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Identification and Functional Importance of Tyrosine Sulfate Residues within Recombinant Factor VIII

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ABSTRACT: Sulfated tyrosine residues within recombinant human factor VIII were identified by [^{35}S]sulfate biosynthetic labeling of Chinese hamster ovary cells which express human recombinant factor VIII. Alkaline hydrolysis of purified [^{35}S]sulfate-labeled factor VIII showed that greater than 95% of the [^{35}S]sulfate was incorporated into tyrosine. [^{3}H]Tyrosine and [^{35}S]sulfate double labeling was used to quantify the presence of 6 mol of tyrosine sulfate per mole of factor VIII. Amino acid sequence analysis of thrombin and tryptic peptides isolated from [^{35}S]sulfate-labeled factor VIII demonstrated tyrosine sulfate at residue 346 in the factor VIII heavy chain and at residues 1664 and 1680 in the factor VIII light chain. In addition, the carboxyl-terminal half of the A2 domain contained three tyrosine sulfate residues, likely at positions 718, 719, and 723. Interestingly, all sites of tyrosine sulfation border thrombin cleavage sites. The functional importance of tyrosine sulfation was examined by treatment of cells expressing factor VIII with sodium chlorate, a potent inhibitor of tyrosine sulfation. Increasing concentrations of sodium chlorate inhibited sulfate incorporation into factor VIII without affecting its synthesis and/or secretion. However, factor VIII secreted in the presence of sodium chlorate exhibited a 5-fold reduction in procoagulant activity, although the protein was susceptible to thrombin cleavage. These results suggest that tyrosine sulfation is required for full factor VIII activity and may affect the interaction of factor VIII with other components of the coagulation cascade.

Hemophilia A is a bleeding disorder caused by a deficiency or abnormality in factor VIII. Factor VIII functions in the intrinsic pathway of coagulation. After proteolytic activation, it serves as the cofactor for the factor IXa dependent proteolytic activation of factor X. The isolation of the factor VIII gene and its expression in mammalian cells have greatly enhanced the understanding of the structure and synthesis of factor VIII (Vehar et al., 1984; Wood et al., 1984; Toole et al., 1984). The deduced amino acid sequence revealed a domain structure of A1-A2-B-A3-C1-C2 (Figure 1). The A-domains occur twice in the heavy chain and once in the light chain and share homology to the coagulation protein factor V and to the plasma copper binding protein ceruloplasmin (Kane & Davie, 1988; Mann et al., 1990). The C-domains are repeated twice in the light chain and are homologous to the C-domains in factor V and other phospholipid binding proteins (Kane & Davie, 1988; Stubbs et al., 1990). The B-domain does not share homology with other known proteins and contains 19 of the 25 potential asparagine (N)-linked glycosylation sites. Previous studies have demonstrated that the B-domain is not required for procoagulant activity (Toole et al., 1986; Eaton et al., 1986b; Kaufman et al., 1987). Two regions containing a high content of acidic amino acids occur between domains A1 and A2 and domains B and A3.

Since there are no known naturally occurring cell lines which produce factor VIII, analysis of the biosynthesis, processing, and secretion of factor VIII has required the heterologous expression of the human factor VIII cDNA in mammalian cells (Kaufman et al., 1988). Factor VIII is synthesized as a 2351 amino acid precursor from which a 19 amino acid signal peptide is cleaved. Upon transit into the Golgi compartment, factor VIII is modified by addition of O-linked oligosaccharides to serine and threonine and addition of complex structures to N-linked oligosaccharides (Kaufman et al., 1988). Also in the Golgi compartment the polypeptide is cleaved after residues 1313 and 1648 to generate the heavy

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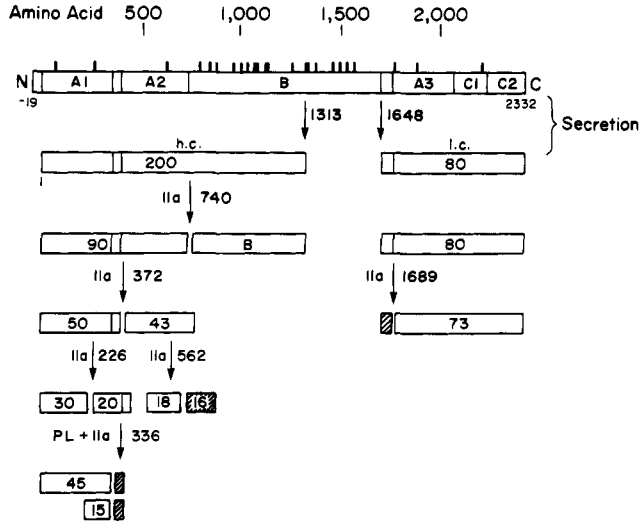

FIGURE 1: Domain structure and proteolytic processing of factor VIII. The structural domains of factor VIII deduced from the primary amino acid sequence are shown with the potential N-linked glycosylation sites depicted by the vertical bars above. The acidic regions are depicted between domains A1 and A2, and between the B-domain and the A3-domain. The thrombin (IIa) cleavage products are shown. The heavy chain is cleaved to generate a 90-kDa polypeptide which is subsequently cleaved to a 50- and a 43-kDa polypeptide. Concomitantly, the 80-kDa light chain is cleaved to a 7- and a 73-kDa polypeptide. The 30-, 20-, 18-, and 16-kDa polypeptides are derived from the 90-kDa heavy-chain fragment with extensive thrombin digestion. In the presence of phospholipid, thrombin also cleaves after residue 336 to yield 45- or 15-kDa fragments and the 5-kDa acidic peptide. The peptide fragments which are labeled with [^{35}S]sulfate in this study are shown by hatching.

chain of 200 kDa in a metal ion complex with the light chain of 80 kDa (Kaufman et al., 1989a) (Figure 1). In the plasma, this complex is stabilized by association with another plasma protein, von Willebrand factor (vWF)[1] (Weiss et al., 1977; Kaufman et al., 1988).

Upon treatment of factor VIII with thrombin, there is an initial rapid activation of procoagulant activity and then subsequent first-order decay of procoagulant activity. The activation coincides with proteolysis of the heavy chain initially after residue 740 to yield a 90-kDa polypeptide and then after residue 372 to yield 50- and 43-kDa polypeptides (Figure 1) (Fulcher et al., 1984; Eaton et al., 1986a). Concomitantly, the 80-kDa light chain is cleaved after arginine residue 1689 to generate a 73-kDa polypeptide (Fulcher et al., 1984; Eaton et al., 1986a). Upon extensive digestion with thrombin, cleavages also occur after residues 226 (Vehar et al., 1984) and 562 (reported herein) within the factor VIII heavy chain to yield polypeptides of 30, 20, 18, and 16 kDa. In this report, we show that in the presence of phospholipid, thrombin additionally cleaves after residue 336 to yield either a 45- or a 15-kDa polypeptide derived from domain A1 and a 36 amino acid peptide (Figure 1).

Tyrosine sulfation is a posttranslational modification that occurs on a number of secretory proteins as they transit the trans Golgi apparatus (Huttner & Baeuerle, 1988). Tyrosine sulfation is mediated by the tyrosylprotein sulfotransferase which utilizes the sulfate donor 5′-phosphoadenosine 3′-phosphosulfate (PAPS) (Niehrs & Huttner, 1990). We report here that factor VIII is also modified by the addition of sulfate to tyrosine residues 346, 1664, and 1680, as well as three tyrosine residues in the carboxyl-terminal end of the A2 domain. Using an inhibitor of PAPS synthesis, we demonstrate that proper sulfation of factor VIII is required for full functional factor VIII activity but not for synthesis or secretion.

[1] Abbreviations: vWF, von Willebrand factor; PAPS, 5′-phosphoadenosine 3′-phosphosulfate; IIa, thrombin; HPLC, high-performance liquid chromatography; MTX, methotrexate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; LA, B-domain-deleted factor VIII; CHO, Chinese hamster ovary.

Experimental Procedures

Reagents. [^{35}S]Sulfuric acid (1050–1600 Ci/mmol) and [^{3}H]tyrosine (50 Ci/mmol) were purchased from Dupont—New England Nuclear Corp., Boston, MA. [^{35}S]Methionine (1000 Ci/mmol) was purchased from Dupont—New England Nuclear or Amersham, Arlington Heights, IL. Alpha medium, sulfate-free alpha medium, and Ham's F12 medium were obtained from Gibco Corp., Grand Island, NY. Methionine-free medium was purchased from Flow Labs, ICN, Costa Mesa, CA, and sulfate- and tyrosine-free alpha medium was obtained from Specialty Media, Lavellete, NJ. Soybean trypsin inhibitor, phenylmethanesulfonyl fluoride, aprotinin, and rabbit brain cephalin were purchased from Sigma Chemical, St. Louis, MO. A monoclonal antibody, F8, which reacts with the A2 domain within the factor VIII heavy chain was kindly provided by W. B. Foster (Genetics Institute, Cambridge, MA). Sodium chlorate was obtained from Fluka Chemie AG, Ronkonkoma, NY. The synthesis of tyrosine sulfate was according to G. Hortin (Hortin et al., 1986a). *N*-Glycanase was purchased from Genzyme, Boston, MA. Human thrombin was a gift from K. Mann (University of Vermont, Burlington, VT) and also purchased from Sigma Chemical. Factor VIII deficient plasma was obtained from George King Biomedical, Overland Park, KA. Trypsin (TPCK) was acquired from Worthington, Freehold, NJ. Immobilon-P transfer membranes were from Millipore, Bedford, MA. All other reagents were of highest quality and commercially available.

Cell Lines. Chinese hamster ovary (CHO) cell lines producing wild-type factor VIII designated H9 (Dorner et al., 1989) and 10A1 (Kaufman et al., 1988) have been described previously. The B-domain deletion mutant, designated LA, deletes residues 760–1639, and is functionally similar to wild-type factor VIII (Toole et al., 1986; Kaufman et al., 1987). One LA-producing cell line, designated clone LA3-5, was previously described (Dorner et al., 1987). A second cell line, designated M18, produces high levels of factor VIII LA and also coexpresses vWF. M18 was derived by coamplification of the LA expression vector with a DHFR gene by selection for resistance to 0.1 μM MTX and then selection for vWF expression by coamplification with an adenosine deaminase gene by selection for resistance to 1.0 μM 2′-deoxycoformycin as described (Kaufman et al., 1989b). M18 produces factor VIII at 20 units mL^{-1} (10^6 cells)$^{-1}$ day^{-1}.

Factor VIII Assay. Factor VIII activity was measured by a chromogenic assay (Kabi Coatest) (Toole et al., 1984), and clotting activity was measured using the one-stage clotting assay with factor VIII deficient plasma (Proctor & Rapaport, 1961). One unit of factor VIII activity is that amount measured in 1 mL of normal human pooled plasma.

Analysis of Factor VIII Synthesis. All CHO cells were grown in complete alpha medium supplemented with 10% dialyzed fetal bovine serum, 100 units/mL penicillin–streptomycin, 2 mM glutamine, and 0.1% aprotinin at 37 °C in a 5% CO_2 atmosphere. For labeling experiments, the same supplements were added to all media. 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, the conditioned medium was collected and cen-

trifuged to remove cellular debris. In parallel, cells were labeled with [^{35}S]methionine (300 μCi/mL) as described (Dorner et al., 1989). 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. The conditioned medium was immunoprecipitated with anti-factor VIII monoclonal antibody F8 coupled to Sepharose CL-4B (Pharmacia Inc., Piscataway, NJ) as described (Dorner & Kauman, 1990). Pellets were resuspended in 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 2.5 mM $CaCl_2$, and 5% (v/v) glycerol. Samples were digested with thrombin, denatured, and loaded onto an SDS–8% polyacrylamide gel (Dreyfus et al., 1984) for analysis by autoradiography after treatment with En^{3}Hance (Dupont—New England Nuclear) as described (Pittman & Kaufman, 1988). Aliquots of immunoprecipitated protein were denatured and digested with *N*-glycanase under conditions described (Tarentino et al., 1985).

Sodium Chlorate Treatment of Cells. Factor VIII-producing 10A1 cells were pretreated with alpha medium or sulfate-free alpha medium with increasing concentrations of sodium chlorate for 1.5 h at 37 °C. The medium was removed, and fresh sulfate-free alpha medium containing 167 μCi/mL [^{35}S]sulfate and the indicated concentration of sodium chlorate was added. Cells were incubated for 24 h at 37 °C. In parallel, cells were labeled with 200 μCi/mL [^{35}S]methionine in methionine-free alpha medium with increasing concentrations of sodium chlorate with the above medium components for 1 h. Complete alpha medium was added with sodium chlorate, and the cells were incubated overnight. Conditioned medium was harvested, and factor VIII activity was measured by a chromogenic assay (Toole et al., 1984). Incorporation of [^{35}S]sulfate and [^{35}S]methionine into total secreted protein was monitored by precipitation with trichloroacetic acid. Soybean trypsin inhibitor (1 mg/mL) and phenylmethanesulfonyl fluoride (0.1 mM) were added to the conditioned medium, and factor VIII was immunoprecipitated as above. Half of the immunoprecipitate was digested with thrombin (10 units/mL) for 1 h at 37 °C, and samples were analyzed by SDS–PAGE.

Purification of [^{35}S]Sulfate-Labeled Factor VIII LA. CHO cells expressing the B-domain-deleted factor VIII LA (either LA3-5 or M18) were labeled with sulfate-free medium containing 30 μCi/mL [^{35}S]sulfuric acid. After 24 h, Ham's F12 (10 mL) medium was applied, and 24 h later, the conditioned medium was harvested by centrifugation and addition of soybean trypsin inhibitor (1 mg/mL) and phenylmethanesulfonyl fluoride (0.1 mM). Where indicated, purified recombinant wild-type factor VIII (2000 units) was added as carrier. Factor VIII was purified by monoclonal antibody affinity chromatography as described (Nesheim et al., 1990). The purified protein was digested with 2–6 units/mL human thrombin for 20–60 min at 37 °C. Where indicated, thrombin digestion was performed in the presence of 10% (v/v) rabbit brain cephalin. Following digestion, the peptides were applied to a 0.46 × 25 cm Vydac C4 column in 0.1% trifluoroacetic acid, and the column was developed with a gradient to 95% acetonitrile/0.1% trifluoroacetic acid. Fractions were collected and aliquots subjected to scintillation counting.

M18 cells were also propagated in the presence of sodium chlorate, and the medium was harvested as above for purification of factor VIII. The specific activity of factor VIII LA prepared from M18 cells grown in the presence and absence of sodium chlorate was determined using the one-stage clotting assay (Proctor & Rapaport, 1961) or the Kabi Coatest assay (Toole et al., 1984), and protein concentration was determined by the micro BCA method (Pierce, Rockford, IL).

Tryptic Peptide Analysis. Thrombin-generated peptides isolated by reverse-phase HPLC were vacuum-dried and resuspended in 0.1 M ammonium bicarbonate (pH 8.3). The peptides were digested with trypsin [1:50 (w/w)] overnight at 37 °C and fractionated by reverse-phase HPLC as described above. Fractions were collected, and the sequence was determined on an Applied Biosystems 470A protein sequencer (Hewick et al., 1982).

Stoichiometry of Tyrosine Sulfation. M18 cells were rinsed with sulfate- and tyrosine-free alpha medium and fed fresh medium containing 114 μCi/mL [^{3}H]tyrosine and 285 μCi/mL [^{35}S]sulfuric acid. After 24 h, the conditioned medium was harvested, and factor VIII LA was immunoprecipitated with anti-factor VIII monoclonal antibody F8 as above. Half of the immunoprecipitate was digested with 7 units/mL thrombin for 3 h at 37 °C. The proteins were subjected to SDS–PAGE and transferred to Immobilon-P for visualization by autoradiography. The labeled bands were excised and subjected to scintillation counting using the fluor Aquasol. The ratio of [^{35}S]sulfate to [^{3}H]tyrosine in tyrosine sulfate was determined by acetone precipitation of the labeled conditioned medium followed by barium hydroxide hydrolysis for 18 h in a Tuftainer (Pierce) under vacuum at 110 °C. Samples were neutralized, and two-dimensional electrophoresis was performed as described below. The ratio of [^{35}S]sulfate/[^{3}H]-tyrosine was determined by scintillation counting using the fluor Aquasol.

Thin-Layer Electrophoresis. Purified [^{35}S]sulfate-labeled LA was subjected to alkaline hydrolysis with barium hydroxide as described (Huttner, 1984). The lyophilized pellet was resuspended in 5% acetic acid/0.5% pyridine, and 20 volumes of acetone were added. Unlabeled tyrosine sulfate (3 μg) was added to the sample, and the sample was spotted onto 20 × 20 cm plastic-backed 100-μm cellulose thin-layer sheets (Kodak, Rochester, NY). After two-dimensional electrophoresis, the thin-layer sheet was dried, the tyrosine sulfate was detected with 0.2% ninhydrin in ethanol, and autoradiography was performed.

RESULTS

Identification of Tyrosine Sulfate Residues within Factor VIII. Tyrosine sulfate residues within factor VIII were identified by measuring the incorporation of [^{35}S]methionine and [^{35}S]sulfate into wild-type or a B-domain deletion mutant of factor VIII, designated LA (Figure 2). CHO cells which express either wild-type factor VIII or the B-domain deletion molecule LA were radiolabeled as described under Experimental Procedures. Factor VIII was immunoprecipitated from the conditioned medium and subjected to SDS–PAGE before (–) and after (+) thrombin digestion (Figure 2A). Immunoprecipitation of [^{35}S]methionine-labeled wild-type factor VIII detects the 200-kDa heavy-chain and 80-kDa light-chain polypeptides (Figure 2A, lane 7). Following thrombin digestion, [^{35}S]methionine label was detected in polypeptides derived from the heavy chain migrating at 45 kDa (*) and 43 kDa (□) and in the 73-kDa doublet generated from the light chain (○) (Figure 2A, lane 8). Under these conditions of thrombin digestion, the majority of the 50-kDa heavy-chain fragment was cleaved to a 45-kDa species by cleavage after residue 336 (Eaton et al., 1986a). This thrombin cleavage likely resulted from the presence of residual phospholipid derived from the fetal bovine serum in the conditioned medium (see below). A discrete band was not detected for the B-do-

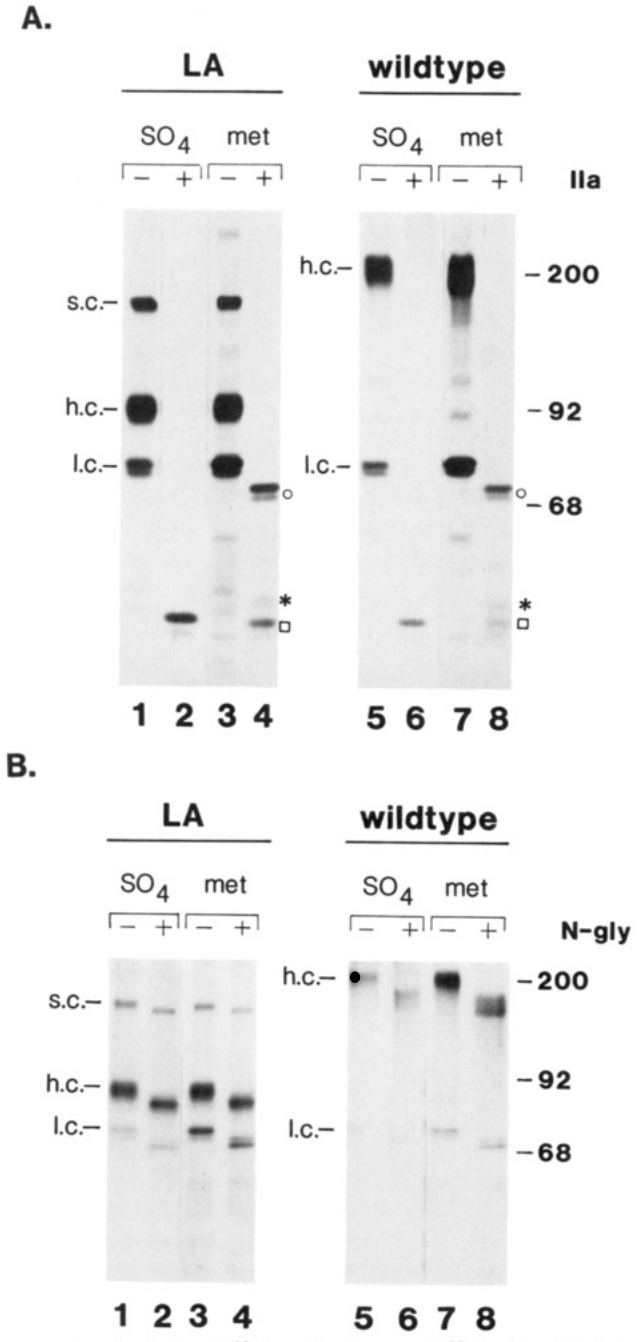

FIGURE 2: Analysis of [^{35}S]methionine- and [^{35}S]sulfate-labeled wild-type and B-domain-deleted factor VIII. CHO cells expressing wild-type factor VIII (clone H9) and B-domain deletion mutation LA (clone LA3-5) were labeled with [^{35}S]methionine (lanes 3, 4, 7, and 8) or [^{35}S]sulfate (lanes 1, 2, 5, and 6) for 24 h, and the factor VIII in the conditioned medium was immunoprecipitated. Equal aliquots of the immunoprecipitates were digested with thrombin (IIa, +; panel A) or with *N*-glycanase (N-gly, +; panel B) and analyzed by SDS–PAGE. The single chain (s.c.), the heavy chain (h.c.), and the light chain (l.c.) are identified. Specific thrombin cleavage products are indicated: the 73-kDa light chain (open circle); the 45-kDa heavy-chain polypeptide (asterisk), and the 43-kDa heavy-chain polypeptide (open box). Molecular mass markers are indicated on the right (kDa).

main fragment due to its diffuse mobility resulting from heterogeneity in glycosylation (Kaufman et al., 1988). By comparison, [^{35}S]sulfate was also incorporated into the 200-kDa and the 80-kDa polypeptides of wild-type factor VIII (Figure 2A, lane 5). After thrombin digestion, [^{35}S]sulfate label was only detected in the 43-kDa peptide derived from the heavy chain and not in the 45-kDa peptide from the heavy chain or the 73-kDa peptide from the light chain (Figure 2A, lane 6). A similar analysis performed with the B-domain-deleted factor VIII LA showed incorporation of [^{35}S]-methionine and [^{35}S]sulfate into the heavy-chain and light-chain polypeptides as well as a single-chain polypeptide of the intact molecule (Figure 2A, lanes 1 and 3). After thrombin treatment, [^{35}S]methionine label was observed in the 73-, 45-, and 43-kDa polypeptides (Figure 2A, lane 4), whereas [^{35}S]sulfate label was detected only in the 43-kDa polypeptide (Figure 2A, lane 2). The results demonstrated [^{35}S]sulfate incorporation into the intact heavy and intact light chains of factor VIII. The absence of detectable [^{35}S]sulfate incorporation into either the 73- or the 45-kDa polypeptides can be explained is sulfate is incorporated into the acidic amino acid rich regions between amino acids 336 and 372 of the heavy chain and amino acids 1648–1689 of the light chain (Figure 1). These fragments released by thrombin digestion are not detected by this gel system due to their small size.

Sulfate can be added to carbohydrate or tyrosine residues within proteins (Huttner & Baeuerle, 1988; Huttner, 1984; Hortin et al., 1986b). To investigate whether [^{35}S]sulfate was incorporated into N-linked carbohydrate residues, *N*-glycanase was used to remove the N-linked carbohydrate from [^{35}S]-sulfate-labeled factor VIII (Tarentino et al., 1985). The amounts of radiolabel detected in polypeptides before or after *N*-glycanase digestion of [^{35}S]sulfate-labeled (Figure 2B, lanes 5 and 6) and [^{35}S]methionine-labeled (Figure 2B, lanes 7 and 8) wild-type factor VIII or [^{35}S]methionine- and [^{35}S]-sulfate-labeled B-domain-deleted factor VIII LA (Figure 2B, lanes 1–4) were the same regardless of the label. In all cases, treatment with *N*-glycanase (+) increased the mobility of the polypeptides, demonstrating the removal of N-linked oligosaccharides. Since *N*-glycanase-treated and control preparations exhibited similar levels of [^{35}S]sulfate incorporation, removal of N-linked carbohydrate did not significantly remove the [^{35}S]sulfate present within factor VIII. This suggested that the sulfate incorporation observed was primarily into tyrosine residues rather than carbohydrate.

Tyrosine sulfate was directly detected in [^{35}S]sulfate-labeled factor VIII LA by barium hydroxide hydrolysis and two-dimensional thin-layer electrophoresis (Huttner, 1984). Barium hydroxide treatment and neutralization with sulfuric acid precipitate the alkali-labile sulfated carbohydrate residues and proteoglycans, whereas tyrosine *O*-sulfate remains in solution (Huttner, 1984). Greater than 95% of the [^{35}S]sulfate incorporated into factor VIII was recovered in the supernatant after alkaline hydrolysis. Upon two-dimensional thin-layer electrophoresis, the majority of radioactivity comigrated with the cold tyrosine sulfate marker (Figure 3), demonstrating the presence of tyrosine sulfate in factor VIII.

Quantitation of Sulfated Tyrosine Residues in Factor VIII. The precise number of sulfated tyrosine residues within factor VIII was determined by double labeling cells expressing high levels of B-domain-deleted factor VIII LA with [^{3}H]tyrosine and [^{35}S]sulfate. Radiolabeled factor VIII was immunoprecipitated, and polypeptides were separated by SDS–PAGE before and after thrombin digestion. After transfer to Immobilon-P, radioactive bands were excised and subjected to scintillation counting. The specific radioactivity of tyrosine sulfate (^{35}S/^{3}H) incorporated into secreted protein was quantitated by purifying tyrosine sulfate from the conditioned medium using barium hydroxide hydrolysis and two-dimensional thin-layer electrophoresis. Table I shows the [^{35}S]-sulfate:[^{3}H]tyrosine ratio of incorporation into each of the different polypeptides derived from factor VIII LA. Although this analysis was performed with a clone which expresses factor

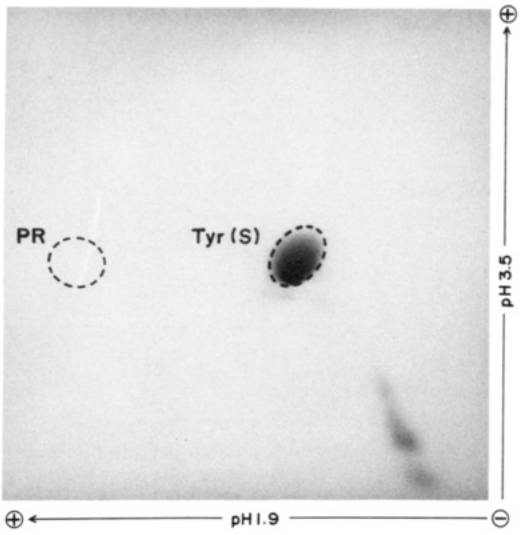

FIGURE 3: Thin-layer electrophoresis to detect tyrosine sulfate. Purified [^{35}S]sulfate-labeled B-domain-deleted factor VIII LA was subjected to alkaline hydrolysis with barium hydroxide and analyzed by two-dimensional thin-layer electrophoresis at pH 1.9 and 3.5. The autoradiograph of the cellulose thin-layer sheet is shown with identification of the cold tyrosine sulfate marker [Tyr(S)] and phenol red (PR).

VIII LA at a high level (M18), quantitatively similar results were obtained with a clone expressing less factor VIII LA (LA3-5). Quantitation of the moles of tyrosine sulfate per mole of polypeptide demonstrated intact factor VIII contains six sulfated tyrosine residues. Four tyrosine sulfate residues were present within the factor VIII 90-kDa heavy chain, and two were present within the factor VIII 80-kDa light chain. Analysis of the thrombin cleavage products demonstrated the presence of 1 mol of tyrosine sulfate/mol of peptide within the 50-kDa polypeptide. The thrombin-generated 43-kDa polypeptide migrated as a doublet upon SDS–PAGE (see Figure 2, lane 2). Quantitation of the radioactivity in the upper and lower species of the doublet showed that both species contain three tyrosine sulfate residues. Thus, the difference in the mobility of the two species of the 43-kDa fragment did not result from quantitative differences in tyrosine sulfation.

Identification of the Sites of Tyrosine Sulfation. Tyrosine sulfate cannot be detected by amino acid sequence analysis since the sulfate group is removed during Edman degradation (Hortin et al., 1986a). The identification of the specific sulfated tyrosine residues within factor VIII was accomplished by the isolation of [^{35}S]sulfate-labeled peptides and amino acid sequence analysis. Since greater than 95% of [^{35}S]sulfate was incorporated into tyrosine, it was possible to infer sites of sulfation to those tyrosine residues within labeled peptides that contain one tyrosine. This characterization was performed with the B-domain-deleted VIII LA because it is more efficiently expressed (Toole et al., 1986; Dorner et al., 1987) and more easily purified than wild-type factor VIII. Factor VIII LA exhibits in vitro and in vivo cofactor activity (Toole et al., 1986; Kaufman et al., 1987), thrombin cleavage products (Toole et al., 1986), and [^{35}S]sulfate incorporation into tyrosine (Figure 2) similar to wild-type factor VIII.

Purified [^{35}S]sulfate-labeled factor VIII LA was treated with thrombin, and the resulting peptides were separated by reverse-phase HPLC (Figure 4). The peptides in each fraction were identified by N-terminal sequence analysis (Figure 4) and SDS–PAGE (Figure 7). Peaks of radioactivity were found in fractions 15–17, 33–34, and 38–41. N-Terminal sequence

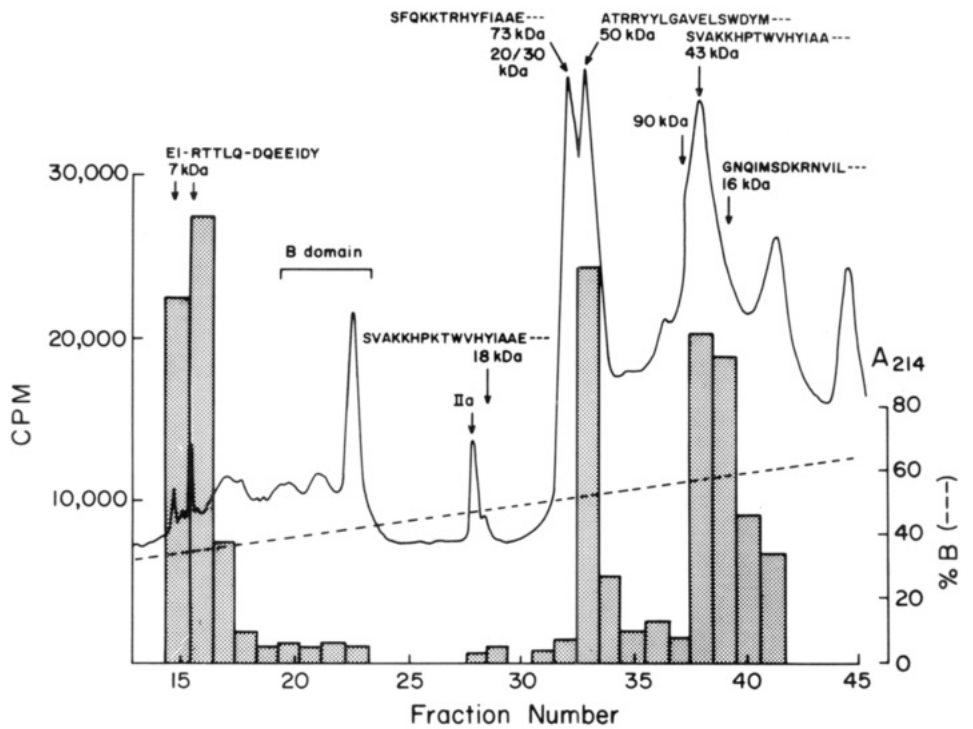

FIGURE 4: Analysis of thrombin-digested factor VIII by reverse-phase HPLC. [^{35}S]Sulfate-labeled conditioned medium was prepared from CHO cells expressing B-domain deletion mutant LA. Recombinant wild-type factor VIII was added to conditioned medium and factor VIII purified as described under Experimental Procedures. The protein was digested with thrombin, and the resulting peptides were resolved by reverse-phase HPLC. Fractions (1 mL) were subjected to scintillation counting and N-terminal sequence analysis. The protein elution profile is indicated by A_{214}. Bars represent [^{35}S]sulfate cpm/fraction above background. Amino acid sequences were identified by N-terminal sequence analysis and correspond to the thrombin cleavage products indicated by molecular masses in Figure 1. The 73-kDa light-chain peak also contains residual amounts of peptides identified as the 20- and 30-kDa thrombin cleavage products from the heavy chain. Fractions 15 and 16 both yielded the sequence of the 7-kDa light-chain thrombin cleavage fragment. Fraction 16 sequence initiated at residue 1648 (sequence shown above fraction 16) whereas fraction 15 was missing nine amino acids (sequence not shown), initiating at residue 1657. The acetonitrile gradient elution profile is indicated on the right.

Table I: Stoichiometry of Tyrosine Sulfation of Factor VIII[a]

polypeptide	[^{35}S]:[^{3}H] cpm ratio	no. of mol of Tyr/mol	no. of mol of Tyr-SO_4/mol
single chain	0.068	68	6.3
90 kDa	0.082	38	4.3
80 kDa	0.053	30	2.2
50 kDa	0.055	14	1.1
43 kDa			
a	0.088	24	2.9
b	0.086	24	2.8

[a] The number of moles of tyrosine sulfate per mole of peptide for each of the polypeptide species of factor VIII LA was determined as described under Experimental Procedures. The [^{35}S] cpm:[^{3}H] cpm ratio for isolated tyrosine sulfate (0.73) was used to determine the number of moles of tyrosine sulfate per mole of polypeptide = $[([^{35}S]/[^{3}H]_{peptide})/([^{35}S]/[^{3}H]_{Tyr\ standard})]$ (no. of Tyr/peptide).

analysis of peptides in these fractions identified the presence of the 7-kDa acidic peptide derived from the 80-kDa light chain, the 50-kDa peptide from the heavy chain, and the 90- and 43-kDa peptides from the heavy chain, respectively (Figure 4).

The sites of tyrosine sulfation in the factor VIII light chain were identified by sequencing tryptic fragments of the purified [^{35}S]sulfate-labeled 7-kDa thrombin cleavage product of the factor VIII light chain. Trypsin digests after lysine-1673, separating the two tyrosine residues at positions 1664 and 1680. Upon reverse-phase HPLC, two peaks of absorbance were observed, and both contained radioactivity (Figure 5). NH_2-terminal sequence analysis of fractions 30 and 35 identified the COOH-terminal half and the NH_2-terminal half of the 7-kDa peptide, respectively (Table II). These results demonstrated that both tyrosines at residues 1664 and 1680 are sulfated.

The tyrosine sulfate residue within the acidic region between domains A1 and A2 was identified by isolation of [^{35}S]-sulfate-labeled factor VIII LA and digesting it with thrombin in the presence of phospholipid. In the course of our studies, we observed that in the presence of phospholipid, thrombin cleaves factor VIII after residue 336 to generate a 45-kDa A1-domain fragment and a 36 amino acid peptide. Addition of phospholipid to the thrombin digestion significantly increased the amount of conversion of the 50-kDa polypeptide

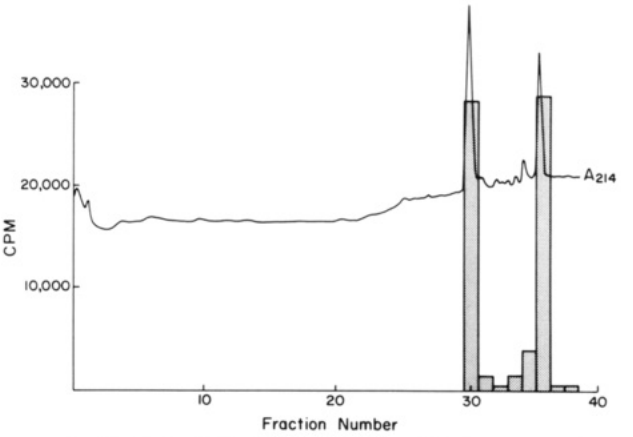

FIGURE 5: Analysis of the 7-kDa acidic peptide by reverse-phase HPLC. The [^{35}S]sulfate-labeled 7-kDa peptide generated by thrombin digestion was isolated by reverse-phase HPLC as described in Figure 4 (fraction 16), digested with trypsin, and separated by reverse-phase HPLC. The bars correspond to radiolabeled peptides. Fractions containing radioactivity were subjected to N-terminal sequence analysis.

to the 45-kDa species [compare (+) and (−) PL in Figure 6 inset]. [^{35}S]Sulfate-labeled factor VIII LA was cleaved with thrombin in the presence of phospholipid and subjected to reverse-phase HPLC. A fraction of peak radioactivity was identified by NH_2-terminal sequence analysis as the 36 amino acid peptide from residues 336–372 (Figure 6, Table II). Identification of tyrosine sulfate at residue 346 was unambiguous because this peptide contains only one tyrosine residue.

Quantitation of tyrosine sulfate within the 43-kDa peptide identified the presence of 3 mol of tyrosine sulfate/mol of peptide (Table I). On the basis of consensus features for tyrosine sulfation (Huttner & Baeuerle, 1988), 4 of the total 24 tyrosine residues within the 43-kDa polypeptide may serve as potential sites for tyrosine sulfation. These tyrosines occur at residues 395, 718, 719, and 723 (Table III). The 43-kDa thrombin cleavage fragment was digested with trypsin and subjected to reverse-phase HPLC. However, a distinct peak of radioactivity was not detected. A tryptic peptide containing tyrosine-395 was identified by sequence analysis; however, it was not radioactive. Thus, tyrosine sulfation at amino acid

Table II: Sequential Edman Degradation of Tyrosine Sulfate Containing Peptides[a]

	heavy-chain Fxn 38		light-chain Fxn 35		light-chain Fxn 30	
cycle	residue (no.)	yield (pmol)	residue (no.)	yield (pmol)	residue (no.)	yield (pmol)
1	Met (337)	186	Thr (1653)	108	Lys (1674)	140
2	Lys	167	Thr	106	Glu	136
3	Asn	116	Leu	161	Asp	126
4	Asn	117	Gln	131	Phe	128
5	Glu	104	Ser	73	Asp	104
6	Glu	101	Asp	132	Ile	93
7	Ala	113	Gln	104	Tyr	87
8	Glu	89	Glu	97	Asp	92
9	Asp	66	Glu	94	Glu	76
10	Tyr	59	Ile	93	Asp	74
11	Asp	61	Asp	91	Glu	64
12	Asp	61	Tyr	79	Asn	46
13	Asp	62	Asp	78	Gln	42
14	Leu	70	Asp	76	Ser	24
15	Thr	24	Thr	21	Pro	23
16	Asp	41	Ile	38	Arg	12
17			Ser	22		
18			Val	18		
19			Glu	13		
20			Met	7		
21			Lys	1		

[a] NH_2-terminal amino acid sequence analysis was performed on isolated thrombin and tryptic cleavage peptides isolated in Figures 5 (light chain) and 6 (heavy chain). The amino acid residue numbers are presented with respect to the mature factor VIII amino acid sequence (Toole et al., 1984).

Table III: Consensus Tyrosine Sulfation Sites within Factor VIII[a]

residue	sequence	acidic residue −2	3 acidic residues −5/+5	turn-inducing residues −7/+7	no Cys, no N-gly −7/+7	Tyr-SO_4
346	RMKNNEEAED<u>Y</u>DDDLTDSEMD	+	+	+	+	+
395	YIAAEEEDWD<u>Y</u>APLVLAPDDR	+	+	+	+	−
718/719	VSSCDKNTGD<u>YY</u>EDSYEDISA	+	+	+	+	?
723	KNTGDYYEDS<u>Y</u>EDISAYLLSK	+	+	+	+	?
1664	TLQSDQEEID<u>Y</u>DDTISVEMKK	+	+	+	+	+
1680	VEMKKEDFDI<u>Y</u>DEDENQSPRS	+	+	+	+	+

[a] The consensus sequences surrounding the potential sites of tyrosine sulfation within factor VIII as proposed by Huttner and Baeuerle (1988). The sulfated tyrosine residue is underlined. The features include the presence of an acidic residue (Glu or Asp) within −2, at least three acidic amino acids between −5 and +5, the presence of turn-inducing amino acids (at least one Pro or Gly or at least three of Ser, Asn, or Asp), and the absence of Cys residues or N-linked carbohydrates between −7 and +7.

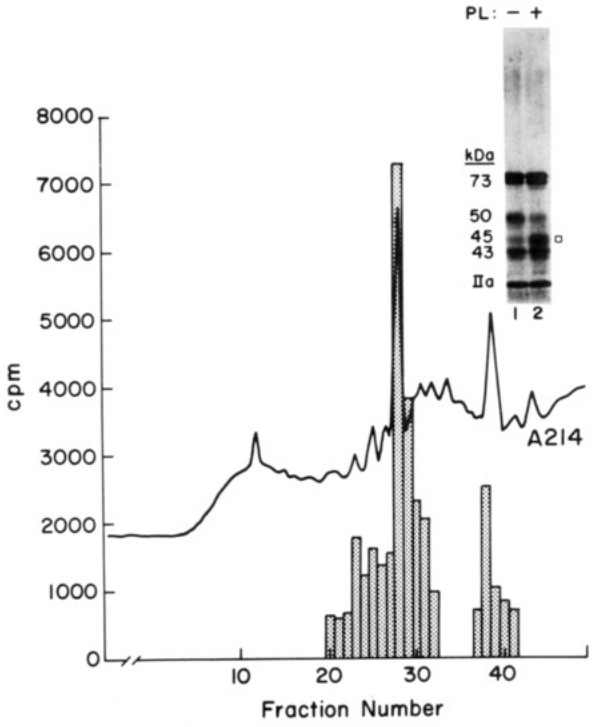

FIGURE 6: Reverse-phase HPLC analysis of the 36 amino acid acidic peptide from the heavy chain. [^{35}S]Sulfate-labeled factor VIII LA was purified from conditioned medium of M18 cells in the absence of added carrier wild-type factor VIII, digested with thrombin in the presence of (+) and absence (−) of phospholipid, and analyzed by SDS–PAGE and silver staining as described under Experimental Procedures [inset, (□) = 45-kDa A1-domain thrombin cleavage fragment]. The reaction was chromatographed on a reverse-phase HPLC column, and fractions (200 μL) were collected. The polypeptides present in the peak fractions were identified by NH_2-terminal amino acid sequence analysis (Table II). The elution profile (A_{214}) in the region where the 36 amino acid peptide elutes is shown. Fraction 28 represents the 7-kDa acidic thrombin cleavage fragment from the light chain. Fraction 38 represents the 36 amino acid peptide from residue 336 to residue 372. The bars represent [^{35}S]sulfate cpm in each fraction.

residue 395 did not occur to a detectable extent. Other isolated tryptic peptides derived from the 43-kDa thrombin cleavage fragment were sequenced; however, we did not detect the presence of the peptide expected to be generated by trypsin cleavage after lysine residue 713. It is possible that under the conditions employed, this peptide is blocked at the amino terminus. To determine the region of the 43-kDa polypeptide which contains [^{35}S]sulfate, fractions from a reverse-phase HPLC fractionation were analyzed by SDS–PAGE to evaluate the smaller peptides derived from the heavy chain upon extensive thrombin digestion. Comparison of a Coomassie Blue stained gel with the autoradiogram confirmed [^{35}S]sulfate incorporation into the 90-kDa [Figure 7B, lanes 5–9 (□)], the 50-kDa (Figure 7B, lane 4), and the 43-kDa (Figure 7B, lanes 6–9) polypeptides, but not the 73-kDa (Figure 7B, lanes 4–9) polypeptide. [^{35}S]Sulfate label was also detected in the 20-kDa fragment from the COOH-terminus of the 50-kDa polypeptide (Figure 7B, lane 3). The 18-kDa NH_2-terminal fragment derived from the 43-kDa polypeptide was visible only by Coomassie Blue staining (Figure 5A, lane 1) whereas [^{35}S]-sulfate radioactivity was detected in the 16-kDa COOH-terminal fragment from the 43-kDa polypeptide (Figure 7B, lane 8). Since [^{35}S]sulfate was only detected in the COOH-terminal 16-kDa fragment of domain A2 (Figure 7B, lane 8), and the only tyrosine residues within this fragment which fit the consensus features are at residues 718, 719, and 723, it is likely that these three tyrosine residues represent the three sites of sulfation in the 43-kDa polypeptide.

Inhibition of Tyrosine Sulfation Reduces Procoagulant Activity without Altering Synthesis or Secretion of Factor VIII. The requirement for tyrosine sulfation for factor VIII secretion or activity was studied by treating wild-type factor VIII-expressing cells with increasing concentrations of sodium chlorate. Sodium chlorate is a potent inhibitor of sulfation in intact cells (Baeuerle & Huttner, 1986; Friederich et al., 1988). Cells were treated with increasing concentrations of sodium chlorate, and sulfate incorporation into total protein was monitored. As the concentration of sodium chlorate increased to 100 mM, factor VIII activity decreased proportionally to the inhibition of [^{35}S]sulfate incorporation (Figure 8). At 100 mM sodium chlorate, sulfate incorporation was inhibited to 12% and factor VIII activity was inhibited to 20% of the levels obtained with untreated cells. Control experiments demonstrated that addition of sodium chlorate to the assay did not interfere with determination of factor VIII activity (data not shown). Although the data in Figure 8 were obtained using a chromogenic activity assay for factor Xa generation, similar degrees of inhibition of factor VIII activity were detected by measuring procoagulant activity using factor VIII deficient plasma (data not shown).

To determine whether the synthesis and/or secretion of factor VIII was affected by addition of sodium chlorate, cells were labeled in the presence of sodium chlorate with either [^{35}S]sulfate or [^{35}S]methionine, and factor VIII in the conditioned medium was analyzed by immunoprecipitation and SDS–PAGE. Concentrations of sodium chlorate greater than 20 mM inhibited the incorporation of [^{35}S]sulfate into both the heavy and light chains of factor VIII (Figure 9, lanes 1–5) with little effect on [^{35}S]methionine incorporation into secreted factor VIII (Figure 9, lanes 6–10). In addition, quantitation of factor VIII antigen by Western blot analysis of the same samples demonstrated that sodium chlorate treatment did not reduce the amount of factor VIII protein in the conditioned

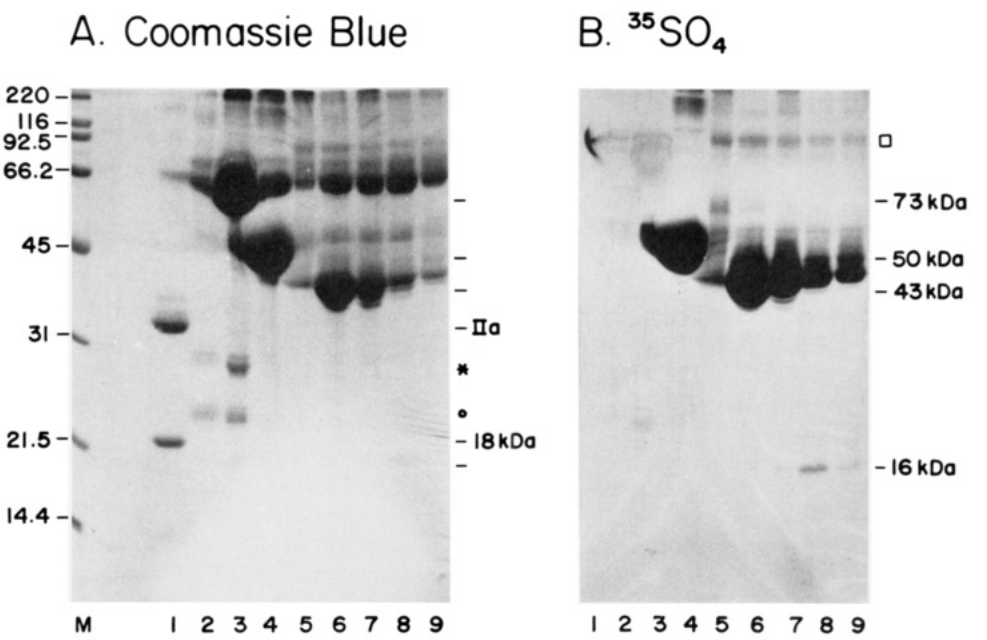

FIGURE 7: SDS–PAGE analysis of thrombin-digested factor VIII isolated by reverse-phase HPLC. [^{35}S]Sulfate-containing fractions isolated by reverse-phase chromatography as described in Figure 4 were analyzed by reducing SDS–PAGE. The gel was stained with Coomassie Blue (A) and exposed for autoradiography (B). Markers, lane M; thrombin cleavage products are denoted as follows: 73-kDa light chain (lanes 3–9), 90-kDa polypeptide (lanes 5–9, □), 50-kDa polypeptide (lane 4), and the intact 43-kDa polypeptide (lanes 5–9). The 18-kDa (lane 1) and 16-kDa (lane 8) thrombin cleavage products from the 43-kDa A2-domain are identified. The 20-kDa (○) and 30-kDa (*) polypeptides derived from the 50-kDa A1-domain polypeptide are shown in lanes 2 and 3. The migration of thrombin (IIa) in lane 1 is also shown. On the left of panel A are molecular mass markers (kDa). The marks on the right of panel A correspond to those polypeptides identified on the right of panel B.

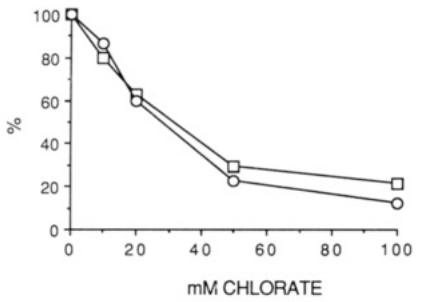

FIGURE 8: Effect of sodium chlorate concentration on sulfate incorporation and factor VIII activity. 10A1 CHO cells expressing wild-type factor VIII were treated with increasing concentrations of sodium chlorate in the presence of [^{35}S]sulfate as described under Experimental Procedures. After 24 h, the conditioned medium was harvested for the factor VIII activity assay (Kabi Cotest). Incorporation of [^{35}S]sulfate into total protein in the conditioned medium was determined by trichloroacetic acid precipitation. Values represent the percent of control of sulfate incorporation (○) and of factor VIII activity (□) in the absence of sodium chlorate.

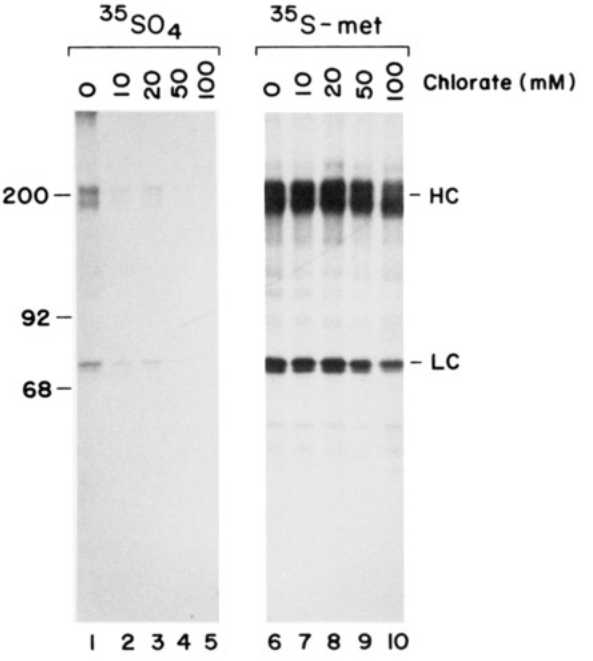

FIGURE 9: Effect of sodium chlorate on factor VIII secretion. 10A1 CHO cells expressing wild-type factor VIII were treated for 24 h with increasing concentrations of sodium chlorate. In parallel, cells were labeled with [^{35}S]sulfate (lanes 1–5) or [^{35}S]methionine (lanes 6–10). Conditioned medium was harvested, factor VIII was immunoprecipitated, and immunoprecipitates were analyzed by SDS–PAGE. Migration of the heavy chain (HC) and light chain (LC) is indicated.

medium (data not shown). At 100 mM sodium chlorate, [^{35}S]methionine incorporation into trichloroacetic acid precipitable protein was inhibited approximately 10% (data not shown). These results show that inhibition of tyrosine sulfation did not inhibit factor VIII synthesis or secretion.

The thrombin cleavage products of factor VIII synthesized in the presence of sodium chlorate were analyzed by immunoprecipitation of [^{35}S]methionine-labeled factor VIII and thrombin digestion prior to SDS–PAGE (Figure 10). No dramatic differences in thrombin cleavage products were observed. At 100 mM sodium chlorate, some partial thrombin cleavage products were detected (Figure 10, lane 10). In the absence of sodium chlorate treatment, the 43-kDa polypeptide migrated as a doublet (Figure 10, lane 2). In the presence of sodium chlorate, the mobility of this doublet was increased (Figure 10, arrow). This shift in migration of the 43-kDa species likely resulted from the absence of sulfate in the polypeptide (Mikkelsen et al., 1991).

The specific activity of factor VIII synthesized in the presence of sodium chlorate was determined by purification of factor VIII LA from conditioned medium and analysis of activity in the one-stage clotting assay and a chromogenic assay for factor Xa generation. Factor VIII synthesized in the presence of sodium chlorate exhibited a 5-fold reduction in procoagulant activity as measured in either assay (Table IV).

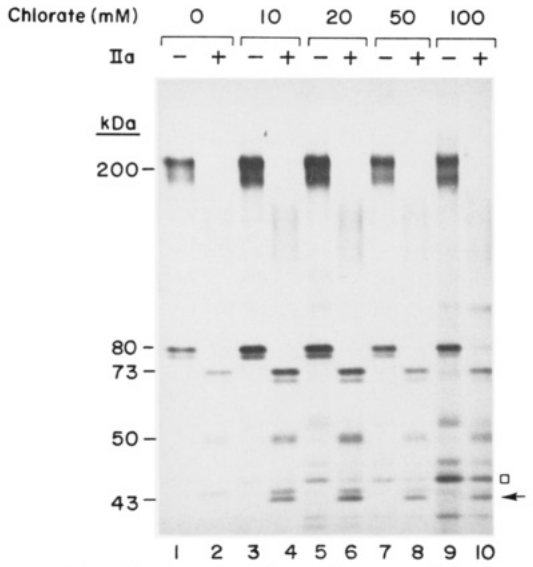

FIGURE 10: Thrombin cleavage of factor VIII secreted in the presence of sodium chlorate. 10A1 CHO cells expressing wild-type factor VIII were labeled with [^{35}S]methionine in the presence of increasing concentrations of sodium chlorate. Conditioned medium was harvested and factor VIII immunoprecipitated. Prior to SDS–PAGE, half of the immunoprecipitate was digested with thrombin (IIa). The factor VIII polypeptides and cleavage products are indicated. The species migrating at 45 kDa (□) is a background band (likely actin), which becomes more predominant at sodium chlorate concentrations which affect cell viability. The arrow identifies the 43-kDa polypeptide obtained from factor VIII expressed in the presence of sodium chlorate.

Table IV: Specific Activity of Factor VIII Synthesized in the Presence and Absence of Sodium Chlorate[a]

	sp act. (units/mg)	
	one-stage clotting	coatest
LA −	2691	2048
LA +	449	384

[a] Factor VIII LA was purified from conditioned medium of cells (clone M18) grown in the presence (LA +) or absence (LA −) of 100 mM sodium chlorate. Factor VIII specific activity (units/mg) was determined as described under Experimental Procedures.

Discussion

A consensus sequence for tyrosine sulfation was proposed on the basis of the amino acids adjacent to sulfated tyrosine residues found in a number of different proteins (Huttner & Baeuerle, 1988; Hortin et al., 1986b). Within factor VIII, there are seven tyrosine residues (at positions 346, 395, 718, 719, 723, 1664, and 1680) which meet the consensus sequence requirements for sulfation (Table III). Recombinant factor VIII contains 6 mol of tyrosine sulfate per mole of polypeptide. Although tyrosine-395 meets the criteria for sulfation, our analysis did not detect sulfation at residue 395. Tyrosine sulfate was demonstrated at amino acid residues 346, 1664, and 1680. In addition, three tyrosine sulfate residues were identified in the COOH-terminal half of the 43-kDa thrombin cleavage fragment. Although we were not able to definitively demonstrate tyrosine sulfation at residues 718, 719, and 723 in the COOH-terminal half of the 43-kDa polypeptide, site-directed mutagenesis of these tyrosines to phenylalanine residues demonstrated that all three mutations are required to inhibit [^{35}S]sulfate incorporation into the 43-kDa thrombin cleavage fragment of factor VIII (D. Michnick and D. Pittman, unpublished results). Thus, it is likely that all three tyrosine residues are sulfated.

Quantitation of tyrosine sulfate within the different polypeptides of factor VIII expressed in CHO cells demonstrated that these six tyrosine residues are sulfated with high efficiency. This analysis was performed with a B-domain deletion mutant of factor VIII which is expressed at a very high level, indicating that posttranslational sulfation of tyrosine was not limiting. This finding is in contrast to a recent report by Mikkelsen et al. (1991) which concluded inefficient tyrosine sulfation at one or more of the residues 718, 719, and 723 was the source for the microheterogeneity in the 43-kDa thrombin cleavage fragment. The 43-kDa fragment generated by thrombin digestion of recombinant factor VIII, and not plasma-derived factor VIII, migrated as a doublet upon SDS–PAGE (Mikkelsen et al., 1991). Although this analysis did not demonstrate the presence of tyrosine sulfate within the 43-kDa fragment, mild acid hydrolysis, which removes the labile sulfate from tyrosine, converted the doublet to a single species (Mikkelsen et al., 1991). It is difficult to know the cause for the difference between their results and those reported here. Our characterization demonstrated that the 43-kDa thrombin-generated fragment also migrates as a doublet; however, the ratio of [^{35}S]sulfate to [^{3}H]tyrosine incorporated into each species of the doublet was identical, and each species of the doublet contained three tyrosine sulfate residues. Inhibition of tyrosine sulfation by sodium chlorate treatment did increase the mobility of the 43-kDa-derived polypeptide (Figure 10). A factor VIII mutant with tyrosine to phenylalanine mutations at all three tyrosine residues at 718, 719, and 723 also yielded a 43-kDa thrombin cleavage fragment which migrated as a doublet upon SDS–PAGE (D. Michnick, unpublished results). These results suggest that the doublet of the thrombin-generated 43-kDa fragment is due to some other yet unknown modification, or carboxyl-terminal heterogeneity.

Tyrosine sulfation is a frequent posttranslational modification that occurs on a number of secretory proteins (Huttner & Baeuerle, 1988). Despite the frequency of this modification, there are few examples where tyrosine sulfation is required for the biological activity of the molecule. Tyrosine sulfation is required for the biological activity of cholecystokinin (Mutt, 1980). Several reports have suggested that tyrosine sulfation alters protein binding of gastrin (Brand et al., 1984), Leu-enkephalin (Unsworth & Hughes, 1982), and chorionic gonadotropin (Gordon & Ward, 1985) with their respective receptors, and of fibronectin with fibrin (Paul & Hynes, 1984). Interestingly, tyrosine sulfate occurs in many proteins which interact with proteases. For example, tyrosine sulfation occurs within α_2-antiplasmin at the site of interaction with plasmin (Hortin et al., 1987). Inhibition of tyrosine sulfation within C4 complement reduces its hemolytic effect by 50% (Hortin et al., 1989). More significantly, many proteins that interact with thrombin, such as hirudin (Braun et al., 1988), fibrinogen (Jevons, 1963; Farrell et al., 1991), heparin cofactor II (Hortin et al., 1986c), bovine factor X (Morita & Jackson, 1986), vitronectin (Jenne et al., 1989), factor V (Hortin, 1990), and, as shown here, factor VIII, contain tyrosine sulfate. Tyrosine sulfation at the carboxyl terminal of hirudin increase its binding affinity to the anion binding exosite of thrombin (Niehrs et al., 1990; Rydel et al., 1990). It is interesting that all the sulfated tyrosine residues within factor VIII as well as those characterized in factor V (Hortin, 1990) border sites cleaved by thrombin, factor Xa, and activated protein C.

An earlier speculation on the role of tyrosine sulfation was that it was required for protein secretion (Huttner & Baeuerle, 1988). Inhibition of tyrosine sulfation retarded the transport of a yolk protein through the Golgi apparatus (Friederich et al., 1988). In contrast, secretion of complement C4 protein or α_2-antiplasmin was not affected by inhibition of tyrosine sulfation (Hortin & Graham, 1988). Similarly, inhibition of

tyrosine sulfation by sodium chlorate treatment of cells did not alter factor VIII secretion. Factor VIII expressed in the presence of sodium chlorate was purified and shown to have a 5-fold-reduced specific activity measured in either a one-stage clotting assay or a factor Xa generation assay. This result is in contrast to recently published observations which suggest there is no change in the specific activity of factor VIII synthesized in the presence of sodium chlorate (Leyte et al., 1991). The discrepancy may result from the lesser degree of inhibition of tyrosine sulfation or the difficulty in accurately quantitating the specific activity of the low level of factor VIII secreted into the conditioned medium in the study of Leyte et al. Alternatively, the antibody used to quantitate factor VIII antigen may have a different reactivity toward sulfated and nonsulfated factor VIII.

Factor VIII expressed in the presence of sodium chlorate was susceptible to thrombin digestion, suggesting no gross alteration in its ability to serve as a substrate for thrombin cleavage. However, further studies are required to study the affinity of nonsulfated factor VIII with thrombin as well as other components of the factor Xa-generating complex. Recently we have also shown that inhibition of tyrosine sulfation of factor V secreted from CHO cells also reduces its procoagulant activity (Pittman et al., 1991). Thus, tyrosine sulfation of factors V and VIII may play a unique role in the interaction of these molecules with other components of the coagulation cascade. It is interesting to speculate that the rare form of hemophilia resulting from combined deficiency of both factor VIII and factor V (Seligshohn et al., 1982) may result from defects in protein tyrosine sulfation.

One of the sulfated tyrosine residues within factor VIII occurs at amino acid residue 1680. This region has previously been shown to be a primary site for interaction with vWF (Foster et al., 1988; Leyte et al., 1989). Mutation of tyrosine-1680 to phenylalanine resulted in a factor VIII molecule that was defective in high-affinity factor VIII binding to vWF (Leyte et al., 1991; Pittman & Kaufman, 1989). In addition, a patient with moderate hemophilia A was identified with the same mutation of tyrosine to phenylalanine at 1680. This patient had factor VIII circulating antigen activity reduced to 20% and factor VIII activity reduced to 10% (Higuchi et al., 1990). Since vWF is required to stabilize factor VIII in plasma, the defect in this patient may result from a factor VIII with decreased vWF binding properties. Further studies using site-directed mutagenesis of the tyrosine residues within factor VIII are required to further evaluate the functional significance of tyrosine sulfation within factor VIII.

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Registry No. Sulfated tyrosine, 956-46-7; factor VIII, 109319-16-6; thrombin, 9002-04-4.

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