Sulfation of Tyrosine Residues in Coagulation Factor V

By G.L. Hortin

Sulfation of human coagulation factor V was investigated by biosynthetically labeling the products of HepG2 cells with [35S]sulfate. There was abundant incorporation of the sulfate label into a product identified as factor V by immunoprecipitation, lability to proteases, affinity for the lectin jacalin, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Two or more sites in factor V incorporated sulfate as indicated by labeling of different peptide chains of factor Va. The 150-Kd activation fragment of factor Va incorporated the greatest amounts of sulfate. This fragment of factor Va was bound selectively by jacalin-agarose, reflecting its content of O-linked oligosaccharides. Analysis of an alkaline hydrolysate of sulfate-labeled factor Va by anion-exchange chromatography showed that the sulfate occurred partly in tyrosine sulfate residues and partly in alkaline-labile linkages. Sulfate groups are potentially important structural and functional elements in factor V, and labeling with [35S]sulfate provides a useful approach for examining the biosynthesis and processing of this protein. The hypothesis is advanced that sites of sulfation in factor V and several other plasma proteins contribute to the affinity and specificity of thrombin for these molecules, just as it does for the interaction of thrombin with the potent inhibitor hirudin from leeches.

© 1990 by The American Society of Hematology.

MATERIALS AND METHODS

[4,5-3H]Leucine (60 Ci/mmol) was obtained from Amersham (Arlington Heights, IL), and carrier-free [35S]sulfate from ICN Radiochemicals (Irvine, CA). Rabbit antiserum to human factor V was purchased from Accurate (Westbury, NY), and antiserum to human fibrinogen from Calbiochem (San Diego, CA). Protein A-Sepharose was from Pharmacia (Piscataway, NJ). Factor V-deficient plasma, other reagents for assay of factor V activity, and thrombin were obtained from Sigma Chemical (St Louis, MO). Jacalin-agarose was purchased from Vector Laboratories (Burlingame, CA). Hirudin purified from leeches (activity > 10,000 U/mg) was from American Diagnostica (New York, NY). Other reagents were purchased from Sigma Chemical.

HepG2 cells, a human hepatoma-derived cell line,17 were grown to confluence in Earl's medium with 10% fetal calf serum and 4 mmol/L glutamine. Biosynthetic labeling of proteins was performed for 4 to 16 hours with 1 mcCi [35S]sulfate or 0.25 mcCi [3H]leucine in 10 ml serum-free medium deficient in sulfate and leucine. There was no chase incubation. Procedures for immunoprecipitation, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of samples under reducing conditions, and autoradiography have been described previously.24

Lectin affinity chromatography of culture medium from HepG2 cells was performed at room temperature using a column of jacalin-agarose with 1 ml bed volume, 0.6 cm in diameter. Samples of 5 ml serum-free culture medium containing labeled products were passed through the column with gravity flow. The column was washed with 10 vol of 0.15 mol/L NaCl or 0.5 mol/L NaCl, each buffered with 20 mmol/L Hepes (pH 7.4). Specifically bound components were then eluted with 5 ml of 0.1 mol/L melibiose in the corresponding wash buffer. Factor V in the eluate was purified and concentrated by immunoprecipitation. Human plasma in acid-citrate-dextrose was separated from whole blood 1 day after collection, and was fractionated at 4°C on a 10-ml column of jacalin-agarose in 0.5 mol/L NaCl with 20 mmol/L Hepes (pH 7.4). Fractions of 4 ml were collected, and specific elution was effected with 20 mmol/L a-methyl galactopyranoside in the same buffer. Factor V activity was determined with a clotting assay25 using a Precision photometer.

From The Edward Mallinckrodt Department of Pediatrics, Washington University School of Medicine, St Louis, MO.

Submitted October 27, 1989; accepted May 9, 1990.

Supported by a grant from the National Institutes of Health.

Address reprint requests to Glen L. Hortin, MD, PhD, Box 8116, Department of Pediatrics, Washington University School of Medicine, 490 S Kingshighway, St Louis, MO 63110.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1990 by The American Society of Hematology.

0006-4971/90/7505-002283.00/0

Blood, Vol 76, No 5 (September 1), 1990: pp 946-952

946
SULFATION OF FACTOR V

Sites of linkage of sulfate were assessed by elution of [35S]sulfate-labeled peptides from polyacrylamide gel by excision of labeled bands and incubation with 10 μg/mL trypsin in 0.1 mol/L NH4HCO3 for 1 day. Base hydrolysis of eluted peptides was then performed under vacuum in Teflon vessels (Tuf-tainers from Pierce Chemical, Rockford, IL). Peptides were hydrolyzed to amino acids by heating for 20 hours at 110°C in 1 mL of 1 mol/L KOH. Hydrolysates were neutralized and desalted by passage through a 2-mL column of Dowex 50 (H+ form) at 4°C eluted with water. Analysis for tyrosine sulfate was performed by high-performance liquid chromatography on a 150 × 4.6 mm anion-exchange column (8 μm PL-SAX 1000A column from Polymer Laboratories, Amherst, MA). Elution was performed with a linear gradient of phosphate buffers over 16 minutes, beginning with 20% buffer B and proceeding to 100% buffer B. After 16 minutes, elution was with 100% buffer B. Buffer A was 5% acetonitrile/0.02 mol/L NaH2PO4 adjusted to pH 2.5 with phosphoric acid, and buffer B was 0.25 mol/L NaH2PO4, pH 2.5.

RESULTS

Biosynthetic sulfation of human factor V was examined using HepG2 cells in culture, a cell line previously demonstrated to synthesize factor V30 and to add sulfate to tyrosine residues in other proteins.28,31-34 [35S]Sulfate-labeled and [3H]leucine-labeled products synthesized and secreted by HepG2 cells were analyzed by immunoprecipitation and SDS-PAGE (Fig 1). Lanes 1 and 2 show proteins precipitated from culture medium by antiserum to factor V after 4- and 16-hour labeling with [35S]sulfate. At 4 hours, the major labeled product has a molecular weight considerably greater than 205,000 (the size of polypeptide chains of myosin, the largest molecular weight marker). After 16 hours, a much greater amount of labeled product was recovered and the predominant bands occurred at apparent molecular weights of approximately 150,000 and 105,000. Factor V is highly labile protein11-13 and apparently is slowly converted to factor Va during prolonged incubations in the culture medium. The major sulfate-labeled bands in lanes 1 and 2 were identified as intact factor V, which has a molecular weight of 330,000; the connecting peptide of 150,000 daltons; and the 105,000-dalton heavy chain of factor Va14-15 by comparison with leucine-labeled peptides precipitated by antiserum to factor V (lanes 5 and 6). The light chain of factor Va and the small activation peptide both have a molecular weight of about 70,000.1 Small amounts of sulfate-labeled product of this size (lane 2 and more apparent with longer exposures as in Fig 3, below) and a doublet of leucine-labeled products (lane 6) of this size were precipitated by antiserum to factor V. It is not clear whether the sulfate label corresponds to one or both bands of the doublet. There was relatively high incorporation of sulfate label into factor V-related products in comparison with the fibrinogen chain and to other products studied previously, such as the fourth component of complement18 and α2-antiplasmin.31 In Fig 1, lanes depicting sulfate-labeled products were exposed for 1 day and lanes with leucine-labeled products were exposed for 12 days.

Specificity of immunoprecipitations was evaluated by comparison of products precipitated by antiserum to factor V and by antiserum to fibrinogen. Precipitation of the sulfate-labeled products in lanes 1 and 2 was shown to be specific for the antiserum to factor V by comparison with immunoprecipitations using antiserum to fibrinogen, which isolate a major labeled product of about 55,000 daltons (lanes 3 and 4). Previous studies identify the sulfated fibrinogen polypeptide as a structural variant of the γ-chain termed the γ-chain.34 Foregoing studies of several sulfate-labeled proteins produced by HepG2 cells have not observed immunoprecipitation of products corresponding in size to factor V or the peptide chains of factor Vα.28,32-35 Among leucine-labeled products, factor V and derived peptides were precipitated specifically by the antiserum to factor V (lanes 5 and 6), and not by antiserum to fibrinogen (lanes 7 and 8). However, there was recovery of small amounts of fibrinogen in precipitations with antiserum to factor V, with the Aα, Bβ, and γ-chains at molecular weights of about 70,000, 55,000, and 50,000, respectively. Also, there was an unidentified protein with a molecular weight of about 250,000 that was recovered nonspecifically in precipitations with antiserum to factor V and fibrinogen.

The relative amounts of intact factor V and fragments derived from it in culture medium were variable from experiment to experiment. This may reflect varying levels of thrombin or other proteolytic activities in the culture me-
specifically bind most proteins containing O-linked oligosaccharides, even if there is occurrence of only one such oligosaccharide in the protein. Proteins can be eluted from the lectin by α-galactosides such as melibiose or α-methyl galactoside. It was found that jacalin bound the sulfate-labeled products derived from factor V, and these products were eluted by solutions containing melibiose (Fig 3).

![Figure 2](image-url)

**Figure 2.** Cleavage of sulfate-labeled factor V by thrombin. HepG2 cells were incubated for 16 hours with 1 mCi [35S]sulfate in 10 mL of serum-free medium either lacking (lanes 1 and 2) or containing (lanes 3 and 4) 0.1 U/mL of hirudin. Medium was collected and aliquots were incubated for 30 minutes at 37°C, with additions of 0.2 U/mL thrombin as indicated. Factor V was isolated by immunoprecipitation and analyzed by PAGE.

Further verification of the identities of sulfate-labeled factor V and the 150,000-dalton activation peptide was obtained by lectin-affinity chromatography on jacalin-agarose. Jacalin is the lectin from *Artocarpus integrifolium* with specificity for O-glycosidically linked oligosaccharides. A recent study has determined that this lectin will bind the sulfate-labeled products derived from factor V, and these products were eluted by solutions containing melibiose.
SULFATION OF FACTOR V

ty of the products recovered varied depending on the column washing procedure. If the column was washed with 0.5 mol/L NaCl, elution with melibiose yielded the sulfate-labeled 150,000-dalton activation fragment (lane 2), but not the 105,000-dalton heavy chain of factor Va precipitated from the total labeled product (lane 1). Other studies have found that jacalin-agarose retains its affinity for O-linked oligosaccharides at high ionic strength and that nonspecific binding is decreased under such conditions. Use of high ionic strength appeared to be necessary for highly specific fractionation of factor V and its derivative peptides. If the column was washed with 0.15 mol/L NaCl before elution with melibiose, then the recovered products (lane 3) appear similar to those from the total product with respect to the presence of the 150,000-dalton and 105,000-dalton polypeptides.

These results are consistent with the known structure of factor Va. The 150,000-dalton activation peptide, which is recognized to be the predominant site for attachment of O-linked oligosaccharides, does not remain associated with the heavy and light chains of factor Va. At high ionic strength without calcium ions, the heavy and light chains should also dissociate, and only peptide chains containing O-linked oligosaccharides would be retained by the lectin. The observed results provide further evidence that the sulfate-labeled product of 150,000 daltons was, in fact, the large activation fragment, and suggest that this is the only segment in factor V containing O-linked oligosaccharides.

Examination of the total profile of leucine-labeled proteins secreted by HepG2 cells (lane 4) and the products bound to jacalin and eluted with melibiose (lane 5) showed that very few leucine-labeled products bound to jacalin. Results are shown for a separation performed at high ionic strength to minimize nonspecific binding. Factor Va was a relatively minor product, and the leucine-labeled 150,000-dalton product was apparent only after immunoprecipitation, which resulted in a 50-fold concentration of products from eluate (lane 6). The upper band is the nonspecifically bound product shown in Fig 1. The observation that very few products from the cultured cells are retained by jacalin-agarose suggests that this lectin may serve as an affinity matrix for isolating factor V or other secreted products bearing O-linked oligosaccharides.

The binding of factor V to jacalin-agarose was confirmed by fractionation of human plasma on jacalin-agarose and monitoring the elution of factor V with an activity assay (Fig 4). The major peak of factor V activity was eluted by solutions containing α-methyl galactopyranoside (top panel). A smaller peak of activity was in flow-through fractions, and no activity was eluted by lactose, which is a β-galactopyranoside. Only 7% of total protein as measured by absorbance at 280 nm bound to the column and was eluted with α-methyl galactopyranoside (bottom panel; note a 10-fold lower dilution of specifically eluted fractions relative to flow-through fractions). Total recovery of factor V activity from the column was approximately 20% of the total activity of plasma applied to the column. Incomplete recovery may reflect the lability of factor V and failure to completely displace bound protein from the lectin column. The major components in plasma that are known to bind to jacalin are immunoglobulin A and CI inhibitor, but this lectin also appears to be useful for fractionation of factor V.

The nature of the linkage of sulfate to the 150,000-dalton activation peptide was examined by base hydrolysis of the [35S]sulfate-labeled peptide eluted from an SDS-PAGE. The hydrolysate was neutralized then analyzed by anion-exchange high-performance liquid chromatography (Fig 5). The sulfate label eluted as two major peaks (A), which corresponded to the elution positions of authentic tyrosine sulfate and of free sulfate ions in a parallel analysis (B). The peak widths are broader for the hydrolysate due to the more complex sample. An internal standard of tyrosine sulfate was monitored to ensure that there was no significant change in retention time of this analyte (not shown). The total number of counts in the sulfate peak was 30% greater than that in the tyrosine sulfate peak, indicating that slightly less than half of the label is linked to tyrosine residues, and the remainder to alkali-labile sites. Although tyrosine sulfate residues are not 100% stable during base hydrolysis, a previous study has found that only a small amount of the sulfate linked to tyrosine in fibrinogen is released as free sulfate, using the same conditions for hydrolysis. Unless there is marked sequence-specific variation in the lability of tyrosine sulfate to base hydrolysis, most of the free sulfate derived on base hydrolysis of factor V originated from structures other than tyrosine sulfate.
The precise sites of sulfation in factor V remain to be established, but examination of the primary amino acid sequence of factor V permits prediction of the tyrosine residues that are probable sites of sulfation. Analysis of the amino acid sequences of known sites of sulfation in other proteins has shown several common structural features, and criteria for predicting sites of sulfation have been developed. Based on these criteria, the amino acid sequence of factor V contains six tyrosines that are likely sites of sulfation: residues 696, 698, 1494, 1510, 1515, and 1565. The first two sites are located at the carboxy-terminal end of the 105,000-dalton heavy chain. The next three sites are near the carboxy-terminus of the 150,000-dalton activation peptide, and the final site is within the light chain segment of factor V. The distribution of predicted sites is consistent with the incorporation of labeled sulfate into the 150-Kd activation peptide and the 105-Kd heavy chain in this study. The potential site in the light chain must undergo sulfation inefficiently, if at all, considering that only small amounts of labeled product of this size were observed.

Thrombin cleavage sites and predicted sites of sulfation along the linear structures of factor V and the homologous protein factor VIII are diagramed in Fig 6. There is striking apposition of sites of sulfation and cleavage by thrombin. All of the tyrosine sulfate residues are predicted to be located 10 to 42 residues amino-terminal to the points of cleavage. The amino acid sequences of the segments preceding points of cleavage by thrombin are presented in Fig 6. All of the sites of cleavage required for activation of factors V and VIII are

**HUMAN FACTOR V**

\[
\begin{align*}
\text{NH}_2 & \quad 658 \quad \text{DDDDSYTELPEPESTMTARKKHD} \quad \text{EY} \quad 938 \quad \text{RDQGGKSKLQSGPLRTRKKREKHHPALPSPYRTFH} \quad 1507 \quad \text{EDDYALADVVPKTDVRTNNSSRDPDNI} \quad \text{SN} \\
& \quad 717 \quad \text{Q} \quad \text{Q} \quad \text{Q} \quad \text{S} \quad \text{S} \\
& \quad 1060 \quad \text{REDYDTISTVEMKEDPHTDEDENGSPYRFS} \\
& \quad 1070 \quad \text{SF} \quad \text{GF} \\
\end{align*}
\]

**HUMAN FACTOR VIII**

\[
\begin{align*}
\text{NH}_2 & \quad 50 \quad \text{S} \quad \text{L} \quad \text{W} \quad \text{R} \quad \text{A} \quad \text{R} \quad \text{Y} \quad \text{K} \quad \text{H} \quad \text{Q} \quad \text{S} \quad \text{F} \quad \text{W} \quad \text{F} \\
& \quad 717 \quad \text{DDYEDYDISAYLSSKKNAIEPRF} \quad \text{S} \quad \text{F} \\
& \quad 1060 \quad \text{REDYDTISTVEMKEDPHTD} \quad \text{SN} \quad \text{S} \quad \text{F} \\
\end{align*}
\]

In addition to describing a significant structural modification of factor V, the present study provides new approaches for biosynthetic labeling of this protein and its partial purification by lectin affinity chromatography on jacalin agarose. These approaches should be useful in examining the synthesis and processing of factor V in multiple cell types, including megakaryocytes, aortic endothelial cells, and macrophages.

**DISCUSSION**

Evidence is presented here that factor V undergoes biosynthetic sulfation of at least two sites along its peptide chain. Sulfate label was added to factor V in the segments corresponding to both the 150-Kd activation peptide and the 105-Kd fragment of factor Va, with the greatest amount of sulfate added to the activation peptide. Part of the sulfate was linked to tyrosine residues and part to alkali-labile sites. The alkali-labile sites possibly correspond to sulfates bound to serine and threonine in the polypeptide backbone have not been detected in nature. Linkage of sulfate to mucinous-type proteins with multiple O-linked oligosaccharides is commonly observed, and HepG2 cells have been observed to transfer sulfate to N-linked oligosaccharides of other proteins.

In addition to describing a significant structural modification of factor V, the present study provides new approaches for biosynthetic labeling of this protein and its partial purification by lectin affinity chromatography on jacalin agarose. These approaches should be useful in examining the synthesis and processing of factor V in multiple cell types, including megakaryocytes, aortic endothelial cells, and macrophages.
SULFATION OF FACTOR V

preceded by clusters of acidic amino acid residues (underlined) embodying predicted tyrosine sulfate residues. Cleavage at the one site, following Arg, in factor V, that is not preceded by a cluster of acidic amino acids apparently is not essential for activation of factor V to factor Va. Functional significance of the tyrosine sulfated residues and the surrounding clusters of acidic amino acids is suggested by identification of an anion-binding site on thrombin's surface that participates in interactions with fibrinogen and hirudin. This functional element, termed the "anion-binding exosite," is located a significant distance from the enzyme's catalytic site, such that a minimal-length peptide segment of approximately 10 amino acid residues would be required to span the intervening space. Interactions of the anion-binding exosite with peptide ligands have been best defined with regard to a carboxyl-terminal segment of hirudin. The final 12 residues of hirudin contain multiple acidic residues, which contribute to binding to the anion-binding exosite. Sulfation of the tyrosine residue near the carboxyl-terminus of hirudin increases its affinity for thrombin 10-fold, and sulfation has similar effects on the affinity for thrombin of synthetic peptides corresponding to carboxy-terminal segments of hirudin. There has been extensive study of the relationship of peptide structure to this binding site on thrombin, and it is observed that considerable sequence variation is permitted with retention of affinity for thrombin. Recent work has provided evidence that the anion-binding exosite also binds to an acidic segment in the plasma proteinase inhibitor, heparin cofactor II, that contains two tyrosine sulfated residues. The acidic segment of heparin cofactor II does not have a high degree of sequence homology with the carboxy-terminal segment of hirudin. Thus, the thrombin's anion-binding exosite may be able to interact with a variety of polyanionic peptide segments, such as those that occur in factors V and VIII preceding points of cleavage by thrombin. A surprising number of proteins that interact with thrombin are recognized to contain acidic regions with tyrosine sulfated residues, including coagulation factors V and VIII, hirudin, heparin cofactor II, fibrinogens (within B fibrinopeptides) of many animal species, human fibrinogen (within B fibrinopeptides), and vitronectin. Thus, thrombin's interactions with many proteins may be directed, at least in part, by binding to these sites.

Location of ligands for thrombin's anion-binding exosite within substrates and inhibitors of thrombin offers the potential for substantial increases in the efficiency and specificity of interactions with thrombin. The local concentration of thrombin would be increased and the enzyme's catalytic site may be aligned with appropriate points of cleavage or reactive sites of inhibitors. A more unexpected consequence of interaction of the anion-binding exosite with peptide ligands is that there is a substantial conformational change of the thrombin molecule. The conformational change produces functional changes at the catalytic site that are reflected by altered kinetic parameters for chromogenic substrates and increased affinity for the amino-terminal segment of hirudin. These results suggest that conformational changes of thrombin induced by interactions with polyanionic peptide segments of a natural substrate or inhibitor may produce in situ increases in the enzyme's catalytic efficiency.

The proposed role for polyanionic sequences, and particularly tyrosine sulfated residues, in the interactions of factors V and VIII and other proteins with thrombin targets a number of sites for further investigation by site-directed mutagenesis, by use of inhibitors of sulfation, and by production of model synthetic peptides. Understanding these interactions will be important for determining the molecular basis for thrombin's specificity and for delineating the mechanism of a new family of anticoagulants based on the carboxyl-terminal sequence of hirudin.

ACKNOWLEDGMENT

I thank James Graham and Barbara Benutto for technical assistance.

REFERENCES


From www.bloodjournal.org by guest on October 23, 2017. For personal use only.


44. Monkovic DD, Tracy PB: Activation of human factor V by factor Xa and thrombin. Biochemistry 29:1118, 1990


Sulfation of tyrosine residues in coagulation factor V

GL Hortin