Posttranslational Sulfation of Factor V Is Required for Efficient Thrombin Cleavage and Activation and for Full Procoagulant Activity

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ABSTRACT: Factor VIII and factor V function as cofactors in the blood coagulation cascade to accelerate the rate of activation of factor X and prothrombin, respectively. Both cofactors require proteolytic activation by either activated factor X or thrombin for functional activity. Human factor VIII and factor V expressed in mammalian cells are both modified by posttranslational sulfation of tyrosine residues. In the present study, the posttranslational addition of sulfate in factor V expressed in transfected Chinese hamster ovary (CHO) cells was demonstrated by [^{35}S]sulfate incorporation into the thrombin-cleaved 94-kDa heavy chain and the 150-kDa activation peptide. The presence of tyrosine sulfate in recombinant factor V was confirmed by barium hydroxide hydrolysis and two-dimensional thin-layer electrophoresis. The importance of sulfation for factor V secretion and activity was evaluated by characterizing factor V expressed in Chinese hamster ovary cells grown in the presence of sodium chlorate, a potent inhibitor of posttranslational sulfation in intact cells. Increasing concentrations of sodium chlorate inhibited the incorporation of [^{35}S]sulfate into factor V but did not inhibit the synthesis or secretion of factor V. However, the specific activity of factor V secreted in the presence of sodium chlorate was reduced 5-fold. The reduced activity was attributed to (1) slower cleavage and activation by thrombin and (2) a reduced intrinsic activity of factor Va. In contrast, sulfation of factor V did not affect the rate of activation mediated by factor Xa. These results show that sulfation of factor V is required for efficient thrombin activation but not for activation by factor Xa. In addition, factor V isolated from a patient with homozygous combined factor V and factor VIII deficiency is not defective in posttranslational sulfation.

Coagulation factor V is a 330-kDa single-chain plasma glycoprotein that plays a critical role in hemostasis as a cofactor for the factor Xa dependent proteolytic cleavage of prothrombin to thrombin (Esmon, 1979; Nesheim et al., 1979; Dahlback, 1980; Kane & Majerus, 1981). The amino acid sequence deduced from the cDNA showed that factor V is synthesized as a 2224 amino acid precursor containing a 28 amino acid signal peptide (Kane & Davie, 1986; Jenny et al., 1987). Intact single-chain factor V has little intrinsic cofactor activity (Katzman et al., 1981; Monkovic & Tracy, 1990). Factor V is cleaved by thrombin after residues 709, 1018, and 1545 to generate activated factor Va that is composed of a 94-kDa amino-terminal-derived fragment in a metal–ion association with a carboxy-terminal-derived 74-kDa fragment (Esmon, 1979; Nesheim et al., 1979; Dahlback, 1980; Kane & Majerus, 1981; Katzman et al., 1981). In contrast, factor V activation by factor Xa cleavage yields the 94-kDa fragment and a larger 220-kDa carboxy-terminal-derived fragment (Monkovic & Tracy, 1990). Factor Va generated by either process effectively combines with factor Xa on a phospholipid surface in the presence of calcium ions and accelerates the factor-Xa-mediated proteolysis of prothrombin to thrombin by 10 000-fold (Krishnaswamy et al., 1987).

Factor V shares sequence homology with factor VIII (Vehar et al., 1984; Toole et al., 1984; Mann et al., 1988), and both proteins have a domain organization of A1:A2:B:A3:C1:C2. The triplicated A domains are homologous to the A domains in the plasma copper binding protein ceruloplasmin (Ortel et al., 1984). The C domains repeated twice in the carboxy terminus share homology with phospholipid binding proteins (Stubbs et al., 1990). The B domains have significantly diverged, share no homology with other known proteins, and are both encoded by unusually large single exons (Gitschier et al., 1984; Cripe et al., 1992). The B domain of factor V contains 25 of the potential 37 asparagine-linked oligosaccharide addition sites in the molecule.

Within the secretory pathway, factor V is posttranslationally modified by proteolytic processing of the signal peptide, addition of high mannose and complex asparagine-linked oligosaccharides (Bruin et al., 1987), addition of serine- and threonine-linked oligosaccharides, and addition of sulfate to tyrosine and carbohydrate residues (Hortin, 1990). There are examples of posttranslational sulfation of tyrosine residues affecting protein secretion (Friederich et al., 1988; Huttner & Baeuerle, 1988), biological activity (Mutt, 1980), and protein binding affinities (Brand et al., 1984; Unsworth & Hughes, 1982; Gordon & Ward, 1985). Tyrosine sulfation occurs in several proteins that interact with proteases and affects their activity. For example, sulfated tyrosine residues in the plasmin interactive sties of α2-antiplasmin (Hortin et al., 1987) and in the thrombin interactive site of heparin cofactor II (Hortin et al., 1986a, 1989) and hirudin (Braun et al., 1988) increase binding affinities of the respective proteases. In addition, many proteins that interact with

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thrombin, such as fibrinogen (Jevons, 1963; Farrell et al., 1991), bovine factor X (Morita & Jackson, 1986), vitronectin (Jenne et al., 1989), and factor VIII (Pittman et al., 1992), contain sites of tyrosine sulfation. In particular, factor VIII contains six sites of tyrosine sulfatuion that are required for full procoagulant activity (Pittman et al., 1992) and for high-affinity binding to von Willebrand factor (Pittman et al., 1987; Leyte et al., 1991). Since sulfated tyrosine residues influence factor VIII procoagulant activity (Pittman et al., 1992), we have characterized the functional significance of sulfation on factor V activity. In this paper we show that factor V requires sulfation for full procoagulant activity and for efficient thrombin cleavage and activation. However, the efficiency of factor V activation by factor Xa or Russell's viper venom was not affected by sulfation. In addition, we show that defective sulfation does not appear to be the cause for the reduced activity of factor V and factor VIII in a patient with homozygous combined factor VIII and factor V deficiency.

EXPERIMENTAL PROCEDURES

Materials. Methionine-free medium was purchased from Flow Laboratories, ICN (Costa Mesa, CA). Sulfate-free alpha medium was purchased from Specialty Media (Lavallette, NJ). Human thrombin, human factor V, human factor Xa, and Russell's viper venom were purchased from Hematological Technologies (Burlington, VT). Thromboplastin, soybean trypsin inhibitor, and aprotinin were acquired from Sigma Chemical Co. (St. Louis, MO). [^{35}S]Methionine (>1000 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). [^{35}S]Sulfuric acid (1050–1600 Ci/mmol) and En^{3}Hance were obtained from DuPont-New England Nuclear (Boston, MA). Factor V deficient human plasma was obtained from George B. King Biomedical (Overland Park, KS). *N*-Glycanase, *O*-glycanase, deoxymannojirimycin (DMJ),[1] and endoglycosidase H (endo-H) were obtained from Genzyme (Boston, MA). Rabbit polyclonal anti-factor V antibody was obtained from Dako Corp. (Carpinteria, CA). The monoclonal antibody HV-9 was obtained from Hematological Technologies. Sodium chlorate was purchased from Fluka Chemie (Buchs, Switzerland).

Cell Lines. The full-length human factor V cDNA was subcloned into the expression vector pED (Kaufman et al., 1991) to obtain pED-V. pED-V was linearized with *Nde*I and transfected into dihydrofolate reductase (DHFR) deficient Chinese hamster ovary (CHO DUKX; Urlaub & Chasin, 1980) cells by lipofection. The cell clone designated T9 was derived by selection for DHFR gene amplification by propagation in increasing concentrations of methotrexate to 0.1 μM (Kaufman, 1990). This cell line produces approximately 1 unit of factor V 10^6 cells mL^{-1} (10^6 cells)$^{-1}$ day^{-1}.

Factor V Expression and Activity. Factor V synthesis and secretion were measured by growing cells in complete alpha medium supplemented with 10% dialyzed fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, and 0.1% aprotinin. Cells were rinsed and incubated for 30 min at 37 °C in sulfate-free alpha medium. The medium was removed, and fresh sulfate-free alpha medium containing 330 μCi/ml [^{35}S]sulfuric acid was applied. After 24 h, conditioned medium was collected and centrifuged at low speed to remove cellular debris. In parallel, cells were labeled with [^{35}S]methionine (300 μCi/ml) as described (Dorner & Kaufman, 1990). After incubation for 2 h, the medium was removed and fresh alpha medium was added. After incubation for 12 h, the medium was harvested and centrifuged to remove cellular debris, and soybean trypsin inhibitor (1 mg/mL) and phenylmethanesulfonyl fluoride (0.1 mM) were added. Cells were pretreated with 1 mM DMJ for 1 h at 37 °C. Medium was removed, and fresh medim containing 200 μCi/ml [^{35}S]sulfuric acid or [^{35}S]methionine was added. After 24 h, conditioned medium was harvested and centrifuged and protease inhibitors were added. Factor V was immunoprecipitated with anti-factor V monoclonal or polyclonal antibody, and immunoprecipitated proteins were analyzed by SDS–PAGE as described (Pittman & Kaufman, 1993). Aliquots of immunoprecipitated protein were treated with thrombin (Pittman & Kaufman, 1993), *N*-glycanase (Tarentino et al., 1985), *O*-glycanase, or endo-H (as recommended by supplier) prior to electrophoresis. Gels were analyzed by autoradiography after treatment with En^{3}Hance (Pittman & Kaufman, 1988). Band intensities were quantitated by scanning using an XL-ultroscan laser densitometer (Pharmacia Inc., Uppsala, Sweden).

Factor V activity was measured in a clotting assay using factor V deficient human plasma. The standard curve was preapred by dilution of normal pooled plasma into 50 mM Tris-HCl, pH 7.2, 0.15 M NaCl, and 1% bovine serum albumin. Factor V activity was determined from peak activation after treatment with thrombin (1 unit/mL), factor Xa (1 μg/mL), or Russell's viper venom (2 μg/mL) for increasing periods of time. Experiments conducted with conditioned medium from control nontransfected CHO cells that do not express factor V demonstrated that addition of thrombin, factor Xa, or RVV did not increase the clotting time of factor V deficient plasma. The specific activity of factor V expressed in T9 CHO cells was estimated by comparing the amount of activity after peak thrombin activation to the band intensity observed by Western immunoblot analysis using as standard a dilution series of purified human plasma-derived factor V. By this analysis the specific activity of purified plasma-derived factor V (710 units/mg) was similar to that of T9 CHO-derived factor V (360 units/mg).

Thin-Layer Electrophoresis. Factor-V-expressing CHO cells were labeled for 24 h with [^{35}S]sulfuric acid, and the conditioned medium was immunoprecipitated with a factor V specific polyclonal antibody. The pellet was resuspended in 65 mM Tris-HCl, pH 6.8, 1% SDS, 2.5% 2-β-mercaptoethanol, and 25 mM dithiothreitol and heated for 5 min at 95 °C. The protein was precipitated with 20 volumes of cold 80% acetone, dried, and resuspended in 50 mM Tris-HCl, pH 6.8. The immunoprecipitated protein was subjected to alkaline hydrolysis with barium hydroxide (Huttner, 1984). The lyophilized pellet was resuspended in 5% acetic acid/0.5% pyridine. Unlabeled tyrosine sulfate (3 μg) marker (kindly provided by Dr. Glen Hortin) was added, and the sample was spotted onto a 20 × 20 cm 100-μm cellulose thin-layer sheet (Kodak, Rochester, NY). Two-dimensional thin-layer electrophoresis was performed at pH 1.9 and 3.5. The thin-layer sheet was dried, and unlabeled tyrosine sulfate was detected with 0.2% ninhydrin in ethanol and radiolabeled tyrosine sulfate by autoradiography.

Detection of Factor V Thrombin Cleavage Fragments. The thrombin cleavage of factor V expressed in CHO cells propagated in the presence or absence of sodium chlorate was evaluated by diluting conditioned medium into 50 mM Tris-

[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DMJ, deoxymannojirimycin; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; Endo-H, endoglycosidase H.

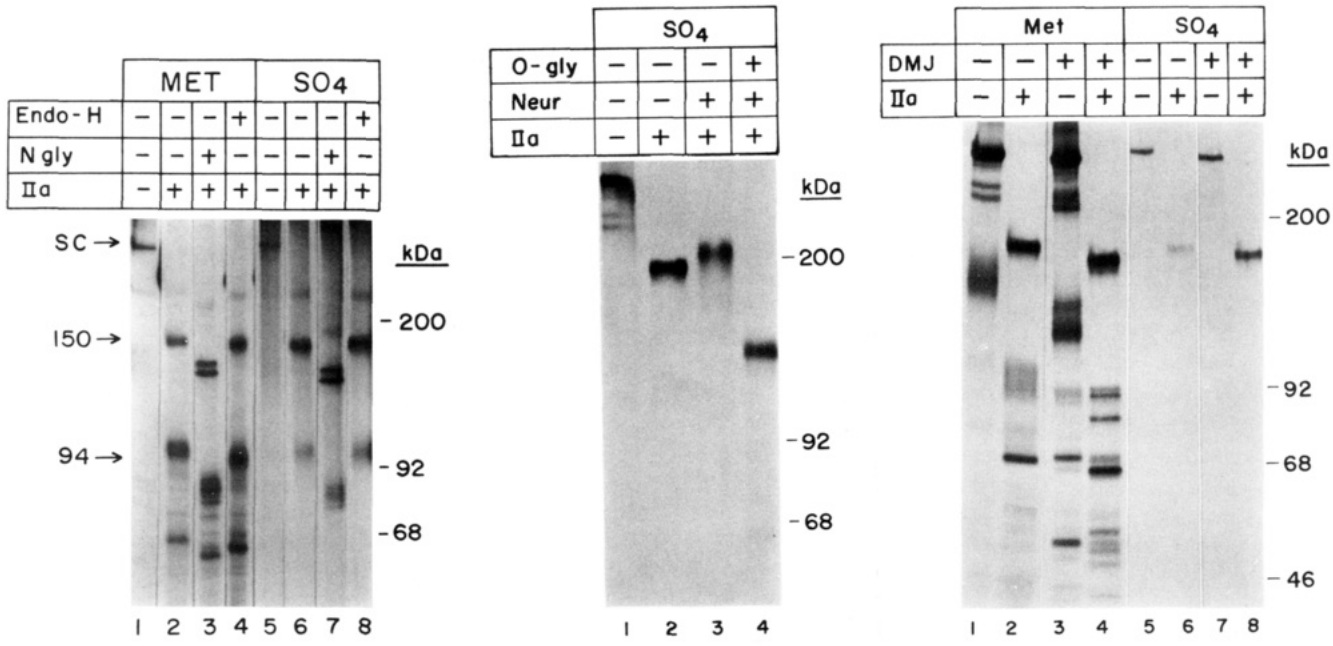

FIGURE 1: Sulfate is not incorporated into N-linked or O-linked oligosaccharides of factor V. T9 CHO cells were labeled with [^{35}S]sulfate or [^{35}S]methionine for 24 h, and the conditioned medium was immunoprecipitated with anti-factor V polyclonal antibody. Equal aliquots of the immunoprecipitates were digested with thrombin (IIa, +), *N*-glycanase (N-gly, +), or endoglycosidase H (Endo-H, +) (panel A, left) or neuraminidase (Neur) and *O*-glycanase (O-gly) (panel B; middle). Samples were analyzed by SDS–PAGE and autoradiography. Cells were labeled with [^{35}S]sulfate or [^{35}S]methionine in the presence of DMJ, and conditioned medium was harvested and analyzed by SDS–PAGE and autoradiography as described under Experimental Procedures (panel C, right).

HCl, pH 7.5, 0.15 M NaCl, and 5% glycerol and adding thrombin (1 units/mL) at room temperature for the indicated periods of time. 2-β-Mercaptoethanol (2.5%) and SDS (1%) were added, and the samples were analyzed by SDS–PAGE and immunoblotting using a rabbit polyclonal anti-factor V antibody and a donkey anti-rabbit antibody coupled to horseradish peroxidase for detection. The polypeptides reacting with the antibody complex were detected by the enhanced chemiluminescence (ECL) method (Amersham). Thrombin cleavage fragments from factor V obtained from normal pooled plasma or from plasma from a factor V and factor VIII deficient patient (BG-A) were immunoprecipitated with anti-factor V HV-9 monoclonal antibody and analyzed after thrombin digestion by SDS–PAGE and immunoblotting using anti-factor V polyclonal antibody and a donkey anti-rabbit horseradish peroxidase-coupled antibody for detection.

RESULTS

Sulfation of Factor V Expressed in Transfected Cells. Previous studies identified the presence of sulfated tyrosine residues within the 150-kDa activation peptide and the 94-kDa heavy-chain-derived thrombin cleavage fragments of factor V secreted from the hepatocellular carcinoma HepG2 cell line (Hortin, 1990). Thus, we characterized whether similar sulfation of factor V occurs in transfected CHO cells. Factor V expression in T9 CHO cells was obtained by stable integration of a factor V expression vector with the selectable and amplifiable genetic marker, DHFR. These cells were radiolabeled with [^{35}S]sulfate or [^{35}S]methionine, and the factor V was immunoprecipitated from the conditioned medium with a polyclonal antibody specific to factor V. The immunoprecipitated radiolabeled polypeptides were analyzed by SDS–PAGE prior to and after thrombin digestion. Immunoprecipitation of the [^{35}S]methionine-labeled factor V detected a single-chain polypeptide present in the medium of transfected CHO (Figure 1A, lane 1) cells. After thrombin treatment, the 150-, 94-, and 74-kDa thrombin cleavage products were generated (Figure 1A, lane 2). In this analysis, the 71-kDa B-domain fragment was not detected, likely due to heterogeneity resulting from glycosylation. [^{35}S]Sulfate was incorporated into factor V expressed in CHO cells (Figure 1A, lane 5). After digestion with thrombin, [^{35}S]sulfate was detected in the 150-kDa activation peptide and to a lesser extent in the 94-kDa-derived heavy-chain fragment (Figure 1A, lane 6). [^{35}S]Sulfate was not detected in the 74-kDa light chain. These results demonstrate incorporation of [^{35}S]-sulfate into the 150- and 94-kDa polypeptides of recombinant factor V and are consistent with results on factor V secreted from HepG2 cells (Hortin, 1990).

Posttranslational sulfation may occur on either carbohydrate or tyrosine residues. To examine if sulfate is incorporated into oligosaccharide residues, samples were treated with specific glycosidases. The factor-V-expressing CHO cells were labeled with [^{35}S]methionine or [^{35}S]sulfate. The conditioned medium samples were immunoprecipitated with factor V specific polyclonal antibody, digested with thrombin, and then treated with *N*-glycanase or endoglycosidase H (Endo-H) prior to SDS–PAGE. Treatment with *N*-glycanase significantly increased the mobility of the 150-, 94-, and 74-kDa thrombin cleavage products, indicating the removal of asparagine (N)-linked carbohydrate residues (Figure 1A, lanes 3, 7). In contrast, Endo-H digestion of thrombin-cleaved factor V slightly increased the mobility of the 94- and 74-kDa polypeptides (Figure 1A, lane 4) compared to that of control treated samples (Figure 1A, lane 2), indicating that the majority of the N-linked oligosaccharides present on recombinant factor V were resistant to Endo-H digestion and were composed of complex or hybrid-type structures. Compared to the thrombin-treated factor V (Figure 1A, lane 6), *N*-glycanase treatment did not remove the [^{35}S]sulfate detected in the 150- and 94-kDa polypeptides, suggesting that the sulfate is not incorporated into N-linked oligosaccharides (Figure 1A, lane 7).

The presence of [^{35}S]sulfate in serine- and threonine-linked oligosaccharides was characterized by treating thrombin-digested factor V with neuraminidase and *O*-glycanase prior to analysis by SDS–PAGE. Treatment with neuraminidase decreased the mobility of the 150-kDa activation peptide (Figure 1B, lane 3), consistent with removal of negatively charged sialic acid residues. Treatment with *O*-glycanase increased the mobility of the 150-kDa polypeptide (Figure 1B, lane 4) compared to that of control factor V (Figure 1B, lane 2), demonstrating the removal of O-linked oligosaccharides. Treatment with neuraminidase or *O*-glycanase did not remove the [^{35}S]sulfate label, suggesting that sulfate is not incorporated into O-linked oligosaccharide residues.

Sulfate incorporation into N-linked oligosaccharides was also characterized by treating cells with deoxymannojirimycin (DMJ), a mannose analogue that inhibits the Golgi α-1,2-mannosidase and therefore inhibits processing of N-linked oligosaccharides to hybrid and complex structures (Fuhrmann et al., 1984). DMJ treatment should therefore inhibit addition of sulfate into N-linked oligosaccharides. T9 CHO cells were labeled with [^{35}S]methionine or [^{35}S]sulfate in the presence or absence of DMJ. The conditioned medium was immunoprecipitated and analyzed by SDS–PAGE before and after thrombin digestion. Factor V activity in the conditioned medium from cells grown in the presence of DMJ was increased by 40% (data not shown). Treatment with DMJ did not alter the secretion or thrombin sensitivity of factor V (Figure 1C, lanes 1–4). In addition, the incorporation of [^{35}S]sulfate into factor V was not reduced in the presence of DMJ (Figure 1C, lanes 5 and 7). Analysis of the thrombin-cleaved polypeptides by sensitivity to Endo-H and *N*-glycanase indicated that the DMJ treatment inhibited addition of complex oligosaccharides (data not shown). These results did not detect the presence of sulfate in N-linked oligosaccharides of factor V expressed in CHO cells.

The presence of [^{35}S]sulfated tyrosine residues in factor V expressed in CHO cells was confirmed by alkaline hydrolysis and two-dimensional thin-layer electrophoresis. Barium hydroxide hydrolysis and neutralization precipitates the alkali labile carbohydrate and proteoglycan residues, whereas tyrosine sulfate remains in solution. The solution was lyophilized and subjected to two-dimensional thin-layer electrophoresis. Under these conditions, radioactivity was recovered as tyrosine sulfate. The [^{35}S]sulfate comigrated with the cold tyrosine marker (Figure 2) and demonstrated the presence of tyrosine sulfate residues within factor V.

Sulfation Is Required for Full Factor V Procoagulant Activity. The effect of sulfation on factor V synthesis, secretion, and activity was evaluated by treating T9 CHO cells with increasing concentrations of sodium chlorate in the presence of [^{35}S]sulfate or [^{35}S]methionine. Sodium chlorate is a potent inhibitor of sulfation of both carbohydrate and tyrosine residues in intact cells. Incorporation of label into secreted factor V was monitored after immunoprecipitation and SDS–PAGE. Concentrations of sodium chlorate up to 100 mM did not affect factor V secretion as measured by [^{35}S]methionine incorporation (Figure 3A, lanes 6–10), whereas [^{35}S]sulfate incorporation was inhibited 20-fold by 10 mM sodium chlorate (Figure 3A, compare lanes 1 and 2) and was not detectable upon treatment with 50 mM sodium chlorate (Figure 3A, lane 4). Treatment with 10 mM sodium chlorate decreased the amount of factor V activity to 40%, and increasing the concentration to 100 mM further reduced the factor V activity to 20% of that of control untreated cells (Figure 3B). Western immunoblot analysis with a factor V specific polyclonal antibody demonstrated that sodium chlorate treatment did not reduce the amount of factor V protein in the conditioned medium (data not shown; see Figure 5), consistent with data in Figure 3A showing that sodium chlorate did not affect secretion of factor V. Control experiments demonstrated that the presence of sodium chlorate did not interfere with the factor V assay. In addition, incubation of conditioned medium in the presence of 100 mM sodium chlorate did not significantly affect factor V activity or the rate and extent of thrombin activation (data not shown), indicating that incubation in the presence of sodium chlorate did not inactivate factor V. These results show that the synthesis and secretion of factor V was not inhibited by sodium chlorate. However, the specific activity of factor V produced in the presence of sodium chlorate was reduced to 20%.

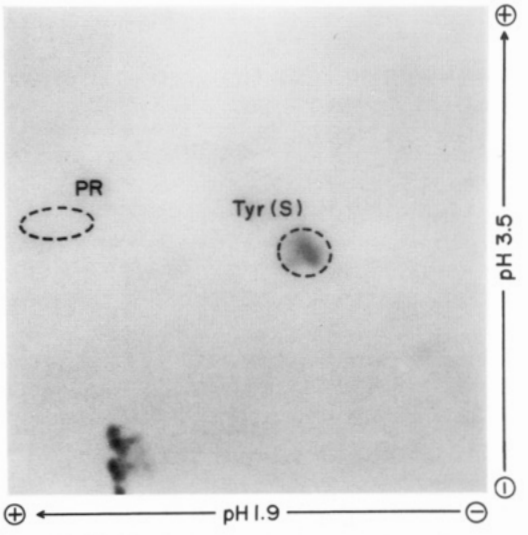

FIGURE 2: [^{35}S]Sulfate is incorporated into tyrosine. T9 CHO cells were labeled for 24 h with [^{35}S]sulfate, and the conditioned medium was immunoprecipitated with anti-factor V polyclonal antibody and subjected to barium hydroxide hydrolysis. Two-dimensional thin-layer electrophoresis was performed at pH 1.9 and 3.5 with cold tyrosine sulfate as a marker. The tyrosine sulfate spot was developed with ninhydrin, and the cellulose sheet was subjected to autoradiography. The cold tyrosine sulfate marker [Tyr(S)] and phenol red (PR) are indicated.

Sulfation Increases the Rate of Factor V Cleavage and Activation by Thrombin. The effect of sulfation on factor V activation by thrombin, factor Xa, and Russell's viper venom was studied by comparing the time course of functional activation of factor V secreted from T9 CHO cells in the presence and absence of 100 mM sodium chlorate. Treatment of factor V with factor Xa yielded peak activation after 30 s. There was no difference in the rate of activation by factor Xa of factor V produced in the absence or presence of 100 mM sodium chlorate (Figure 4B). However, the peak activity for factor V produced in the presence of sodium chlorate was approximately 30% of that obtained in the absence of sodium chlorate. Treatment of T9 cells with sodium chlorate also reduced the peak activity to 40–50% after activation by Russell's viper venom (Figure 4C). In contrast, the rate of activation by thrombin was 5-fold reduced when factor V was produced from T9 cells in the presence of sodium chlorate (Figure 4A). In addition, the peak activity obtained was approximately 30% of that obtained from factor V produced from T9 cells in the absence of sodium chlorate. These results show that sulfation of factor V did not affect the rate of activation by factor Xa but was required for rapid activation by thrombin. In addition, the intrinsic activity of factor V derived after treatment with either factor V activator was

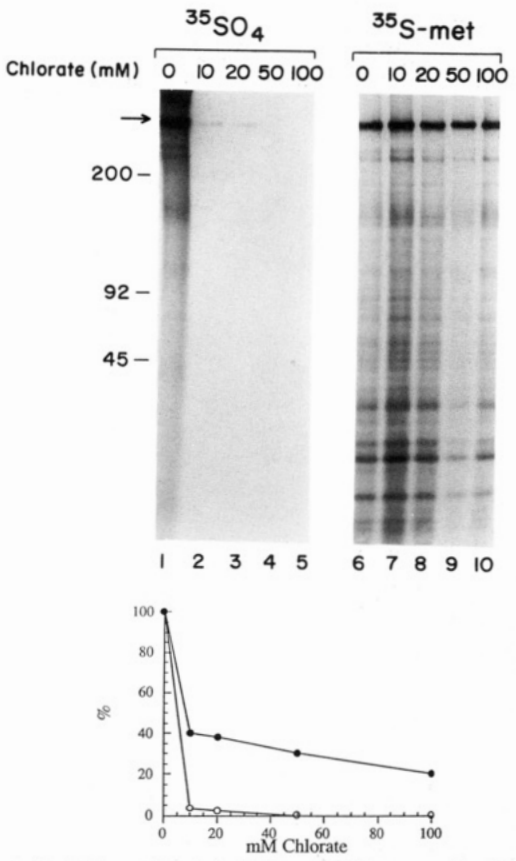

FIGURE 3: Sodium chlorate inhibits sulfate incorporation into factor V and reduces activity. (Panel A, top) T9 CHO cells were labeled with [^{35}S]sulfate (lanes 1–5) or [^{35}S]methionine (lanes 6–10) for 24 h in the presence of increasing concentrations of sodium chlorate. The conditioned medium was harvested and immunoprecipitated with anti-factor V polyclonal antibody and analyzed by SDS–PAGE. The arrow indicates the mobility of factor V. (Panel B, bottom) Conditioned medium from cells in panel A were assayed for factor V clotting activity. Incorporation of [^{35}S]sulfate into factor V in the autoradiogram in panel A was quantitated by laser densitometry. The values represent the percent of control in the absence of sodium chlorate of [^{35}S]sulfate incorporation (○) and factor V activity (●).

reduced to 30–50%. The results show that sulfation of factor V not only affects the rate of activation by thrombin but also is required for optimal cofactor activity.

To further investigate the role of sulfation on thrombin cleavage, the kinetics of thrombin cleavage of factor V synthesized in the presence or absence of 100 mM sodium chlorate was studied. Conditioned medium containing factor V produced from T9 CHO cells propagated in the presence or absence of 100 mM sodium chlorate was treated with thrombin, and aliquots were removed for analysis by SDS–PAGE and Western immunoblotting. Single-chain factor V produced in the absence of sodium chlorate was cleaved to generate the 94-kDa heavy chain fragment after 10 s (Figure 5, lane 3). In contrast, factor V produced in the presence of sodium chlorate did not generate significant amounts of the 94-kDa heavy-chain fragment after 30 min. In addition, there was a delay in the appearance of the cleavage intermediates migrating at 180 and 220 kDa. These results show that sulfation of factor V is required for rapid cleavage by thrombin. The resistance of nonsulfated factor V to thrombin cleavage is likely responsible for the reduced rate of functional activation of the molecule by thrombin.

Combined Factor V and Factor VIII Deficiency Is Not Due to a Defective Sulfation. We have previously postulated that patients with reduced factor V and factor VIII activities and

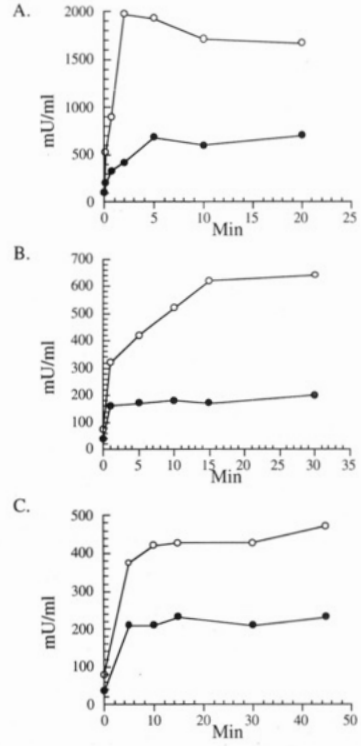

FIGURE 4: Activation of factor V produced in the presence and absence of sodium chlorate. T9 CHO cells were propagated for 24 h in the absence (○) or presence (●) of 100 mM sodium chlorate. Conditioned medium was harvested and assayed for factor V clotting activity prior to (0 time point) and after activation with human α-thrombin (panel A), factor Xa (panel B), or Russell's viper venom (panel C) for the indicated periods of time. Although the results presented are from one set of activation experiments, an independent set of activation curves yielded similar results.

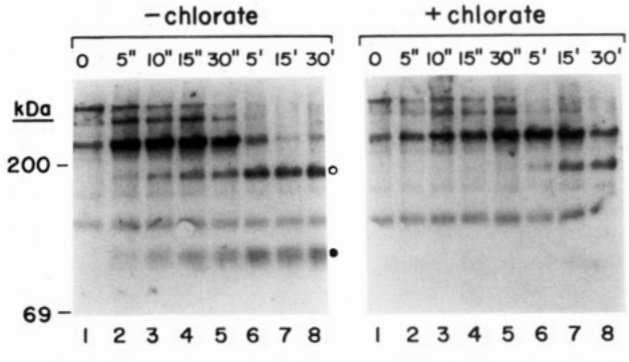

FIGURE 5: Kinetics of thrombin cleavage of factor V produced in the presence and absence of sodium chlorate. T9 CHO cells were incubated 24 h in the presence or absence of 100 mM sodium chlorate and conditioned medium harvested. Conditioned medium was treated with thrombin for the indicated periods of time, and samples were analyzed by SDS–PAGE and Western immunoblot analysis using a factor V polyclonal specific antibody as described under Experimental Procedures. The migrations of the 150- (○) and 94-kDa (●) thrombin cleavage fragments are indicated. The thrombin-cleaved light chain is not detected in this analysis, possibly due to the presence of residual bovine serum albumin in the samples of conditioned medium.

antigens (Seligsohn et al., 1982) may have a deficiency in a posttranslational modification common to both proteins (Pittman et al., 1992). To investigate whether sulfation is defective, we have characterized sulfation of factor V isolated from a patient with homozygous combined deficiency of factor V and factor VIII. We distinguished sulfated from nonsulfated factor V on the basis of their differential susceptibility to thrombin cleavage. Factor V was immunoprecipitated from plasma samples, treated with thrombin for increasing periods

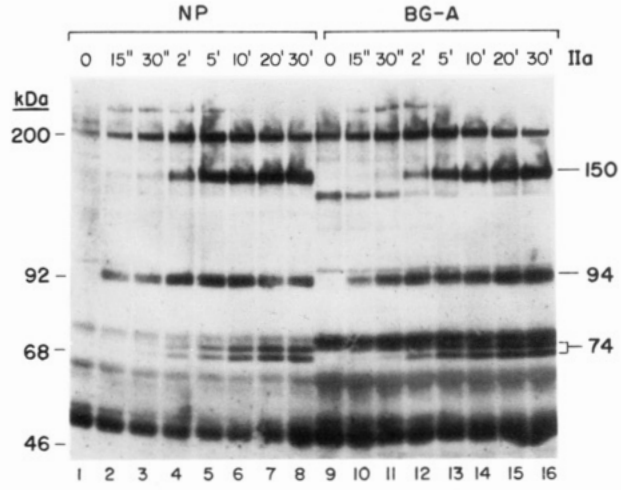

FIGURE 6: Kinetics of thrombin cleavage of factor V isolated from normal plasma and a patient with combined factor V and factor VIII deficiency. Pooled normal human plasma and plasma from a homozygous deficient patient were immunoprecipitated with anti-factor V monoclonal antibody. Immunoprecipitated proteins were treated with α-thrombin for the indicated periods of time, and reactions were analyzed by SDS–PAGE and Western immunoblot analysis using anti-factor V polyclonal antiserum as described under Experimental Procedures. The migrations of the 150-kDa heavy chain, and 74-kDa light chain are indicated. Nonspecific species are more abundant in the patient's sample since 8 times greater amounts of plasma were used for the immunoprecipitation to obtain similar amounts of factor V protein. The lesser reactivity observed in the early time points likely reflects the poor transfer efficiency of single-chain factor V.

of time, and analyzed by SDS–PAGE and Western immunoblotting procedures. To correct for the reduced amount of factor V antigen present in the patient's plasma, 8 times more patient's plasma was immunoprecipitated compared to pooled normal human plasma to yield similar levels of factor V antigen. Analysis of the rate of appearance of the specific thrombin 94- and 74-kDa factor V cleavage fragments, and the rate of appearance and disappearance of the cleavage intermediates, demonstrated that the rate of thrombin cleavage for factor V isolated from normal plasma was indistinguishable from that obtained from an affected patient's plasma (Figure 6). Thus, this analysis did not detect any difference in the kinetics of thrombin cleavage of factor V isolated from a normal or an affected individual.

DISCUSSION

Previous studies demonstrated that factor V expressed in HepG2 cells is posttranslationally modified by addition of sulfate to tyrosine and serine/threonine-linked oligosaccharides (Hortin, 1990). The studies reported here demonstrate that factor V expressed in transfected mammalian cells was also sulfated on tyrosine residues. Our analysis using glycosidases and inhibitors of N-linked oligosaccharide addition did not detect sulfate incorporation into oligosaccharides. The differences may reflect differences in posttranslational processes between CHO cells and HepG2 cells. Although the precise sites of tyrosine sulfation in factor V remain to be elucidated, analysis of the factor V primary amino acid sequence shows that six tyrosine residues (at positions 696, 698, 1494, 1510, and 1565) fit the consensus for tyrosine sulfation (Huttner & Baeuerle, 1988; Hortin et al., 1986b; Huttner, 1988). Tyrosine residues 696 and 698 are located in a region rich in acidic amino acids at the amino-terminal side of the thrombin cleavage site at residue 709, and residue 698 is conserved between bovine and human factor V (Guinto et al., 1992). Tyrosine residues 1494, 1510, and 1515 are located in a region rich in acidic amino acids at the amino-terminal side of the thrombin cleavage site at residue 1545, and residues 1494 and 1510 are conserved between bovine and human factor V (Guinto et al., 1992). The homologous protein, factor VIII, also contains sulfated tyrosine residues on the amino-terminal side of each thrombin cleavage site (Pittman et al., 1992). On the basis of the homologous functions and mechanism of action of these two cofactors (Husten et al., 1987; Mutucumarana et al., 1992), we propose that sulfation of tyrosine residues, and not oligosaccharides, is required for full factor V procoagulant activity.

Further analysis of the kinetics of activation by different factor V activators showed that sulfation of factor V affected procoagulant activity through two different mechanisms. First, sulfation of factor V was required for efficient heavy-chain cleavage and activation mediated by thrombin. However, at present we do not know if efficient thrombin cleavage of the light chain also requires tyrosine sulfation. In contrast, activation by factor Xa was not influenced by the sulfation status of factor V. This result shows that thrombin utilizes interactions with factor V to mediate activation different from those utilized by factor Xa. The unique requirement of thrombin for sulfated factor V is reminiscent of the interaction of the anion-binding exosite on thrombin's surface with heparin cofactor II (Hortin et al., 1986a, 1989) and the carboxy-terminal region of hirudin (Braun et al., 1988; Maraganore et al., 1989). The interaction of hirudin with the anion-binding exosite of thrombin is increased 10-fold by sulfation of a tyrosine residue in the acidic amino acid rich carboxy terminal end of hirudin (Rydel et al., 1990; Niehrs et al., 1990).

Sulfation of factor V also increased the intrinsic activity of factor Va, irrespective of the protease used to activate factor V. Since factor Va displays sulfation of the heavy chain, and not the light chain, it is likely that sulfation of the heavy chain is required in the formation of a stable prothrombinase complex. The heavy chain of factor Va likely mediates binding of the substrate prothrombin (Guinto & Esmon, 1984) and contributes in the light chain's interaction with the enzyme, factor Xa (Guinto & Esmon, 1984; Annamalai et al., 1987). It is tempting to speculate that sulfation of tyrosine residues in the carboxy-terminal end of the heavy chain of factor Va may facilitate binding to the anion-binding exosite in the substrate prothrombin. In support for this hypothesis, it was recently demonstrated that the anion-binding exosite in prothrombin is accessible to peptide binding (Ni et al., 1993).

It was previously shown that tyrosine sulfation of at least one to six tyrosine residues at positions 346, 718, 719, 723, 1664, and 1680 within factor VIII expressed in CHO cells was required for full procoagulant activity (Pittman et al., 1992). There was no detectable sulfation of oligosaccharides within factor VIII expressed in CHO cells. Factor VIII expressed in the presence of sodium chlorate prevented tyrosine sulfation, and the specific activity of the factor VIII was reduced by 5-fold. Recently, tyrosine to phenylalanine site-directed mutagenesis demonstrated that sulfation of tyrosine residues 346 and 1664 within factor VIII increases the rate of thrombin cleavage at residues 372 and 1689, respectively, and also increases the rate of activation of factor VIII by thrombin, whereas there was no effect on the rate of factor VIII activation by factor Xa (Michnick et al., 1994). In addition, tyrosine to phenylalanine mutants at residues 718, 719, and 723 yielded molecules with reduced procoagulant activity, although the rate of activation was similar to that of the wild-type factor VIII (Michnick et al., 1994). These

observations with factor VIII are very analogous to those described here for factor V in that (1) sulfation affected the rate of cleavage and activation by thrombin, and not by factor Xa, and (2) sulfated tyrosine residues at the carboxy-terminal end of the A2 domain increased procoagulant activity of both factor V and factor VIII to similar degrees, suggesting that a common binding interaction occurs.

Combined deficiency of factor V and factor VIII is a rare autosomal chromosome-linked recessive genetic disorder that is accompanied by an approximately 80% reduction in circulating levels of both factor V and factor VIII (Selighsohn, 1982). However, little is known about the molecular mechanism of the disease. On the basis of the unique requirement that factor V and factor VIII display for sulfation of tyrosine residues, it was speculated that altered posttranslational modification may be an underlying cause for the disease (Pittman et al., 1992). We used the ability to differentiate between sulfated and nonsulfated factor V, by their differential sensitivity to thrombin cleavage, to show that factor V isolated from a patient with the combined deficiency is indistinguishable from factor V isolated from normal pooled human plasma. In addition, we previously demonstrated that the 43-kDa A2-domain polypeptide of thrombin-cleaved nonsulfated factor VIII migrated with a greater mobility upon SDS–PAGE compared to the sulfated counterpart (Pittman et al., 1992). Using this differential gel mobility analysis, we also confirmed the similarity of the thrombin-cleaved A2-domain fragments of factor VIII isolated from affected and unaffected individuals (D.M., unpublished observation). These results show that the factor V and factor VIII present in the plasma of homozygous patients with combined factor V and factor VIII deficiency are not defective in tyrosine sulfation. However, at present we cannot rule out that a major portion of factor V and factor VIII is rapidly degraded *in vivo* and therefore not detected in our analysis. There is precedent for rapid clearance of a mutant factor VIII in a patient with moderate hemophilia that resulted from a tyrosine to phenyalanine mutation at residue 1680. This patient had 18% of the level of factor VIII antigen and activity (Higuchi et al., 1990). Since sulfation at tyrosine residue 1680 reduces the affinity of factor VIII binding to von Willebrand factor, factor VIII is not stabilized by von Willebrand factor in the plasma (Weiss et al., 1977; Pittman & Kaufman, 1989; Leyte et al., 1991). Although we cannot rule out this possibility at this time, our results did not detect any nonsulfated factor V or factor VIII in the patient's plasma and suggest that defective tyrosine sulfation is not the molecular basis of combined factor V and factor VIII deficiency.

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