

REFERENCES

1. Tracy, P. B., Eide, L. L., and Bowie, E. J. (1982) *Blood 60*, 59−63.
2. Jenny, R. J., Pittman, D. D., Toole, J. T., Kriz, R. W., Aldape, R. A., Hewick, M. H., Kaufman, R. J., and Mann, K. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4846−4850.
3. Dahlbäck, B. (1980) *J. Clin. Invest. 66* (3), 583−591.
4. Kane, W. H., and Majerus, P. W. (1981) *J. Biol. Chem. 256* (2), 1002−1007.
5. Suzuki, K., Dahlbäck, B., and Stenflo, J. (1982) *J. Biol. Chem. 257* (11), 6556−6564.
6. Monkovic, D., and Tracy, P. (1990) *Biochemistry 29*, 1118−1128.
7. Tans, G., Nicolaes, G. A. F., Thomassen, M. C. L. G. D., Hemker, H. C., Van Zonneveld, A.-J., Pannekoek, H., and Rosing, J. (1994) *J. Biol. Chem. 269*, 15969−15972.
8. Thorelli, E., Kaufman, R. J., and Dahlbäck, B. (1997) *Eur. J. Biochem. 247* (1), 12−20.
9. Esmon, C. T. (1979) *J. Biol. Chem. 254*, 964−973.
10. Guinto, E. R., and Esmon, C. T. (1982) *J. Biol. Chem. 257* (17), 10038−10043.
11. Nesheim, M. E., Taswell, J. B., and Mann, K. G. (1979) *J. Biol. Chem. 254*, 10952−10962.
12. Rosing, J., Tans, G., Govers-Riemslag, J. W. P., Zwaal, R. F. A., and Hemker, H. C. (1980) *J. Biol. Chem. 255*, 274−283.
13. Dahlbäck, B., and Hildebrand, B. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1396−1400.
14. Shen, L., and Dahlbäck, B. (1994) *J. Biol. Chem. 269*, 18735−18738.
15. Rosing, J., Bakker, H. M., Thomassen, M. C. L. G. D., Hemker, H. C., and Tans, G. (1993) *J. Biol. Chem. 268*, 21130−21136.
16. Ortel, T. L., Devore Carter, D., Quinn Allen, M., and Kane, W. H. (1992) *J. Biol. Chem. 267* (6), 4189−4198.
17. Ortel, T. L., Quinn-Allen, M. A., Keller, F. G., Peterson, J. A., Larocca, D., and Kane, W. H. (1994) *J. Biol. Chem. 269*, 15898−15905.
18. Ortel, T. L., Yoo, L., Quinn-Allen, M. A., and Kane, W. H. (1994) *Blood 84* (10, Suppl. 1), 387a.
19. Váradi, K., Rosing, J., Tans, G., Pabinger, I., Keil, B., and Schwarz, H. P. (1996) *Thromb. Haemostasis 76* (2), 208−214.
20. Hoekema, L., Nicolaes, G. A. F., Hemker, H. C., Tans, G., and Rosing, J. (1997) *Biochemistry 36* (11), 3331−3335.
21. Hortin, G. L. (1990) *Blood 76* (5), 946−952.
22. Pittman, D. D., Tomkinson, K. N., Michnick, D. A., Seligsohn, U., and Kaufman, R. J. (1994) *Biochemistry 33*, 6952−6959.
23. Kalafatis, M., Rand, M. D., Jenny, R. J., Ehrlich, Y. H., and Mann, K. G. (1993) *Blood 81* (3), 704−719.
24. Bruin, T., Sturk, A., Ten Cate, J. W., and Cath, M. (1987) *Eur. J. Biochem. 170*, 305−310.
25. Fernandez, J. A., Hackeng, T. M., Kojima, K., and Griffin, J. H. (1997) *Blood 89* (12), 4348−4354.
26. Kane, W. H., Ichinose, A., Hagen, F. S., and Davie, E. W. (1987) *Biochemistry 26* (20), 6508−6514.
27. Bause, E. (1983) *Biochem. J. 209* (2), 331−336.
28. Gavel, Y., and von Heijne, G. (1990) *Protein. Eng. 3* (5), 433−442.
29. Kasturi, L., Eshleman, J. R., Wunner, W. H., and Shakin Eshleman, S. H. (1995) *J. Biol. Chem. 270* (24), 14756−14761.
30. Suzuki, K., Stenflo, J., Dahlbäck, B., and Teodorsson, B. (1983) *J. Biol. Chem. 258*, 1914−1920.
31. Nicolaes, G. A. F., Tans, G., Thomassen, M. C. L. G. D., Hemker, H. C., Pabinger, I., Varadi, K., Schwarz, H. P., and Rosing, J. (1995) *J. Biol. Chem. 270* (36), 21158−21166.
32. Plummer, T. H., Jr., and Tarentino, A. L. (1991) *Glycobiology 1* (3), 257−263.
33. Chase, T., Jr., and Shaw, E. (1969) *Biochemistry 8* (5), 2212−2224.
34. Smith, R. L. (1973) *J. Biol. Chem. 248* (7), 2418−2423.
35. Kaufman, R. J. (1990) *Methods Enzymol. 185*, 487−511.
36. Thorelli, E., Kaufman, R. J., and Dahlbäck, B. (1998) *J. Biol. Chem. 273* (26), 16140−16145.
37. Bollag, D. M., Rozycki, M. D., and Edelstein, S. J. (1996) in *Protein Methods* (Bollag, D. M., Ed.) 2nd ed., Wiley-Liss Inc., New York.
38. Böttcher, C. J. F., van Gent, C. M., and Pries, C. (1961) *Anal. Chim. Acta 24*, 203−207.
39. Laemmli, U. K. (1970) *Nature 227* (259), 680−685.
40. Blake, M. S., Johnston, K. H., Russell Jones, G. J., and Gotschlich, E. C. (1984) *Anal. Biochem. 136* (1), 175−179.
41. Tans, G., Rosing, J., Thomassen, M. C., Heeb, M. J., Zwaal, R. F., and Griffin, J. H. (1991) *Blood 77* (12), 2641−2648.
42. Delorme, E., Lorenzini, T., Griffin, J., Martin, F., Jacobsen, F., Boone, T., and Elliott, S. (1992) *Biochemistry 31*, 9871−9876.
43. Zhang, R., Cai, H., Fatima, N., Buczko, E., and Dufau, M. L. (1995) *J. Biol. Chem. 270* (37), 21722−21728.
44. Bause, E., and Legler, G. (1981) *Biochem. J. 195* (3), 639−644.
45. Pittman, D. D., Tomkinson, K. N., and Kaufman, R. J. (1994) *J. Biol. Chem. 269*, 17329−17337.
46. Villoutreix, B. O., Bucher, P., Hofmann, K., Baumgartner, S., and Dahlbäck, B. (1998) *J. Mol. Model. 4*, 268−275.
47. Lampe, P. D., Pusey, M. L., Wei, G. J., and Nelsestuen, G. L. (1984) *J. Biol. Chem. 259* (15), 9959−9964.
48. Stoylova, S., Mann, K. G., and Brisson, A. (1994) *FEBS Lett. 351*, 330−334.
49. Pellequer, J. L., Gale, A. J., Griffin, J. H., and Getzoff, E. D. (1998) *Blood Cells, Mol. Dis. 24* (4), 448−461.
50. Yau, W. M., Wimley, W. C., Gawrisch, K., and White, S. H. (1998) *Biochemistry 37* (42), 14713−14718.
51. Heijne, v. (1994) *Annu. Rev. Biophys. Biomol. Struct. 23*, 167−192.
52. Woods, R. J., Edge, C. J., and Dwek, R. A. (1994) *Nat. Struct. Biol. 1* (8), 499−501.
53. Shaanan, B., Lis, H., and Sharon, N. (1991) *Science 254* (5033), 862−866.

BI991165R