Carbohydrate Moieties on the Procofactor Factor V, but Not the Derived Cofactor Factor Va, Regulate Its Inactivation by Activated Protein C†

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ABSTRACT: Factor V (FV) is a single-chain plasma protein containing 13−25% carbohydrate by mass. Studies were done to determine if these carbohydrate moieties altered the activated protein C (APC)-catalyzed cleavage and inactivation of both FV and the cofactor which results from its activation by α-thrombin, factor Va_{IIa} (FVa_{IIa}). Treatment of purified FV with *N*-glycanase and neuraminidase under nonprotein-denaturing conditions removed ≈20−30% of the carbohydrate from the heavy chain region of the molecule. When glycosidase-treated FV was analyzed in an aPTT (activated partial thromboplastin time)-based APC sensitivity assay, the APC sensitivity ratio (APC-SR) increased from 2.34 to 3.33. In contrast, when glycosidase-treated FV was activated with α-thrombin, the addition of the resulting FVa_{IIa} to the plasma-based APC sensitivity assay produced no substantial increase in the APC-SR. Additional functional analyses of the APC-catalyzed inactivation of FVa_{IIa} in an assay consisting of purified components indicated that both glycosidase-treated and untreated FVa_{IIa} expressed identical cofactor activities and were inactivated at identical rates. Analyses of the APC-catalyzed cleavage of glycosidase-treated FV at Arg^{306}, the initial cleavage site, revealed a 10-fold rate increase when compared to untreated FV. In contrast, and consistent with functional assays, similar analyses of FVa_{IIa}, derived from those FV species, revealed near-identical rates of APC-catalyzed cleavage at both the Arg^{506} and Arg^{306}sites. These combined results indicate that N-linked carbohydrate moieties play a substantial role in the APC-catalyzed cleavage and inactivation of FV but not FVa_{IIa} at position Arg^{306} and that the Arg^{306} cleavage sites of FV and FVa_{IIa} are distinct substrates for APC.

Factor V is a single-chain plasma glycoprotein that is an essential component of the hemostatic system (*1*−*4*). Proteolytic activation of factor V by α-thrombin produces factor Va (*5*−*8*), which is composed of two polypeptides: a heavy chain (M_r = 105 000) and a light chain (M_r = 74 000), associated noncovalently in the presence of divalent metal ions (*9*, *10*). Factor Va serves as a cofactor in the prothrombinase complex, which converts prothrombin to α-thrombin. The resulting α-thrombin can then cleave fibrinogen to form a fibrin clot. In addition to factor Va, the prothrombinase complex consists of the serine protease factor Xa, an appropriate membrane surface, and Ca^{2+} ions (*11*). Although the procofactor, factor V, possesses virtually no cofactor activity, the presence of factor Va in prothrombinase accelerates the rate of α-thrombin generation by more than 4 orders of magnitude (*12*). Thus, alterations in factor V and factor Va, either positively or negatively, can have a dramatic effect on α-thrombin generation and hemostasis.

Factor V and factor Va are both cleaved by the anticoagulant protease, activated protein C (APC)[1] (*13*−*17*). Factor V is initially cleaved at Arg^{306}, followed by additional cleavages at Arg^{506}, Arg^{679}, and Lys^{994}. The initial cleavage at Arg^{306} results in inactivation of the procofactor and forms fragments of M_r = 280 000 (residues 307−2196) and a M_r = 45 000 (residues 1−306) (*17*). In contrast, initial cleavage of factor Va occurs at Arg^{506}, followed by cleavage at Arg^{306} and Arg^{679} (*17*, *18*). Cleavage at Arg^{506} results in only partial inactivation of the cofactor, whereas subsequent cleavage at Arg^{306}, which is membrane-dependent, results in complete inactivation of factor Va (*16*, *17*).

Factor V/Va is found in two pools in whole blood, with approximately 75−80% contained in the plasma and the remaining 20−25% in the α granules of platelets (*19*). In contrast to plasma-derived factor Va, which can be completely inactivated by APC on a pure phospholipid vesicle or the activated platelet surface, platelet-released and bound factor Va retains a substantial portion of its activity, even after prolonged exposure to APC (*20*).

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[1] Abbreviations: APC, activated protein C; APC-SR, APC sensitivity ratio; Tris, tris[hydroxymethyl]aminomethane; PCPS, phosphatidylcholine/phosphatidylserine vesicles (75%:25%); HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; β-ME, 2-mercaptoethanol; DFP, diisopropyl fluorophosphate; PEG, polyethylene glycol 8000; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; aPTT, activated partial thromboplastin time; BSA, bovine serum albumin.

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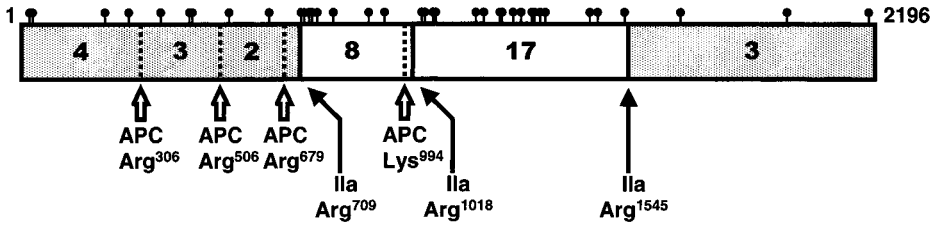

FIGURE 1: Potential N-linked glycosylation sites of factor V. Factor V is indicated as a horizontal bar with the heavy and light chain regions of the molecule indicated by the left and right gray-shaded areas, respectively. Potential N-linked glycosylation sites on the molecule are indicated by the attached filled circles (●). The locations of α-thrombin cleavage sites and APC cleavage sites are indicated on the factor V molecule by solid lines and dashed lines, respectively. Each numeral located inside the schematic represents the number of potential N-linked glycosylation sites in that region of the molecule between the indicated sites of proteolysis.

The term APC resistance defines an abnormality associated with a poor anticoagulant response to APC (*21*, *22*). It is the most common identifiable defect in patients with venous thrombosis, occurring in about 20−60% of this population (*23*−*26*). In most cases, the cause of APC resistance lies in a single nucleotide change in the factor V gene ($G^{1691} \rightarrow A$), resulting in an $Arg^{506} \rightarrow Gln$ (factor V^{Leiden}) substitution in factor V (*27*−*30*). Inactivation of plasma factor Va^{Leiden} is approximately 10−20-fold slower than that in wild-type plasma factor Va because of the mutation at position 506 and the sequential nature of the APC-catalyzed cleavages in the factor Va molecule (*31*−*34*). In marked contrast, the plasma procofactors (wild-type factor V and factor V^{Leiden}), when bound to a membrane surface, are inactivated at the same rate by APC (*34*).

Differences in glycosylation, although perhaps conferring more subtle modifications than an alteration of the APC cleavage site at Arg^{506}, also play a role in factor V/Va function. The carbohydrate content of factor V, which accounts for approximately 13−25% of the entire mass of the protein, is composed of both N-linked and O-linked polysaccharide chains and includes sialic acid (*35*−*37*). The cDNA for human factor V and the deduced amino acid sequence indicate that factor V contains 37 potential N-linked carbohydrate sites: 9 in the heavy chain, 3 in the light chain, and 25 in the connecting region (Figure 1) (*35*, *38*, *39*). Studies have demonstrated that both plasma- and platelet-derived factor V are present in two distinct glycoforms, which can be distinguished by cation exchange chromatography (*40*). Upon their α-thrombin-catalyzed activation, two forms of the factor Va light chain appear with apparent M_r = 74 000 (factor Va_1) and 71 000 (factor Va_2) (*7*, *10*, *41*, *42*). The molecular weight difference arises from the partial glycosylation at Asn^{2181} (*43*, *44*). Functional studies indicate that the two light chain glycoforms of factor Va have different phospholipid binding properties and, therefore, express different cofactor activity in prothrombinase (*40*), are differentially susceptible to inactivation by APC (*45*), and express different cofactor activities in the APC-mediated inactivation of factor VIII (*46*). Studies have also shown that decreasing the level of carbohydrate in human factor V can produce functional changes in the molecule, such as differences in the rate of α-thrombin-catalyzed activation of factor V and changes in the cofactor activity of factor Va (*37*, *47*, *48*). However, absolute comparison of these studies has been difficult, because, in all instances, different conditions were used to effect deglycosylation. For example, a more recent study, which used mild, nonprotein-denaturing conditions at physiological pH to remove a portion of the N-linked carbohydrate from factor V, demonstrated no effect on either factor V's ability to be activated to factor Va or the resulting factor Va cofactor activity (*49*). This partial deglycosylation did, however, result in an increase in its sensitivity to APC, leading the authors to conclude that the loss of protective N-linked carbohydrates from the heavy chain of factor Va resulted in its more rapid inactivation by APC. However, the experimental design of that study did not distinguish between factor V and factor Va, regarding their increased sensitivity to APC-catalyzed inactivation.

Studies have demonstrated that platelet-derived factor Va is more resistant to APC-catalyzed inactivation than its plasma-derived counterpart. We hypothesize that the resistance observed may result from additional posttranslational modification events incurred by the platelet-derived molecule, including, but not limited to, glycosylation (*20*). Therefore, this study was performed to determine if, and how, carbohydrate moieties altered the APC-catalyzed cleavage and inactivation of factor V or factor Va. Deglycosylation conditions were used which had no effect on the rate of the α-thrombin-catalyzed conversion of factor V to factor Va. Consequently, the APC-catalyzed cleavage and inactivation of glycosidase-treated plasma-derived factor V and the derived cofactor factor Va were assessed, using clotting-based and purified assay systems for analysis of factor Va activity and visualization of protein cleavage by SDS−PAGE. Our results demonstrate unequivocally that carbohydrate moieties on the procofactor factor V, but not the derived cofactor factor Va, substantially alter its susceptibility to APC-catalyzed inactivation.

EXPERIMENTAL PROCEDURES

Reagents. Tris[hydroxymethyl]aminomethane (Tris), L-α-phosphatidyl-L-serine [bovine brain] (PS), L-α-phosphatidylcholine [egg yolk] (PC), Tween-20, *N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), Tergitol (type NP-40), 2-mercaptoethanol (β-ME), Coomassie Brilliant Blue R-250 (Coomassie blue), bromophenol blue, and diisopropyl fluorophosphate (DFP) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate and polyethylene glycol 8000 (PEG) were purchased from J. T. Baker (Phillipsburg, NJ). Acrylamide and pure nitrocellulose membrane sheets (0.45 μM) were purchased from Bio-Rad (Hercules, CA). Renaissance western blot chemiluminescence reagent and Kodak X-Omat Blue XB-1 autoradiography film were purchased from DuPont, NEN Research Products (Boston, MA). Automated aPTT reagent was purchased from Organon Teknika Corp. (Durham, NC). Crystallized bovine serum albumin (BSA) was purchased

from ICN ImmunoBiologicals (Aurora, OH). Factor V deficient plasma was purchased from George King Biomedical Inc. (Overland Park, KS), and the chromogenic α-thrombin substrate S-2238 was purchased from Chromogenix (Mölndal, Sweden). The α-thrombin inhibitor hirudin was obtained from Calbiochem (San Diego, CA). Recombinant peptide-*N*-glycosidase F (*N*-glycanase) from *Flavobacterium meningosepticum* (recombinant in *Escherichia coli*), neuraminidase from *Streptococcus* sp., and *endo*-α-*N*-acetylgalactosaminidase (*O*-glycanase) from *Streptococcus pneumoniae* were purchased from Oxford GlycoSystems (Bedford, MA). Phospholipid vesicles composed of 75% (w/w) PC and 25% (w/w) PS (PCPS) were prepared as previously described (*50*), and the concentration of the vesicles was determined by phosphorus assay (*51*).

Preparation of Coagulation Proteins. All proteins were of human origin and purified from fresh frozen plasma unless otherwise noted. Factor V was isolated by immunoaffinity chromatography, as described, and was activated to factor Va with 1 to 2 NIH U/mL (10−20 nM) of α-thrombin for 10 min at 37 °C (*52*, *53*). Factor X and prothrombin were purified by the method of Bajaj and et al. (*54*). Factor X was activated with the factor X activator purified from Russell's viper venom (*55*). α-Thrombin was prepared by activation of prothrombin with Taipan snake venom as described by Owen and Jackson (*56*). Human APC was a gift from Haematologic Technologies Inc. (Essex Junction, VT). All proteins used were >95% pure as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) before and after disulfide bond reduction, according to the method of Laemmli (*57*). Molecular weights and extinction coefficients ($E^{1\%}_{280\ nm}$) of the various proteins used were taken as follows: prothrombin 72 000, 14.2 (*58*); α-thrombin 37 000, 18.3 (*58*, *59*); factor V 330 000, 9.6 (*19*, *53*); factor Xa 50 000, 11.6 (*54*); APC 56 200, 14.5 (*60*).

Glycosidase Treatment of Factor V and Factor Va. Factor V (4 μM) was treated under nonprotein-denaturing conditions with *N*-glycanase (1.32 U/mL) to remove Asn-linked oligosaccharides and neuraminidase (0.165 U/mL) to remove sialic acids in 20 mM Hepes and 150 mM NaCl (pH 7.4) (HBS), containing 5 mM $CaCl_2$ (HBS(Ca^{2+})), for 3 h at 37 °C. After glycosidase treatment, some glycosidase-treated factor V samples (1 μM) were incubated with α-thrombin (2 U/mL, 20 nM) for 15 min at 37 °C to generate glycosidase-treated factor Va. For glycosidase treatment reactions of factor V or factor Va under protein-denaturing conditions, samples of factor V or factor Va (1 μM) in HBS(Ca^{2+}) containing SDS (0.17%) and β-ME (17 mM) were incubated at 90 °C for 5 min, followed by addition of NP-40 (1.25%), *N*-glycanase (5 U/mL), neuraminidase (0.5 U/mL), and *O*-glycanase (0.033 U/mL) for removal of oligosaccharides from serine and threonine. Glycosidase treatment was allowed to proceed at 37 °C for 3 h. All glycosidase treatment reactions were performed in the presence of 1 mM DFP to eliminate any potential effect of contaminating serine proteases. Representative samples (8 μg) were analyzed by SDS−PAGE on 5−15% linear gradient slab gels and visualized with Coomassie blue staining.

APC Sensitivity Assays of Factor V, Factor Va, and Plasma Samples. The APC sensitivity assay (also known as the APC resistance assay) is a modified aPTT (activated partial thromboplastin time) clotting assay in which, after an incubation of sample plasma with aPTT reagent, clot formation is initiated with a $CaCl_2$ solution in the presence or absence of APC. The ratio calculated between the clotting times ± APC is called the APC sensitivity ratio (APC-SR) (*21*, *22*, *61*). To enhance the sensitivity of this assay, and to allow its use on patients receiving oral anticoagulants, a modified APC sensitivity assay, in which patient samples are diluted in factor V deficient plasma, has been described (*62*−*64*). In the aPTT-based APC sensitivity assay, citrated plasma (or purified factor V/Va), is diluted in factor V-deficient plasma and incubated with the aPTT reagent (consisting of a lipid source and contact activator). During this incubation, the intrinsic pathway of blood coagulation is activated, eventually resulting in a portion of the factor V being converted to factor Va. To closely approximate the amount of factor V converted to factor Va in the preincubation step of the assay, varying concentrations of factor Va (0.06−1.0 nM) were added to the aPTT assay in place of factor V. A concentration of 0.2 nM factor Va resulted in a clot time equivalent to that observed upon the addition of 2 nM factor V, indicating that, at most, 10% of the procofactor was activated to factor Va during the preincubation period. Thus, to evaluate the individual contributions of factor V and factor Va to the APC sensitivity assay, additional assays were performed using 0.2 nM factor Va alone.

To determine the effects of factor V glycosidase treatment on the aPTT-based APC sensitivity assay, factor V (0.3 μM) was treated with *N*-glycanase (0.1 U/mL) and neuraminidase (0.0125 U/mL) in HBS(Ca^{2+}). Aliquots of the reaction mixture were removed over time (0, 0.5, 1, 2, 3, and 4 h) and diluted 1:5 in HBS(Ca^{2+}) containing 0.1% BSA. Samples (10 μL) of the diluted factor V (60 nM) were combined with factor V-deficient plasma (100 μL) and aPTT reagent (100 μL) and incubated for 3 min at 37 °C. Clotting was initiated by the addition of 100 μL of a $CaCl_2$ solution (30 mM $CaCl_2$, 10 mM Tris, 50 mM NaCl, and 0.1% BSA) in the presence or absence of 20 nM APC. Clot times were determined on a Stago ST4 coagulometer (Diagnostica Stago, Asnières, France), and the APC-SR was determined as the ratio of clot times with and without APC. To determine how glycosidase treatment affected factor Va behavior in the APC reaction mixture, initial dilutions of the glycosidase-treated factor V were activated with 0.1 U/mL of α-thrombin (1 nM α-thrombin) for 15 min at 37 °C. The reaction was stopped by the addition of 1.5 nM hirudin. Factor Va samples were diluted in HBS(Ca^{2+}), containing 0.1% BSA, and added to the modified APC sensitivity assay (0.2 nM final concentration), as described previously for glycosidase-treated factor V.

APC-Catalyzed Inactivation of Factor Va. Samples of factor Va, untreated or glycosidase-treated under nonprotein-denaturing conditions (20 nM), were incubated with PCPS vesicles (10 μM) and APC (0.1 nM) in HBS(Ca^{2+}) containing 0.1% PEG. Aliquots were removed at various times following APC addition (0, 2, 4, 6, 8, 10, 12, 15, and 30 min) and assayed for factor Va cofactor activity in a prothrombinase assay consisting of purified components. The prothrombinase mixture contained factor Va samples diluted 1:200 (0.1 nM), PCPS vesicles (20 μM), and prothrombin (1.4 μM) in HBS(Ca^{2+}) 0.1% PEG buffer. The reaction was initiated with factor Xa (5 nM final). Aliquots (25 μL) were removed (0,

30, 60, 90, and 120 s) and diluted into 75 μL of an EDTA-containing buffer (20 mM HEPES, 0.15 M NaCl, 50 mM EDTA, and 0.1% PEG (pH 7.4); quench buffer). α-Thrombin concentration was determined using the chromogenic substrate S-2238. Assays were done on a 96-well microtiter plate for analysis on a Molecular Devices (Menlo Park, CA) $Thermo_{MAX}$ kinetic microplate reader. Assay mixtures contained 80 μL of substrate (0.5 mM) in quench buffer and 20 μL of α-thrombin source. The α-thrombin concentration was calculated by determining the rate of chromogenic substrate hydrolysis in the samples, and comparing them to the rates of substrate hydrolysis obtained from a standard curve of known concentrations (0−50 nM) of purified α-thrombin. Alternatively, samples were assayed for cofactor activity in a clotting assay using factor V-deficient plasma.

Analysis of APC-Catalyzed Cleavage of Factor V and Factor Va by SDS−PAGE. Samples of factor V (100 nM), treated with glycosidases under nonprotein-denaturing conditions, were incubated with PCPS vesicles (200 μM) and APC (2 nM) in HBS(Ca^{2+}) containing 0.1% PEG. Hirudin (20 nM) was present in the reaction to inactivate any traces of α-thrombin in the APC preparation. Samples (30 μL) were removed at selected time intervals (0, 1, 3, 5, 10, 30, 60, and 120 min), and added to a 7.5 μL 5X SDS−PAGE sample preparation buffer [62.5 mM Tris HCl, 2% SDS, 10% glycerol, 2% β-ME, and 0.001% bromophenol blue (pH 6.8) (final concentrations)]. The samples were subjected to SDS−PAGE on 4−12% linear gradient slab gels and silver stained by the method of Merril et al. (*65*). Other factor V samples, which were treated with glycosidases under nonprotein-denaturing conditions, were diluted to 1 μM in HBS(Ca^{2+}) and activated with α-thrombin (2 U/mL, 20 nM) for 15 min at 37 °C to factor Va. The reaction was terminated by the addition of hirudin (30 nM). Factor Va (20 nM) was incubated with PCPS vesicles (10 μM) and APC (0.2 nM) in HBS(Ca^{2+}) containing 0.1% PEG. Samples were removed at selected time points (0, 1, 3, 5, 10, 30, 60, and 120 min), placed in sample preparation buffer, and subjected to SDS−PAGE on 5−15% linear gradient slab gels. Following electrophoresis, the factor V and factor Va fragments were transferred to nitrocellulose, as described by Towbin et al. (*66*), and detected by Western blotting using an antihuman factor V monoclonal antibody ($\alpha HFVa_{HC}$#17), as described previously (*67*).

RESULTS

Electrophoretic Analysis of Untreated and Glycosidase-Treated Factor V and Va. Samples of factor V or factor Va were treated with glycosidases and analyzed by SDS−PAGE, followed by Coomassie blue staining (Figure 2). Initial studies established conditions to effect maximal N-linked carbohydrate removal without altering factor Va cofactor activity, as assessed by clotting assays using factor V-deficient plasma (data not shown). Glycosidase treatment of factor V with *N*-glycanase and neuraminidase under nonprotein-denaturing conditions yielded a molecule with slightly increased mobility (Figure 2, lane 2) when compared to untreated factor V (Figure 2, lane 1). As expected, similar treatment under protein-denaturing conditions resulted in additional deglycosylation (Figure 2, lane 3), whereas the addition of *O*-glycanase led to a small additional mobility change (Figure 2, lane 4). Samples of α-thrombin-activated

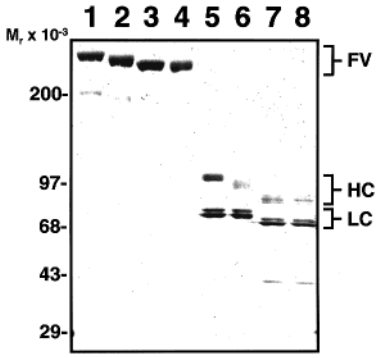

FIGURE 2: Electrophoretic mobility of glycosidase-treated factor V and factor Va. Factor V (lanes 1−4) or factor Va (lanes 5−8) were treated with glycosidases for 3 h at 37 °C under nonprotein-denaturing (HBS; pH 7.4) or protein-denaturing (HBS, 0.17% SDS, 17 mM β-ME, and 1.25% NP-40 (pH 7.4)) conditions as specified in the Experimental Procedures section. Glycosidase-treated factor V and factor Va samples (8 μg) were subjected to SDS−PAGE on 5−15% polyacrylamide gels and visualized using Coomassie blue staining. (Lane 1, untreated factor V; lane 2, factor V (4 μM) treated with neuraminidase (0.165 U/mL) and *N*-glycanase (1.32 U/mL) under nonprotein-denaturing conditions; lane 3, factor V (1 μM) treated with neuraminidase (0.5 U/mL) and *N*-glycanase (5 U/mL) under protein-denaturing conditions; lane 4, factor V treated as in lane 3 with the addition of *O*-glycanase (0.033 U/mL; lanes 5−8, α-thrombin activated factor V (factor Va) treated identically to lanes 1−4, respectively.) The labels to the right of the figure indicate the locations of the single-chain factor V molecule (FV) and the heavy chain (HC) and light chain (LC) of factor Va.

factor Va were treated with glycosidases, as described previously, for factor V (Figure 2, lanes 5−8). Glycosidase treatment of factor Va under nonprotein-denaturing conditions reduced the carbohydrate content of the heavy chain of the molecule without a noticeable effect on the light chain. Again, as anticipated, identical glycosidase treatment under protein-denaturing conditions led to additional and substantial carbohydrate removal from both chains of the factor Va molecule. Combined treatment with *O*-glycanase was without additional effect. Periodic acid Schiff staining of these samples confirmed that virtually all of the carbohydrate was removed from the factor Va heavy chain under protein-denaturing conditions (data not shown). These combined results indicate that, under nonprotein-denaturing conditions, glycosidase treatment removed approximately 20−30% of the carbohydrate moieties from the heavy chain of factor Va, while leaving the carbohydrate moieties of the light chain intact and yielding a fully functional cofactor molecule.

Effect of Factor V/Va Deglycosylation on its APC-Catalyzed Inactivation. Purified factor V, untreated or glycosidase-treated under nonprotein-denaturing conditions for various periods of time, was analyzed in an aPTT-based APC sensitivity assay, as detailed in the Experimental Procedures section, to determine if carbohydrate content altered the susceptibility of factor V to APC-catalyzed inactivation. The time-dependent glycosidase treatment of factor V under nonprotein-denaturing conditions resulted in a maximum increase in the APC-SR from 2.34 to 3.33 within 1 h of treatment (Figure 3A, open squares). The APC-SR obtained with the untreated factor V sample showed no substantial change over time (2.34 to 2.51 at 4 h; Figure 3A, filled squares). To determine if the increased APC-SR was influenced by the presence of factor Va (0.2 nM) formed during the assay prior to APC addition, an analogous

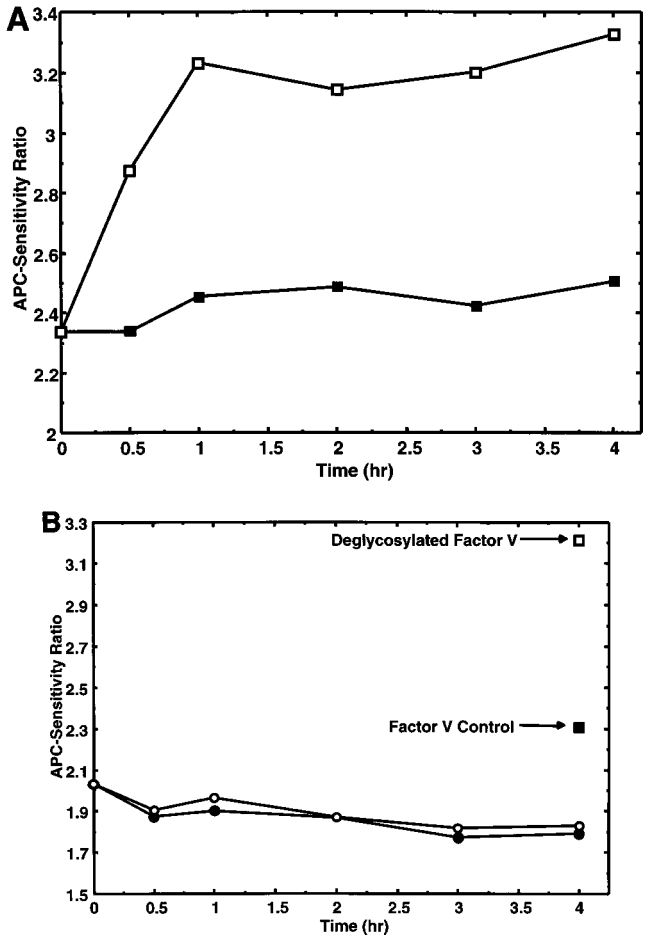

FIGURE 3: aPTT-APC sensitivity assay of glycosidase-treated factor V and factor Va. (A) Untreated factor V (■) and factor V treated with glycosidases under nonprotein-denaturing conditions (□) were assayed in the modified APC sensitivity assay (2 nM factor V final) in the presence or absence of APC (20 nM) in the clot-initiating $CaCl_2$ solution (100 μL) over the course of glycosidase treatment at 0, 0.5, 1, 2, 3, and 4 h. (B) The samples of factor V used in panel A were activated to factor Va with α-thrombin and analyzed in the APC sensitivity assay (0.2 nM factor Va final). Factor Va (●) and glycosidase-treated factor Va (○) were analyzed at the time points specified above. At 4 h, unactivated samples of factor V (■) and glycosidase-treated factor V (□) were analyzed as those in panel A. The observed APC-SR values are reported as the clot time with APC added to the assay system over the clot time without APC.

experiment was done with untreated and glycosidase-treated factor Va. Experimental protocols were as those described for factor V with the following exceptions. At various time points, the untreated and glycosidase-treated factor V samples were activated with α-thrombin to form factor Va prior to its assay at 0.2 nM. This concentration of factor Va was chosen because our initial determinations indicated that this concentration of factor Va was present at the time of clot initiation in the APC sensitivity assay containing 2 nM factor V. The data shown in Figure 3B depict virtually no change in the APC-SR over a 4-h time period, even though effective deglycosylation was achieved within 1 h. These results indicate that partial deglycosylation of factor V increased its sensitivity to APC.

Effect of Glycosidase Treatment on the APC-Catalyzed Cleavage of the Procofactor Factor V and the Derived Cofactor Factor Va. To confirm that the increased APC-SR observed in both clotting assays was a direct consequence

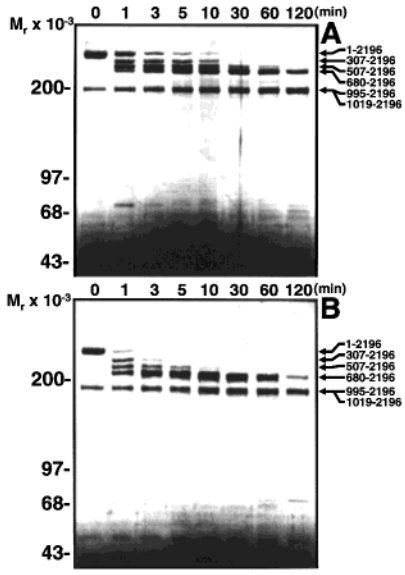

FIGURE 4: APC-catalyzed cleavage of untreated and glycosidase-treated factor V. Factor V (4 μM) was treated with *N*-glycanase (1.32 U/mL) and neuraminidase (0.165 U/mL) for 3 h at 37 °C to generate glycosidase-treated factor V. For untreated factor V, samples were incubated under identical conditions without glycosidase addition. Untreated (A) and glycosidase-treated (B) factor V samples were diluted to 100 nM, and subjected to cleavage by APC (2 nM) in the presence of PCPS vesicles (200 μM) at 37 °C. Samples were removed at 0, 1, 3, 5, 10, 30, 60, and 120 min and quenched in an SDS-sample preparation buffer. The factor V samples were separated by SDS−PAGE on 4−12% polyacrylamide gels and visualized by silver staining. The numbers and arrows correspond to the amino acid sequences of the proteolytically derived fragments produced by sequential APC cleavage. The 200 kD band visible in the starting samples (lanes 0) represents factor V that has been cleaved at position 1018 and is a normal constituent of preparations of purified human factor V (*52*).

of more rapid cleavage of factor V by APC, the influence of the factor V carbohydrate moieties on the APC-catalyzed cleavage of factor V was assessed in a purified system. Samples of factor V, which had been treated with glycosidases under nonprotein-denaturing conditions, as well as untreated factor V samples, were incubated with PCPS vesicles and APC as detailed in Figure 4. Hirudin was present in the reaction to inhibit any traces of α-thrombin in the APC preparation and prevent unwanted activation of factor V. The formation of factor V fragments via its APC-catalyzed proteolysis over time was monitored by SDS−PAGE followed by silver staining. Under these conditions, untreated factor V was cleaved within 1 min of incubation to form four high molecular weight products consistent with its ordered cleavage at Arg^{306}, Arg^{506}, Arg^{679}, and Arg^{994} (Figure 4A, lane 1). The identification of the four high molecular weight fragments was based on their comigration with the three high molecular weight fragments generated by the APC-catalyzed cleavage of factor V^{Leiden} corresponding to residues 307−2196, 680−2196, and 995−2196 (*34*). Although monitoring the appearance of the low molecular weight cleavage products was not possible in this gel/staining system, the sequential nature of the disappearance of the high molecular weight fragments (Figure 4A) was evident. Comparison of the time-dependent APC-catalyzed cleavage of glycosidase-treated (B) versus untreated (A) factor V indicated that even 20−30% carbohydrate removal had a dramatic and substantial effect on the rate of cleavage.

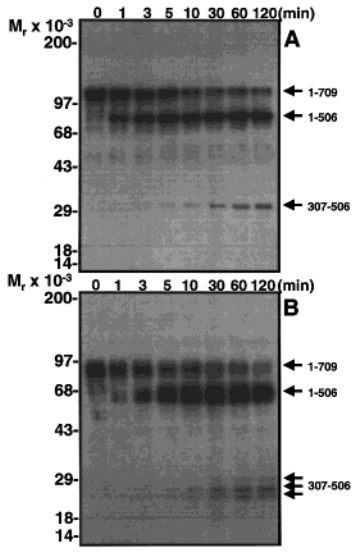

FIGURE 5: APC-catalyzed cleavage of untreated and glycosidase-treated factor Va. Factor V was glycosidase-treated as described in Figure 4 and diluted to 1 μM. This was followed by activation to factor Va with α-thrombin (2 U/mL, 20 nM) at 37 °C for 15 min and termination of the α-thrombin activation with hirudin (30 nM). Samples of untreated (A) and glycosidase-treated (B) factor Va (20 nM) were treated with APC (0.2 nM) in the presence of PCPS vesicles (10 μM) at 37 °C. Samples were removed at 0, 1, 3, 5, 10, 30, 60, and 120 min and quenched in an SDS-sample preparation buffer. Factor Va samples were resolved on 5−15% polyacrylamide gels, transferred to nitrocellulose, and subjected to Western blotting with an antihuman factor V monoclonal antibody (αHFVa$_{HC}$#17), as described in the Experimental Procedures section. The numbers and arrows correspond to the amino acid sequences of the proteolytically derived fragments produced by APC cleavage. The multiple arrows associated with fragment 307−506 in panel B indicate multiple glycoforms of the 30 kD polypeptide.

Whereas near complete proteolysis of the untreated procofactor at its first cleavage site, Arg^{306}, required approximately 10 min, equivalent cleavage of the glycosidase-treated procofactor was obtained within as little as 1 min. These data indicated that partial deglycosylation of factor V resulted in a ≈10-fold increase in the rate of APC-catalyzed cleavage at Arg^{306}, a cleavage that is associated with full inactivation of the factor V molecule.

In marked contrast, similar experiments with factor Va derived from the α-thrombin-catalyzed activation of glycosidase-treated factor V (Figure 5) indicated that the rate of factor Va cleavage by APC was not influenced by glycosidase treatment. The rate of untreated and glycosidase-treated factor Va cleavage by APC on PCPS was determined by SDS−PAGE and Western blotting as described in the Experimental Procedures section. The rate of disappearance of the heavy chain, corresponding to its initial cleavage at Arg^{506}, was the same for both untreated (A) and glycosidase-treated (B) factor Va. However, the rate of disappearance of the 75 kD fragment, corresponding to its cleavage at Arg^{306}, was more difficult to assess because of the multiple glycoforms present in the glycosidase-treated sample. These multiple forms of the 75 kD fragment were most likely derived from differential susceptibility of factor V glycoforms to glycosidase treatment under nonprotein-denaturing conditions. Multiple glycoforms of the 30 kD fragment associated with cleavage of the 75 kD fragment glycoforms at Arg^{306} were also evident in the glycosidase-treated sample (Figure 5B; Figure 6, inset). Because the cleavage of the 75 kD fragment at Arg^{306} and the appearance of the resulting 30 kD fragment is associated with full inactivation of factor Va, densitometric analyses were done to compare the rates of appearance of the resulting 30 kD glycoforms in the glycosidase-treated sample (Figure 6). These analyses revealed that each glycoform band represented a constant fraction of the total density in each lane over the time course of proteolysis (Figure 6: band 1, filled squares, 13%; band 2, filled circles, 54%; band 3, filled triangles, 33%). These results indicated that although the 30 kD fragment glycoforms existed in varying amounts, they were generated at proportionally similar rates. These data are in excellent agreement with data from factor Va inactivation experiments (Figure 7) in which both untreated and glycosidase-treated factor Va showed identical rates of inactivation in a functional cofactor assay system comprised of purified components.

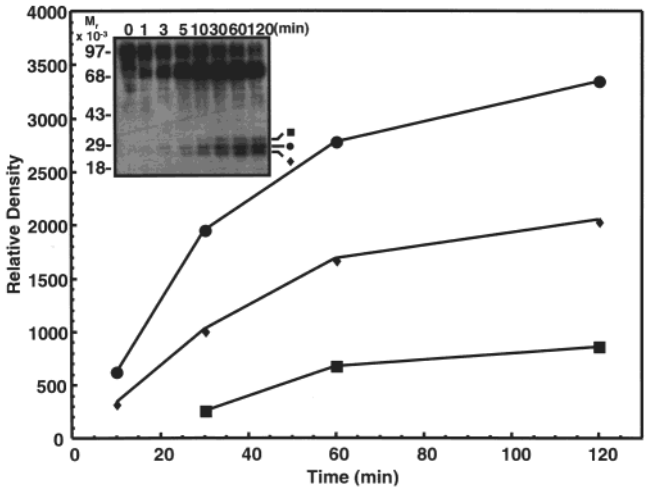

FIGURE 6: Densitometric analysis of the appearance of the 30 kD glycoforms. Densitometric analyses were conducted on a longer exposure of the blot depicted in Figure 5B. The appearance of the glycoforms of the 30 kD fragment, band 1 (■), band 2 (●), and band 3 (◆), was quantitated by densitometry using NIH Image software and plotted against time. The inset depicts the blot exposure used for this analysis.

These combined data indicate that glycosidase treatment of factor V enhanced cleavage at Arg^{306} by APC. In contrast, factor Va derived from glycosidase-treated factor V appeared to be cleaved equivalently at this site as well as at the Arg^{506} site.

DISCUSSION

The data detailed in this study demonstrate that the removal of a portion of the N-linked carbohydrate from factor V increased its susceptibility to cleavage and inactivation by APC. In marked contrast, APC-catalyzed cleavage of factor Va derived from this partially deglycosylated form of factor V was unaffected. Thus, N-linked carbohydrate moieties associated with its heavy chain region appear to protect factor V, but not factor Va, from APC-catalyzed inactivation.

Whereas the presence of carbohydrate moieties has been detected in all α-thrombin-derived fragments of the factor V molecule by periodic acid Schiff staining (*36*), the detailed carbohydrate structure of factor V is unknown. Previous studies indicate that factor V contains N-linked oligosac-

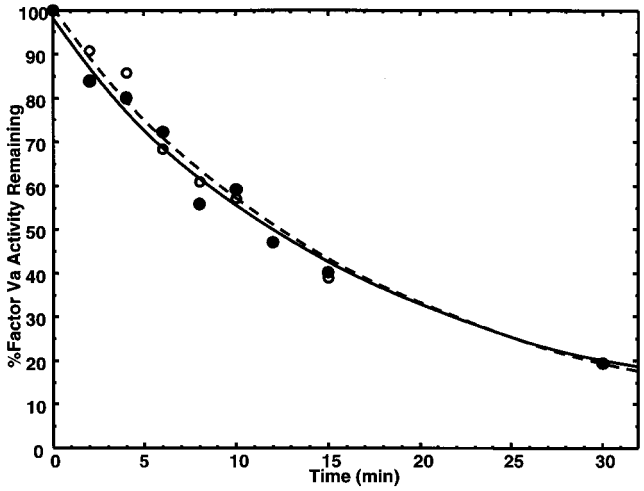

FIGURE 7: APC-catalyzed inactivation of factor Va. Factor V (4 μM) was subjected to glycosidase treatment under nonprotein-denaturing conditions with *N*-glycanase (1.32 U/mL) and neuraminidase (0.165 U/mL) for 3 h at 37 °C. Samples were diluted to 1 μM and activated to factor Va by α-thrombin (2 U/mL, 20 nM) for 15 min at 37 °C, at which point the activation was terminated by the addition of 30 nM hirudin. The resulting factor Va was used at 20 nM and inactivated by APC (0.1 nM) in the presence of PCPS vesicles (10 μM). Samples of the inactivation mixture were removed at 0, 2, 4, 6, 8, 10, 12, 15, and 30 min. Cofactor activity was assessed by prothrombinase assay using purified protein components with saturating amounts of factor Xa (5 nM) and PCPS vesicles (20 μM), as described in the Experimental Procedures section. The percent remaining activity of the glycosidase-treated (○) and untreated (●) cofactor samples were plotted as a function of time.

charides throughout the molecule and O-linked oligosaccharides exclusively in the B domain (*36*, *49*). Our study confirmed these observations and indicated further that there were populations of N-linked carbohydrate that were variably resistant to removal by *N*-glycanase. Under nonprotein-denaturing conditions, only 20−30% of the N-linked carbohydrate moieties attached to the heavy chain of factor V were removed by enzymatic hydrolysis. The total resistance of the light chain N-linked carbohydrate moieties to enzymatic removal under nonprotein-denaturing conditions has been observed previously (*49*). However, once protein-denaturing conditions were employed, nearly all of the carbohydrate was removed from the heavy chain, whereas only a portion of the light chain carbohydrate was removed. Even after 18 h of glycosidase treatment under protein-denaturing conditions, the light chain remained as a doublet, most likely because of its partial glycosylation at Asn^{2181} (*43*, *44*), a carbohydrate moiety highly resistant to enzymatic removal. Consequently, the differential susceptibility of glycosidase-treated factor V and the derived factor Va to APC-catalyzed inactivation observed in this study was the result of relatively small changes in the carbohydrate content of the amino-terminal heavy chain region of the molecules.

After partial deglycosylation of the factor V heavy chain region, we observed a ≈10-fold increase in the rate of its APC-catalyzed cleavage at Arg^{306}. The more rapid cleavage and inactivation observed upon glycosidase treatment of factor V was not observed if the factor V was subsequently activated to factor Va. The mechanism by which select carbohydrate removal promotes an increased rate of cleavage at Arg^{306}, only in factor V, is unclear. However, two possibilities can be ruled out. Despite the fact that the APC-catalyzed cleavage at Arg^{306} requires that the substrate, factor V or Va, is membrane-bound, it is highly unlikely that partial deglycosylation has altered factor V membrane binding in this study. An effect on phospholipid binding would only have been observed if the lipid binding parameters were weakened by greater than 2 orders of magnitude, because high phospholipid concentrations (200 μM) were used in our experiments. Under nondenaturing conditions, no carbohydrate removal was observed in the light chain region of factor V, the region that contributes to the membrane binding of the factor Va molecule (*68*−*70*). Select removal of carbohydrate moieties to expose the cleavage site at Arg^{306} is also unlikely, as well. Substantial heterogeneity is evident with regard to the extent of carbohydrate removal, on the basis of the presence of multiple 75 and 30 kDa fragments observed in factor Va derived from the thrombin-catalyzed activation of glycosidase-treated factor V. Thus, it is highly unlikely that complete carbohydrate removal at a single site was accomplished.

Data, presented in this study or published previously, support the contention that the heavy chain regions of factor V and factor Va are structurally distinct. The data presented in this study demonstrate clearly that N-linked carbohydrate moieties affect the susceptibility of factor V to APC-catalyzed cleavage at Arg^{306}, while cleavage of the factor Va derived from its glycosidase-treated procofactor at either Arg^{506} or Arg^{306} is completely unaffected. This disparity is underscored by the observation that cleavage at Arg^{506} in the partially deglycosylated factor Va molecule yields several differentially glycosylated 75 kDa peptides, which are all cleaved at Arg^{306} at identical rates. Differences in the structure of this region have previously been implied by the order in which the cleavage sites of both molecules are recognized by APC. Initial cleavage of the factor V molecule occurs at Arg^{306} and results in a procofactor species that produces inactive factor Va upon α-thrombin-catalyzed cleavage (*17*). In contrast, initial cleavage of the factor Va molecule occurs at Arg^{506} (*17*, *18*, *31*, *33*, *34*), which appears to facilitate cleavage at Arg^{306}. Thus, the structural differences that dictate the different order of APC-catalyzed cleavage in native factor V and factor Va also appear to be reflected in the different susceptibilities of the glycosidase-treated molecules to cleavage and inactivation by APC.

Fernandez et al. had previously investigated the effect of factor V's carbohydrate moieties on its APC-catalyzed inactivation (*49*). Their study also demonstrated that glycosidase treatment of factor V under nonprotein-denaturing conditions, and its subsequent assay in a clotting based APC sensitivity assay, led to a marked prolongation of the clotting time. However, they concluded that the glycosidase-treated factor Va formed during the assay was substantially more sensitive to APC than the untreated factor Va assayed in the same manner. Results from our studies indicate that approximately 10% of the factor V added to an APC sensitivity assay is converted to factor Va. Because both factor V and Va are APC substrates, an alternative explanation for their data, which was confirmed by our studies, could be offered. Deglycosylated factor V was being cleaved more rapidly by APC present in the assay, thereby limiting the amount of functional cofactor (factor Va) being produced. This conclusion is supported by the demonstration that the APC-catalyzed cleavage of the procofactor, factor V, was sub-

stantially increased following its partial deglycosylation, whereas the APC-catalyzed cleavage of the partially deglycosylated derived cofactor, factor Va, was unaffected.

Other studies have shown that decreases in the carbohydrate content of human factors V and Va have altered select functional and physicochemical properties, although absolute comparisons between studies are difficult because markedly different experimental conditions were used to effect deglycosylation. Bruin et al. reported that deglycosylation of factor V impaired its rate of α-thrombin-catalyzed activation and decreased factor V's clotting activity (*37*). In contrast, factor V secreted from cells treated with deoxymannojirimycin, an inhibitor of complex oligosaccharide addition, possessed increased cofactor activity (*47*, *48*). Additionally, two naturally occurring glycoforms of factor V exists, one of which lacks N-linked carbohydrate at Asn^{2181} (*43*, *44*), and gives rise to a cofactor with increased affinity for phospholipid (*40*, *44*). Because estimates of carbohydrate content suggest that various carbohydrate moieties account for 13−25% of the factor V mass, it is not surprising that these posttranslational modifications regulate its functional and physical properties.

In addition, posttranslational modifications, perhaps unique to platelet-derived factor V, have been hypothesized to distinguish it from plasma-derived factor V for several reasons. A substantial amount (∼20−25%) of the factor V found in human blood is derived from the α-granules of platelets, while the remainder is found in plasma (*19*). These two pools of factor V are distinct substrates for a number of proteases (*5*, *20*, *67*, *71*), including APC. The factor Va obtained from platelets displays a strong resistance to inactivation by APC when compared to its plasma counterpart. Although the presence of the platelet membrane appears to play a role in this resistance, evidence suggests that inherent differences between the platelet-derived and plasma-derived factor V molecules are partly responsible for their different susceptibilities to APC-catalyzed cleavage and inactivation (*20*). Reports from our laboratory indicate that megakaryocytes, the precursor cells of platelets, endocytose the plasma-derived procofactor (*72*, *73*) to create the platelet-derived α-granule sequestered factor V pool (*74*, *75*). While the factor V in plasma circulates as a single-chain molecule (330 kDa), the factor V stored in platelets is composed of a heterogeneous mixture of fragments ranging from 70 to 330 kDa. Furthermore, unlike the factor V in plasma, platelet-derived factor V contains substantial cofactor activity (*5*, *76*). These observations have led us to hypothesize that, after its endocytosis by megakaryocytes, factor V undergoes retrograde transport to the trans-Golgi network, and is posttranslationally retailored to create a physically distinct form of the factor V protein. Although the current study indicates that the N-linked carbohydrate in the heavy chain region of factor V had no affect on the APC-catalyzed cleavage and inactivation of plasma-derived factor Va, it did demonstrate that alterations in glycosylation can significantly regulate the APC-catalyzed cleavage and inactivation of some forms of the factor V molecule. Because platelet-derived factor V has recently been purified by our laboratory (*77*), the functional effects of its carbohydrate moieties can now be studied.

In conclusion, the data presented in this paper clearly indicate that the partial deglycosylation of factor V can have a dramatic effect on its susceptibility to APC-catalyzed cleavage and inactivation. However, this effect was not observed in all forms of the factor V molecule. Two conclusions can be drawn from these collective data. Dramatic alterations in the structure of the factor V molecule occur as it is proteolytically converted to the active cofactor and, in addition, N-linked carbohydrate moieties serve to regulate protease activity toward factor V.

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