Identification and Structural Analysis of the Tetrasaccharide NeuAcα(2→6)Galβ(1→4)GlcNAcβ(1→3)Fucα1→*O*-Linked to Serine 61 of Human Factor IX†

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ABSTRACT: O-Linked fucose has been found attached to Thr/Ser residues within the sequence Cys-X-X-Gly-Gly-Thr/Ser-Cys in the N-terminal EGF domains of several coagulation/fibrinolytic proteins. Carbohydrate composition and mass spectrometric analyses of tryptic and thermolytic peptides containing the corresponding site (Ser-61) in the first EGF domain of human factor IX indicated the presence of a tetrasaccharide containing one residue each of sialic acid, galactose, *N*-acetylglucosamine, and fucose. The Ser-61 tetrasaccharide was not susceptible to α-fucosidase digestion. Fragments generated during mass spectrometric analysis indicated that fucose was the attachment sugar residue. The involvement of fucose in the carbohydrate–peptide linkage was confirmed by two-dimensional ^{1}H NMR spectroscopic analysis of the glycopeptide containing factor IX residues 57–65. The complete structure of the tetrasaccharide was obtained by methylation analysis and two-dimensional ^{1}H TOCSY and ROESY experiments as NeuAcα(2→6)Galβ(1→4)GlcNAcβ(1→3)Fucα1→*O*-Ser^{61}.

Coagulation factor IX (Christmas factor) is the zymogen of a serine protease required for normal hemostasis (Furie & Furie, 1988; Davie et al., 1991). The 57-kDa zymogen contains an N-terminal γ-carboxyglutamic acid (Gla)[1] domain, two EGF domains, and a serine protease region. In the presence of calcium ions, the 12 Gla residues mediate binding to the phospholipid surface of endothelial cells (Stenflo & Suttie, 1977), where activation by either factor XIa or the factor VII/VIIa–tissue factor complex results in the release of a 35-residue peptide and conversion to the two-chain protease (factor IXa). Two potential N-glycosylation sites (Asn-157 and Asn-167) are found within the residue 146–180 activation peptide (Kurachi et al., 1982). Xylα(1→3)Glcβ1→*O* and Xylα(1→3)Xylα(1→3)Glcβ1→*O* saccharides are glycosidically linked to Ser-53 within the first EGF domain (Nishimura et al., 1989). EGF-1 also contains a high-affinity Ca^{2+} binding site with either aspartate or β-hydroxyaspartate (Hya) at position 64 (Fernlund & Stenflo, 1983; McMullen et al., 1983).

O-Linked fucose is an unusual post-translational modification that is found at Thr/Ser residues within the sequence Cys-X-X-Gly-Gly-Thr/Ser-Cys in the N-terminal EGF domains of human urokinase (Buko et al., 1991), t-PA (Harris et al., 1991), and coagulation factors VII and XII (Bjoern et al., 1991; Harris et al., 1992). The function of this modification is unknown. We investigated the possibility that O-linked fucose would be found at the corresponding position (Ser-61) in EGF-1 of human factor IX. Peptides resulting from enzymatic degradation of factor IX were purified by RP-HPLC; those containing Ser-61 consistently showed sialic acid, galactose, and *N*-acetylglucosamine in addition to the expected fucose. By electrospray mass spectrometry of the glycopeptide, a mass 802.8 in excess of the peptide mass was found, consistent with the presence of a tetrasaccharide containing one residue of each of these monosaccharides. Fragments generated during mass spectrometric analysis indicated that fucose is the attachment sugar residue.[2] Nishimura et al. (1992) have recently reported finding the same Ser-61 glycan, with fucose as the reducing end residue of a tetrasaccharide consisting of 1 mol each of galactose, fucose, *N*-acetylglucosamine, and sialic acid. In the present study, methylation analysis and one- and two-dimensional ^{1}H NMR spectroscopic analysis of the glycopeptide containing factor IX residues 57–65 were used to determine that the complete structure of the tetrasaccharide is NeuAcα(2→6)Galβ(1→4)GlcNAcβ(1→3)Fucα1→*O*-Ser^{61}.

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; EGF, epidermal growth factor; EGF-1, first (N-terminal) EGF domain; Xyl, xylose; Glc, glucose; Hya or β, β-hydroxyaspartate; NMR, nuclear magnetic resonance; t-PA, tissue-type plasminogen activator; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; NeuAc, *N*-acetylneuraminic acid (sialic acid); Gal, galactose; GlcNAc, *N*-acetylglucosamine; Fuc, fucose; PTH, phenylthiohydantoin; IFN, interferon; COSY, scalar correlated spectroscopy; DIPSI, decoupling in the presence of scalar interactions; DQF, double-quantum-filtered; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; 1D and 2D, one-dimensional and two-dimensional; ES-MS, electrospray mass spectrometry; FID, free induction decay; GLC–MS, gas–liquid chromatography–mass spectrometry; NOE, nuclear Overhauser effect; ROESY, NOE correlated spectroscopy in the rotating frame; TOCSY, total correlation spectroscopy (homonuclear Hartmann–Hahn spectroscopy); TPPI, time-proportional phase incrementation.

[2] These findings were presented by R. J. Harris et al. on July 28, 1992, in a poster entitled "Identification of a Novel O-Linked Tetrasaccharide Attached to Ser-61 in the First EGF Domain of Human Factor IX" at the Sixth Symposium of the Protein Society, San Diego, CA.

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MATERIALS AND METHODS

Human factor IX was purified from plasma as described (Smith, 1988). TPCK–trypsin was purchased from Worthington (Freehold, NJ); thermolysin was purchased from Boehringer Mannheim (Indianapolis, IN). TFA was purchased from Pierce (Rockford, IL), while acetonitrile was from Burdick & Jackson (Muskegon, MI). Chicken liver α-fucosidase was purchased from Oxford GlycoSystems (Rosedale, NY), and *Clostridium perfringens* neuraminidase was from Sigma (St. Louis, MO).

Peptide Purifications. A sample containing 9.3 mg (200 nmol) of human factor IX was reduced and S-carboxymethylated (Chloupek et al., 1989) and then digested with trypsin at a 1:50 weight ratio for 8 h at 37 °C in a pH 8.3 buffer containing 10 mM Tris, 100 mM NaOAc, and 1 mM CaCl2. A Hewlett-Packard 1090 HPLC system equipped with a Vydac C18 (4.6 × 250 mm) column was equilibrated with 0.1% TFA (solvent A) at 30 °C; 3 min after sample injection, a gradient increasing to 60% solvent B (0.1% TFA in acetonitrile) in 90 min was used to elute tryptic peptides. Twelve injections of 16 nmol of digested factor IX were performed. The peak fractions containing the 44–80 peptide from each run were collected manually and pooled. Peptides were identified by amino acid compositional analysis.

The peptide containing factor IX residues 44–80 (160 nmol) was lyophilized, reconstituted in the pH 8.3 buffer described above, and digested with 250 μg of thermolysin for 4 h at 37 °C. Thermolytic fragments were purified by RP-HPLC as above; peptides were identified by a combination of amino acid compositional and N-terminal sequence analyses. The 57–65 (LNGGSCKDD) and 57–68 (LNGGSCKβDINS) glycopeptides were used for further analyses. The 57–65 fragment was also repurified using the RP-HPLC method described above, except that the C18 column was replaced by a Vydac C4 (4.6 × 250 mm) column.

Glycosidase Digestions. The 57–68 peptide (LNGGSCKβ-DINS) was treated for 18 h with 0.02 unit of neuraminidase per nmol of peptide in a pH 5.6 buffer containing 200 mM NaOAc and 2 mM $CaCl_2$. The sample was repurified by RP-HPLC and subjected to mass spectrometric analysis. A glycopeptide containing t-PA residues 441–449 was purified from trypsin-digested recombinant human t-PA (Genentech) as described (Chloupek et al., 1989). Samples (1 nmol) of the t-PA 441–449 glycopeptide or factor IX 57–68 glycopeptide (with Hya-64) were treated with 0.3 unit of α-fucosidase for 40 h at 37 °C in a 100 mM citrate/phosphate buffer at pH 6.

General Methods. Mass spectrometric analyses were performed using a Sciex API III triple quadrupole electrospray instrument operating in the positive mode (Covey et al., 1988). Samples were concentrated in a Savant SpeedVac and then reconstituted with 50% methanol/0.1% formic acid to a final sample concentration of 5 pmol/μL. The samples were infused into the source using a Harvard syringe pump at a flow rate of 2 μL/min. The orifice potential was set at 90 V. The mass range of the scan was 300–2000 Da, with unit resolution throughout. For β-elimination experiments, glycopeptide samples were incubated in 0.1 N NaOH for 16 h at 55 °C (Anderson et al., 1964) and then neutralized with acetic acid prior to acid hydrolysis. Carbohydrate compositions were determined using a Dionex BioLC high-pH anion-exchange chromatography system with pulsed amperometric detection (Hardy et al., 1988), as described by Spellman et al. (1991). N-Terminal sequence analyses were performed using an Applied Biosystems 477A/120A sequencer.

Glycosyl Linkage Analysis. The oligosaccharide was released from 28 nmol of the 57–65 glycopeptide by automated hydrazinolysis (Merry et al., 1992) on a GlycoPrep 1000 instrument (Oxford GlycoSystems) and was prereduced with $NaBH_4$. The glycosyl linkage composition of the factor IX oligosaccharide was determined by methylation analysis (Lindberg, 1972) using the procedure of Waeghe et al. (1983), except that hydrolysis of the permethylated oligosaccharide alditol was carried out with 4 N trifluoroacetic acid for 1 h at 125 °C. A 40-nmol sample of LS-tetrasaccharide *c* (Oxford GlycoSystems) was derivatized in parallel as a chromatographic reference. Gas–liquid chromatography was carried out on a Hewlett-Packard 5895A gas chromatograph equipped with a Supelco SBP-5 column (30 m × 0.25 mm i.d.; 0.25 mm film thickness) and a flame-ionization detector. GLC–MS analysis of the permethylated alditol acetates generated from the hydrazinolysate of the factor IX glycononapeptide was performed using a Hewlett-Packard 5890/5970 GC/MSD system equipped with a 50-m fused silica methyl silicone column (0.25 mm i.d.) from Quadrex Corporation. The oven temperature was held at 80 °C for 2 min, increased to 180 °C at 20 °C/min, increased again to 240 °C at 2 °C/min, and held at this temperature for 5 min.

Nuclear Magnetic Resonance Spectroscopy. A sample (~20 nmol) of factor IX glycopeptide 57–65 was first dissolved in D_2O containing sodium acetate-d_3 at pH 7.2, lyophilized, and then reexchanged four times with pure D_2O (99.99% D, Cambridge Isotope Laboratories). The final solution of the glycopeptide in 0.7 mL of D_2O contained 40 mM sodium acetate-d_3 (pH 7.2). NMR spectra were recorded at 23 °C on a Bruker AMX-600 spectrometer, operating at a frequency of 600 MHz for ^1H NMR, interfaced with an Aspect X-32 computer. Chemical shifts (δ) are expressed in ppm downfield from DSS with an accuracy of 0.01 ppm, and they were measured relative to internal acetone at δ 2.225. Two-dimensional DQF-COSY (Piantini et al., 1982), TOCSY (Braunschweiler & Ernst, 1983; Bax & Davis, 1985), and ROESY (Bothner-By et al., 1984) data sets were collected in the phase-sensitive mode using the TPPI method (Marion & Wüthrich, 1983). In all experiments, low-power presaturation was applied to the residual HDO signal. In each of the 2D experiments, 800 FIDs of 2048 complex data points were collected. For the DQF-COSY and TOCSY experiments, 64 scans per FID were acquired, the spectral width was set to 3623 Hz, and the carrier was placed at 4.0 ppm. The TOCSY pulse program contained a 108-ms spin-lock pulse consisting of a DIPSI-2 sequence (Rucker & Shaka, 1989). The ROESY experiment used a 200-ms, 2.2-kHz CW spin-lock pulse flanked by two 90° pulses for offset compensation (Griesinger & Ernst, 1987); 160 scans per FID were collected, the spectral width was set to 6024 Hz, and the carrier was placed at 5.8 ppm. The 2D data were processed typically with a Lorentzian-to-Gaussian function applied in the t_2 dimension and a shifted, squared sine bell function and zero-filling applied in the t_1 dimension. Processing was performed with the Felix software package, version 2.0 (Hare Research, Inc.) on a Silicon Graphics Personal Iris workstation.

RESULTS

Preparation and Analysis of Ser-61 Glycopeptides. The HPLC profile of trypsin-digested S-carboxymethylated factor IX is given in Figure 1. The peptide containing residues 44–80 was identified by amino acid analysis (data not shown). Mass spectrometric analysis of this fraction showed a predominant mass of 5626.8 amu; of this, the polypeptide

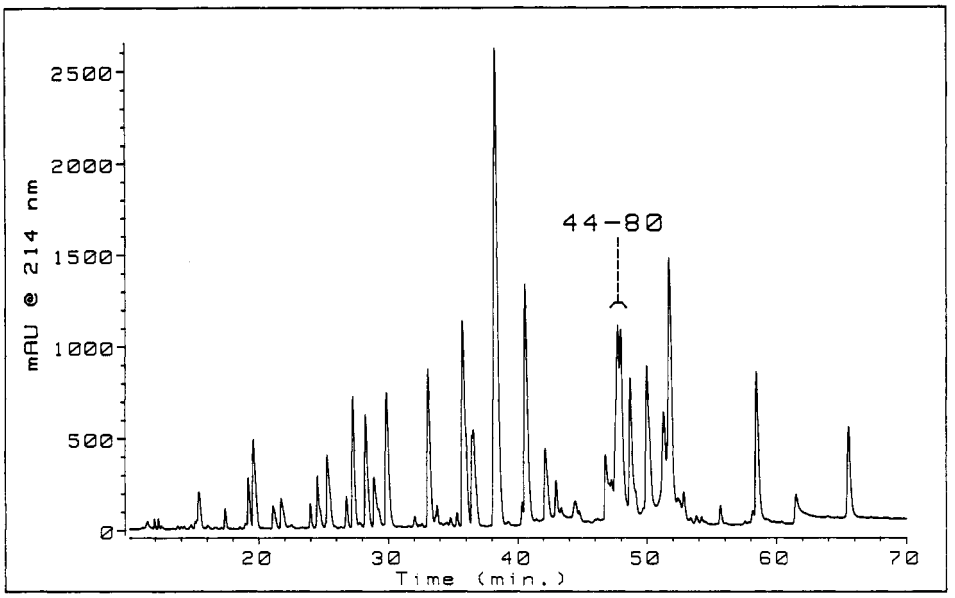

FIGURE 1: RP-HPLC purification of the 44–80 tryptic peptide. Conditions are given in Materials and Methods. The 44–80 peak elutes as a doublet at 48 min. A total of 16 nmol of trypsin-digested S-carboxymethylated human factor IX was injected.

Table I: Carbohydrate Compositional Analysis of Glycopeptides Containing Ser-61[a]

	44–80 glycopeptide	57–65 glycopeptide
NeuAc	1.1	1.0
Gal	1.5	1.0
GlcNAc	1.5	1.0
Fuc	1.1	0.8
Xyl	1.0	0.3
Glc	1.3	0.1

[a] Residues per mole of peptide.

contribution is 4399.5 amu and the principal glycan attached to Ser-53 (Xyl-Glc) contributes 294.3 amu, leaving an unattributed mass of 801.3 amu. The carbohydrate composition showed that approximately one residue each of NeuAc, Gal, GlcNAc (detected as $GlcNH_2$ after acid hydrolysis), and Fuc was present, in addition to Xyl and Glc from the Ser-53 glycans (Table I).

Thermolysis of the 44–80 peptide produced several different fragments containing Ser-61 that were purified by RP-HPLC (Figure 2), including the 57–65 peptide (peak 4) and the 57–68 peptides with or without β-hydroxyaspartate at position 64 (peaks 7 and 10, respectively), as summarized in Table II. Deamidated forms (with Asn-58 converted to isoaspartate) of these peptides were also recovered in peaks 5 and 8 of Figure 2. Peptides with Asp-64 totaled 64 nmol while those with Hya-64 totaled 31 nmol, demonstrating that position 64 is approximately 33% β-hydroxylated, consistent with the 26% value reported by Fernlund and Stenflo (1983).

Carbohydrate compositional analysis showed approximately one residue each of NeuAc, Gal, Fuc, and GlcNAc per mole of the 57–65 peptide (Table I). Mass spectrometric analysis of the 57–65 peptide (Leu-Asn-Gly-Gly-Ser-CMCys-Lys-Asp-Asp) showed a total mass of 1768.8 amu (Figure 3); of this, 966.0 amu are due to the polypeptide, with an additional mass of 802.8 amu due to glycosylation of the peptide. The calculated mass of a tetrasaccharide containing one residue of each of the monosaccharides listed above is 802.7 amu, consistent with the observed mass of 802.8 amu. Other thermolytic fragments containing Ser-61 also showed this additional mass (Table II). None of the remaining thermolytic fragments of the 44–80 peptide (without Ser-61) had an unexpected mass.

Close examination of the mass spectrum of the 57–65 peptide showed minor forms resulting from the partial cleavage of the glycosidic linkages in the ion source of the mass spectrometer (Figure 3). For example, the ions with m/z 885.4 and 739.8 are doubly charged ions corresponding to the masses of the intact glycopeptide (M = 1768.8 amu) and the glycopeptide minus NeuAc (M = 1477.6 amu), respectively. The m/z 1112.5 and 966.4 ions are singly charged fragment ions attributable to the losses of NeuAc, Gal, and GlcNAc and NeuAc, Gal, GlcNAc, and Fuc, respectively. The m/z 1112.5 ion was consistent with the presence of the fucose residue at the reducing end of the tetrasaccharide. Thus, the mass spectrometric fragmentation pattern suggested a NeuAc-(Gal,GlcNAc)-Fuc-*Ser* structure. In addition, mass spectrometric analysis of the 57–68 peptide (with Hya-64) after neuraminidase digestion demonstrated the complete removal of the sialic acid, consistent with the presence of a terminal sialic acid residue (data not shown).

N-Terminal sequence analysis of the 57–65 peptide did not show any unusual PTH–amino acids or "blank" cycles (data not shown). The TFA cleavage steps in the Edman degradation apparently hydrolyzed the glycosidic linkage between the tetrasaccharide and the peptide, allowing nearly normal recovery of PTH-Ser and its β-elimination products during N-terminal sequence analysis. Labile glycosidic linkages have also been reported during N-terminal sequence analysis of fucosylserine (Bjoern et al., 1991) and fucosylthreonine (Harris et al., 1991).

To show that the tetrasaccharide was attached to Ser-61, the 57–65 peptide was incubated at high pH to cause β-elimination of the tetrasaccharide with concomitant conversion of the attachment Ser-61 residue to an acid-labile dehydroalanine residue. Amino acid analysis gave only 0.2 mol of Ser per mol of the base-treated 57–65 peptide, while 0.8 mol was recovered without base treatment. Identical high-pH treatment of an unrelated, unmodified peptide (residues 63–69 of IFN-γ; DDQSIQK) gave 0.8 mol of Ser, with 0.9 mol recovered without base treatment. As there was only one serine residue in the 57–65 peptide, these results demonstrated that the tetrasaccharide was attached to Ser-61.

Samples containing 1 nmol of either the 57–68 peptide of factor IX (with Hya-64) or the 441–449 glycopeptide of t-PA with mainly diantennary fucosylated complex-type structures

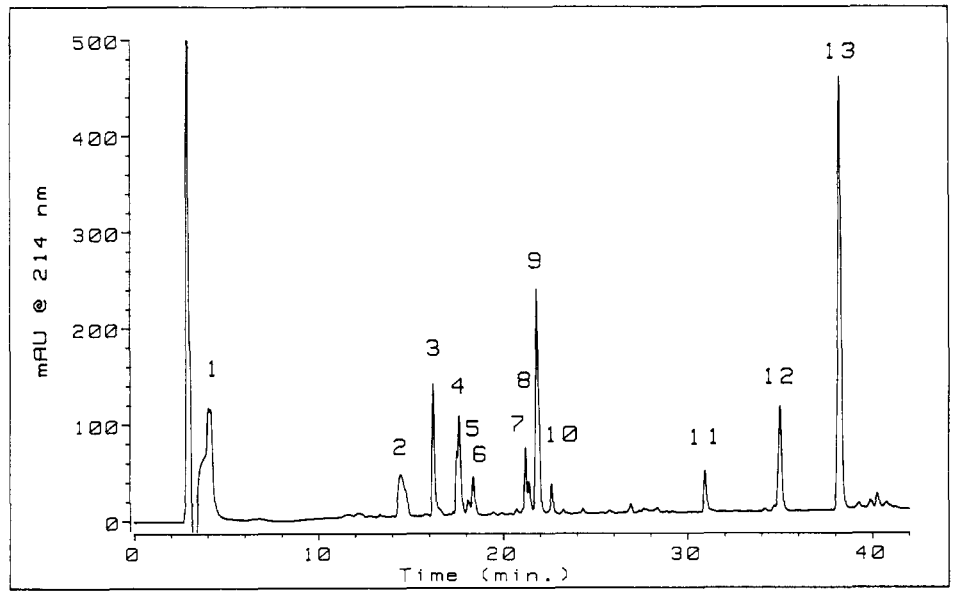

FIGURE 2: RP-HPLC purification of thermolytic fragments of the 44–80 peptide. Chromatographic conditions were the same as for Figure 1. Peptide identities are summarized in Table II.

Table II: Analysis of Thermolytic Fragments of the 44–80 Peptide[a]

peptide	HPLC peak	mass of peptide	observed mass	CHO mass	CHO structure
QY	2	309.1	nd[b]	nd	nd
VDGDQCESNPC	9	1281.5	1575.7	294.2	Xyl-Glc
			1708.2	426.7	Xyl-Xyl-Glc
LNGGSCKDD	4	966.0	1768.8	802.8	tetrasaccharide
LDGGSCKDD	5	967.0	1769.6	802.6	tetrasaccharide
LNGGSCKβDINS	7	1296.3	2098.2	801.9	tetrasaccharide
LNGGSCKDDINS	10	1280.3	2082.4	802.1	tetrasaccharide
YECWCP	13	915.9	915.4	none	none
FG	4	204.1	nd	nd	none
FEGK	3	479.5	479.2	none	none

[a] The 44–80 tryptic peptide sequence is QYVDGDQCESNP-CLNGGSCKDDINSYECWCPFGFEGK with either Asp or Hya (β) at position 64. HPLC peaks refer to Figure 2. Mass values are in amu. [b] nd: not determined.

attached to Asn-448 (Spellman et al., 1989) were digested with α-fucosidase. After RP-HPLC rechromatography was performed to desalt the samples, mass spectrometric analyses showed that the factor IX 57–68 glycopeptide was unaffected by α-fucosidase treatment, while the t-PA 441–449 glycopeptide mass was reduced by 146.2 amu, consistent with the loss of one fucose residue. The inability of α-fucosidase to remove fucose from the Ser-61 tetrasaccharide is consistent with the presence of an internal fucose residue.

Glycosyl Linkage Analysis. The 57–65 fraction that had been collected by C18 RP-HPLC (Figure 2) was rechromatographed using a C4 RP-HPLC column to resolve the coeluting Phe-Gly dipeptide and a minor amount of nonsialylated 57–65 peptide as shown in Figure 4. The oligosaccharide was released from the purified glycopeptide by automated hydrazinolysis and subjected to methylation analysis to determine the glycosyl linkage composition. The factor IX tetrasaccharide was found to contain one residue each of 3-linked prereduced fucose, 6-linked galactose, and 4-linked *N*-acetylglucosamine (Table III). Sialic acid is destroyed by the hydrolysis conditions used in methylation analysis and, therefore, was not detected directly by this analysis. The results were also consistent with a linear tetrasaccharide with fucose at the reducing terminus.

The detection of the 3-linked fucose in addition to the prereduced form of 3-linked fucose (Table III) was unexpected and suggests that a portion of the sample subjected to

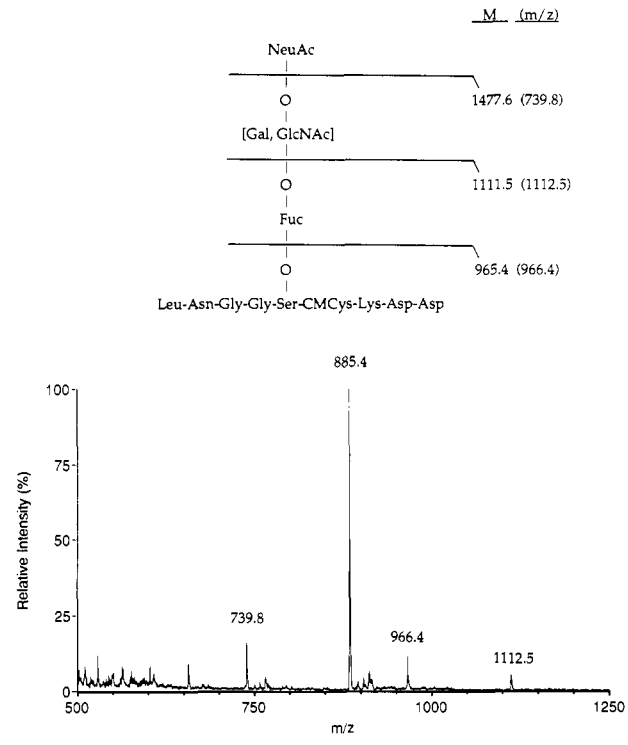

FIGURE 3: Electrospray mass spectrometric analysis of the 57–65 glycopeptide. Calculated masses and (observed ions) for the intact glycopeptide and fragments generated during the analysis are indicated. The sample mass (M) is derived from the formula $M = [(m/z)z - z]$, where m/z is the observed ion and z is the number of associated H^+. For example, the major observed ion at m/z 885.4 is the doubly protonated form of the glycopeptide with a mass of 1768.8 amu. See the Results section for the interpretation of the observed ions and the fragmentation pattern.

methylation analysis was not in the form of a reducing oligosaccharide. The 3-linked fucose could have arisen either from incomplete release of the oligosaccharide by hydrazinolysis or from incomplete hydrolysis of the hydrazide derivative prior to reduction and methylation. A small amount of nonreducing terminal galactose was also detected and presumably arose from desialylation of the oligosaccharide during sample preparation. Peak areas of 6-Gal and 4-GlcNAc from the Ser-61 tetrasaccharide were compared with those

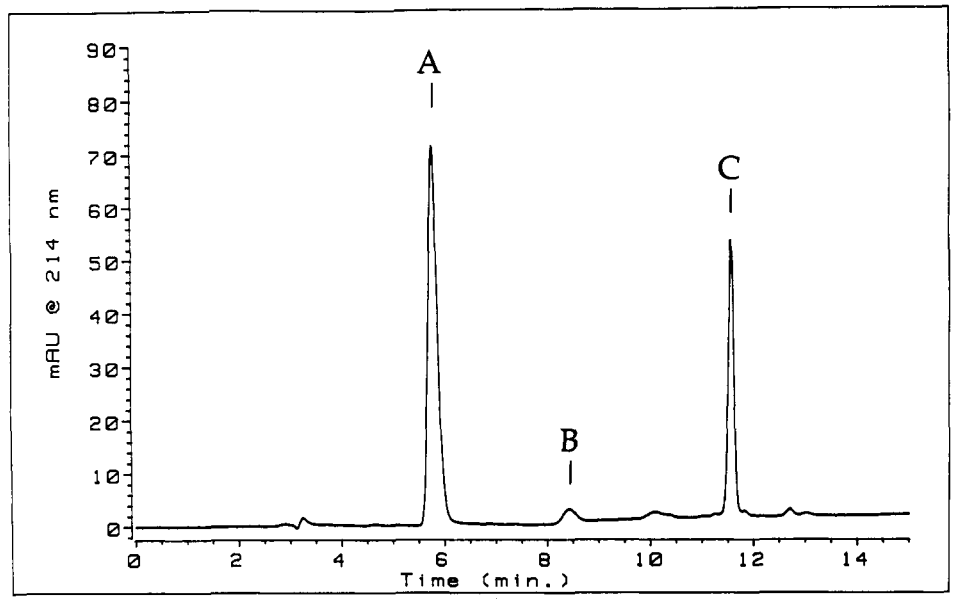

FIGURE 4: C4 RP-HPLC rechromatography of the 57–65 glycopeptide: (A) Phe-Gly dipeptide; (B) asialo 57–65 glycopeptide; (C) sialylated 57–65 glycopeptide.

Table III: Methylation Analysis of the Oligosaccharide Released from Ser-61

residue[a]	deduced glycosyl linkage	area %[b]
fucitol		
1,2,4,5-tetra-*O*-methyl (3-*O*-acetyl)	3-linked (PR)[c]	24
2,4-di-*O*-methyl (1,3,5-tri-*O*-acetyl)	3-linked	14
galactitol		
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	terminal	10
2,3,4-tri-*O*-methyl (1,5,6-tri-*O*-acetyl)	6-linked	30
2-(*N*-methylacetamido)-2-deoxyglucitol		
3,6-di-*O*-methyl (1,4,5-tri-*O*-acetyl)	4-linked	22

[a] Identifications are based on gas chromatographic retention times and on the electron-impact mass spectrometric fragmentation patterns. [b] Values are based on uncorrected flame-ionization detector responses. [c] PR: prereduced.

from the reference (LS-tetrasaccharide *c*) sample; this comparison indicated an overall recovery after hydrazinolysis and methylation for the Ser-61 tetrasaccharide of approximately 70% (data not shown).

NMR Spectroscopic Analyses. A sample of the 57–65 glycopeptide (Leu-Asn-Gly-Gly-Ser-CMCys-Lys-Asp-Asp) was lyophilized and exchanged with D_2O for one- and two-dimensional ^1H NMR analyses. The 1D ^1H NMR spectrum shows the subspectra of the tetrasaccharide and the nonapeptide moieties superimposed on one another (Figure 5). It was not possible to unequivocally assign the carbohydrate structural reporter groups (*i.e.*, the anomeric and deoxy protons; Van Halbeek, 1993a,b) from this relatively crowded 1D spectrum. Therefore, we resorted to the combination of 2D DQF-COSY and TOCSY for identification of the spin systems of the individual glycosyl and amino acid residues. A portion of the TOCSY spectrum of the factor IX glycopeptide is shown in Figure 6A. Typical subspectral features of glycosyl residues were observed in cross-sections through the three anomeric signals at δ 4.92, 4.71, and 4.43 and also through two mutually coupled CH_2 proton signals at δ 1.72 and 2.67 (not shown in Figure 6A), respectively. The chemical shifts of these and other assigned protons are compiled in Table IV.

These subspectra were attributed to the four constituent glycosyl residues (Table I) on the basis of their characteristic, different ring geometries. The signals at δ 1.72 and 2.67 were assigned to H3ax and H3eq of the NeuAc residue by virtue of their chemical shifts, their triplet and doublet-of-doublet patterns, their mutual (geminal) coupling, and the chemical shifts of the other protons in their spin system. The singlet at δ 2.03 is attributed to the NAc methyl protons of this residue. The chemical shifts of H3ax and H3eq, sensitive as they are to the microenvironment of the sialic acid residue, indicate that the NeuAc residue is $\alpha(2\rightarrow6)$-linked to a β-linked *N*-acetyllactosamine moiety (Vliegenthart et al., 1983). The doublet at δ 4.43 was assigned to H1 of the Gal$\beta(1\rightarrow4)$ residue within the sialyl-*N*-acetyllactosamine unit. Its coupling constant (J_{12} = 7.8 Hz) confirms the β-type of the Gal linkage. The positions of the Gal H2, H3, and H4 signals were identified from the TOCSY subspectrum through Gal H1 (see Figure 6A). The H1 signal at δ 4.71 (J_{12} = 8.0 Hz) was attributed to the β-GlcNAc residue. A similar position for GlcNAc H1 around δ 4.7 has been observed before in ^1H NMR spectra of mucin–glycoprotein-derived oligosaccharides (Van Halbeek, 1984), and of milk oligosaccharides (Grönberg et al., 1990) that contain a $\beta(1\rightarrow3)$-linked GlcNAc residue, and is known not to be significantly affected by the type of aglycon. The subspectrum of the GlcNAc residue was so unusually crowded that unambiguous assignment of GlcNAc H2 through H6 solely from the TOCSY spectrum was not possible. However, the NAc methyl protons of GlcNAc were found to resonate as a separate singlet at δ 2.07 (Figure 5). The remaining anomeric proton signal, at δ 4.92, was attributed to the Fuc residue. The chemical shift along with the coupling constant (J_{12} = 4.2 Hz) indicates that Fuc is involved in an O-glycosidic linkage with the α-anomeric configuration. The Fuc C6 methyl protons were found to resonate at δ 1.21 (Figure 5), while both the COSY and TOCSY spectra revealed the Fuc H5 signal to appear at δ 3.86. Two of the three remaining Fuc proton signals (namely, H2 and H3) were assigned from the DQF-COSY and TOCSY spectra (the latter is shown in Figure 6A). From the DQF-COSY and TOCSY spectra of the factor IX glycopeptide sample in D_2O, most of the amino acid residue spin systems were also located (see Table IV). Sequential assignment of the amino acid residues by NMR was not possible due to the impossibility of generating NOE

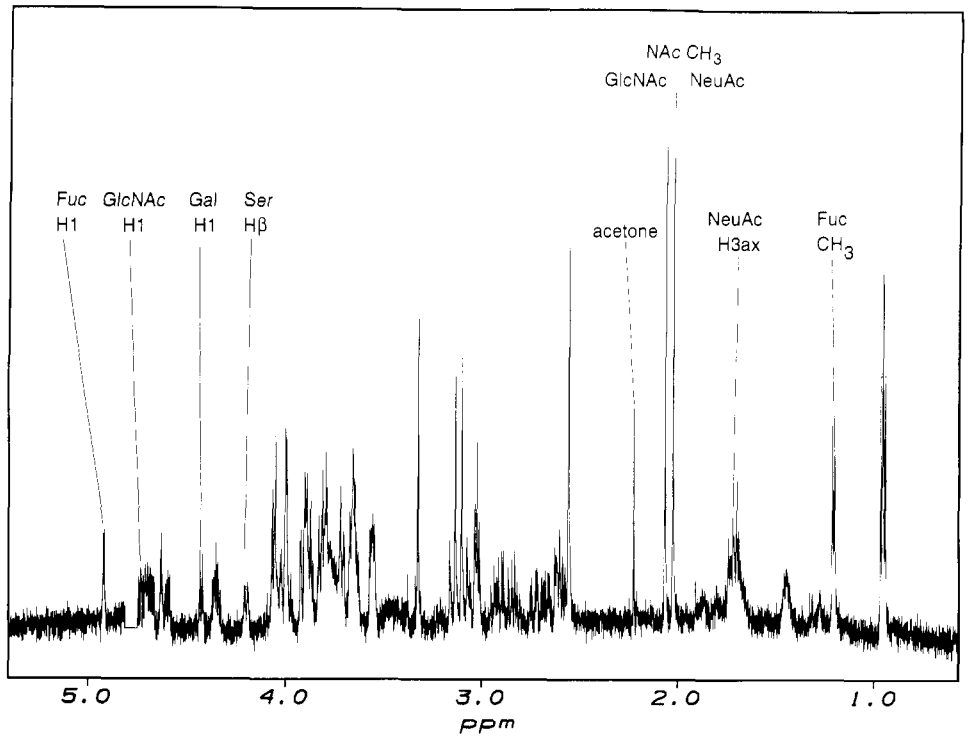

FIGURE 5: 1D ^{1}H NMR spectrum of the factor IX glycononapeptide (~20 nmol) in D_2O at pD 7.2, recorded at 600 MHz. The structural reporter group signals for the carbohydrate moiety are indicated.

data from the low-concentration sample of the glycopeptide in H_2O.

The sequence of the four glycosyl residues in the tetrasaccharide as well as the site of attachment to the peptide were successfully examined by a ROESY experiment in D_2O. The ROESY spectrum (Figure 6B) provided a number of intraglycosyl residue cross peaks that are compatible with the assignments of, for example, the triaxial spin system β-Gal H1, H3, and H5 (with δ H5 ~ 3.82), β-GlcNAc H1, H3 (δ 3.77), and H5 (δ 3.65), and α-Fuc H1 and H2 (at δ 3.90). The assignments for the GlcNAc protons listed in Table IV are in agreement with literature values for the NeuAcα(2→6)Galβ(1→4)GlcNAcβ(1→x) trisaccharide sequence as part of a diantennary milk oligosaccharide (x = 3; Grönberg et al., 1990) and for a diantennary N-type glycoprotein oligosaccharide (x = 2; Wieruszeski et al., 1989). This parallelism further supports the presence of this terminal trisaccharide element in the factor IX 57–65 glycopeptide. When one compares the data obtained for the Fuc ring protons in the factor IX glycopeptide with those published for terminal α-linked Fuc residues [*e.g.*, Vliegenthart et al. (1983)], the downfield shift of Fuc H3 (from δ ~3.85 to 4.06) is immediately obvious. This shift reflects the involvement of Fuc C3 in the glycosidic bond to GlcNAc. The chemical shift of Fuc H5 in the glycopeptide was unusual (δ 3.86); its upfield shift compared to typical values (ranging between δ 4.10 and 4.85) may be caused by the neighboring peptide fragment.

Key to the assignment of the sequence of glycosyl residues in the tetrasaccharide were the interglycosidic NOEs observed between GlcNAc H1 and the Fuc proton signal at δ 4.06 (assigned to H3) and between Fuc H1 and one of the β-protons (at δ 3.72) on Ser-61. The NOE between Gal H1 and GlcNAc H4 (at δ 3.65) overlapped with the intraresidue NOE between Gal H1 and Gal H3 (Figure 6B). The interresidue ROESY connectivities, in conjunction with the results from methylation analysis (Table III), allowed us to unambiguously establish the sequence of the tetrasaccharide. An additional ROESY cross peak, labeled f2 in Figure 6B, at {3.92, 4.71} is most likely indicative of a short distance (<4.5 Å) between GlcNAc H1 and Fuc H2.

In sum, the NMR data are compatible with the following structure: NeuAcα(2→6)Galβ(1→4)GlcNAcβ(1→3)-Fucα1→*O*-Ser[61].

DISCUSSION

Human protein C and factor VII have the consensus sequence required for the β-hydroxylation of Asp residues (Stenflo et al., 1987) in their N-terminal EGF domains; these also have Thr or Ser in the position equivalent to the O-fucosylation site in human urokinase and t-PA. Protein C was found to have β-hydroxyaspartate at residue 71 (Drakenberg et al., 1983) but is not O-fucosylated at Thr-68 (Harris et al., 1992). In contrast, factor VII has O-linked fucose at Ser-60 (Bjoern et al., 1991), but the potential β-hydroxylation site at Asp-63 is unmodified (Thim et al., 1988). These observations led us to propose that these two modifications are mutually exclusive. Human factor IX has the potential for both of these modifications as well (at Ser-61 and Asp-64, respectively), but is only partially β-hydroxylated at the latter site. If these two modifications were mutually exclusive, then we would have found two forms of factor IX, with either O-linked fucose at Ser-61 *or* β-hydroxyaspartate at residue 64.

An alternate explanation is that the confirmed O-linked fucose residues are found within *Gly*-Gly-Thr/Ser-Cys sequences, while the equivalent unmodified site of human protein C has the sequence *His*-Gly-Thr-Cys (Harris et al., 1992). If O-fucosylation were dependent on the presence of two Gly residues in the preceding sequence, then we would expect to find the Ser-61 of factor IX to be completely O-fucosylated, as it falls within the sequence *Gly*-Gly-Ser-Cys. Thus, either possibility could have been demonstrated by analysis of human factor IX.

No evidence for the presence of a simple O-linked fucose residue at Ser-61 was obtained. Instead, we found a novel,

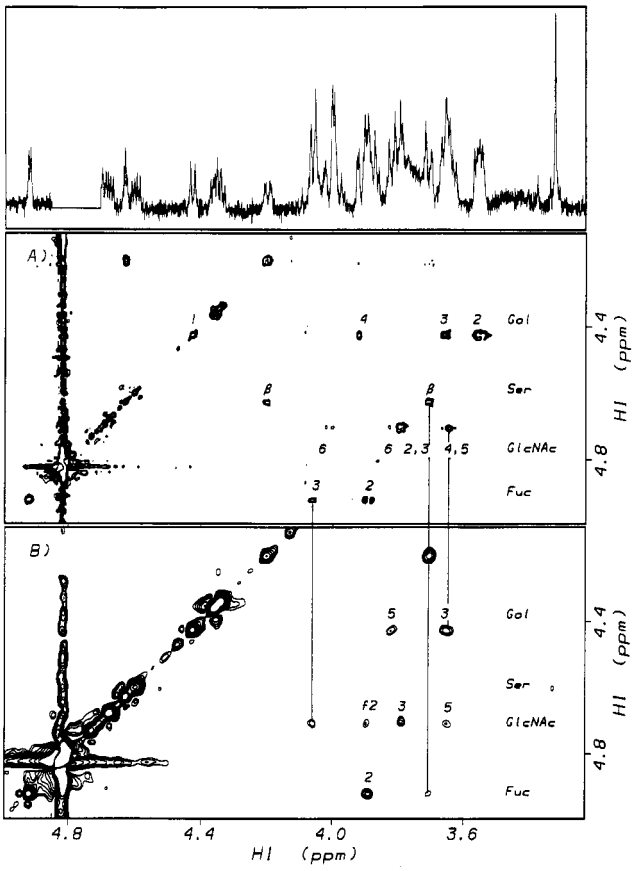

FIGURE 6: Regions of the 2D ^{1}H NMR spectra of factor IX glyconanopeptide in D_2O at pD 7.2, recorded at 600 MHz: (A) TOCSY spectrum (mixing time 108 ms); (B) ROESY spectrum (mixing time 200 ms). The occurrence of the structural entities Galβ(1→4)GlcNAc, GlcNAcβ(1→3)Fuc, and Fucα1→*O*-Ser is indicated by the vertical lines that connect ROESY and TOCSY cross peaks. The numbers in the spectra refer to the protons in the respective glycosyl residues.

Table IV: ^{1}H NMR Chemical Shifts for the Glycosyl Residues and for Pertinent Amino Acids Residues of the Factor IX 57–65 Glycopeptide[a]

	chemical shift						
residue	H1	H2	H3	H4	H5	H6	NAc
Fuc	4.92	3.90	4.06	nd[d]	3.86	1.21	
GlcNAc	4.71	3.79	3.77	3.65	3.65	3.82, 4.01	2.07
Gal	4.43	3.55	3.66	3.92	3.82	nd	
NeuAc			2.67, 1.72	3.65	3.81	3.71	2.03

	chemical shift					
	Hα	Hβ	Hβ′	Hγ	Hδ	Hε
Leu-57	4.04	1.73	1.73	0.95		
Asn-58[b]	4.60	3.04	2.93			
Gly-59[c]	3.22, nd					
Gly-60[c]	3.32, 3.39					
Ser-61	4.63	4.20	3.72			
CMCys-62	3.73	3.13	3.10			
Lys-63	4.34	1.88	1.80	1.45	1.69	3.02
Asp-64[b]	4.73	2.90	2.83			
Asp-65[b]	4.68	2.74	2.56			

[a] Chemical shifts are referenced to internal DSS; data were acquired in D_2O at 23 °C and pD 7.2. [b] Assignments for residues Asn-58, Asp-64, and Asp-65 are interchangeable. [c] Assignments for residues Gly-59 and Gly-60 are interchangeable. [d] nd: not determined.

unbranched tetrasaccharide with one residue each of NeuAc, Gal, GlcNAc, and Fuc. The fragmentation pattern observed during mass spectrometric analysis, the labile glycosidic linkage observed during N-terminal sequence analysis, and the resistance of the tetrasaccharide to α-fucosidase digestion all indicated that fucose was the attachment sugar residue. The tetrasaccharide composition and the Fuc–Ser^{61} linkage were also recently reported by Nishimura et al. (1992) by analyses of a different set of glycopeptides containing Ser-61.

Table V

protein	partial sequence										site
Proposed Consensus Sequences for O-Fucosidic Modification											
	C	X	X	G	G	T	C				
	C	X	X	G	G	S	C				
Sites of Confirmed O-Fucosidic Glycosylation (in bold type)											
t-PA	R	C	F	N	G	G	T	C	Q	Q	61
urokinase	D	C	L	N	G	G	T	C	V	S	18
factor VII	P	C	Q	N	G	G	S	C	K	D	60
factor XII	P	C	L	K	G	G	T	C	V	N	90
factor IX	P	C	L	N	G	G	S	C	K	D	61
Potential Sites for O-Fucosidic Glycosylation[a]											
Agrin[b] (rat) (1)	P	C	L	H	G	G	T	C	Q	D	1234
Agrin[b] (rat) (2)	P	C	L	N	G	G	S	C	V	P	1726
Cripto-1[c]	C	C	L	N	G	G	T	C	M	L	88
endothelial tyrosine kinase[d]	Q	C	Q	N	G	G	T	C	D	R	326
HSPG2/Perlecan[e]	P	C	L	H	G	G	T	C	Q	G	4158
LDL receptor-related[f] (1)	Q	C	F	N	G	G	S	C	F	L	4190
LDL receptor-related[f] (2)	H	C	R	N	G	G	T	C	A	A	4226
LDL receptor-related[f] (3)	H	C	S	N	G	G	S	C	T	M	4367
Neurocan[g]	P	C	E	N	G	G	T	C	I	D	1001
Versican[h]	P	C	L	N	G	G	T	C	Y	P	2116

[a] Proteins are human unless otherwise indicated; two sequences have multiple sites. References for these sequences are as follows. [b] Rupp et al., 1991. [c] Ciccodicola et al., 1989. [d] Partanen et al., 1992. [e] Murdoch et al., 1992. [f] Herz et al., 1988. [g] Rauch et al., 1992. [h] Zimmerman et al., 1989.

In the present study, methylation analysis of the released tetrasaccharide indicated glycosyl linkages of 3-Fuc, 6-Gal, and 4-GlcNAc. Identification of the anomeric signals of the glycosyl residues allowed us, by virtue of their chemical shifts and coupling constants, to determine the configurations of the glycosidic linkages. ROESY connectivities permitted us to establish the sequence of the tetrasaccharide as NeuAc→Gal→GlcNAc→Fuc→Ser. NMR chemical shifts of the anomeric and nonanomeric ring protons, as determined by the combination of DQF-COSY, TOCSY, and ROESY experiments, and the observed interglycosidic ROESY cross peaks suggested the structure of the tetrasaccharide to be NeuAcα(2→6)Galβ(1→4)GlcNAcβ(1→3)Fucα1→*O*-Ser^{61}. Unambiguous determination of the positions of the glycosidic linkages was possible by combining the NMR results with those from methylation analysis.

The O-fucosidic linkage at Ser-61 suggests that it is related to the O-linked fucose found at the equivalent positions of urokinase, t-PA, factor VII, and factor XII. This tetrasaccharide appears to be present regardless of the presence of β-hydroxyaspartate. Therefore, O-fucosyl modifications at such sites may depend solely on the presence of a Cys-X-X-Gly-Gly-Thr/Ser-Cys sequence within an EGF domain [also discussed by Nishimura et al. (1992)]. To date, these O-fucosyl modifications have all been found within EGF domains. If we assume that a Cys-X-X-Gly-Gly-Ser/Thr-Cys sequence within an EGF domain is sufficient, additional candidate proteins for O-fucosyl modifications include several cell surface receptors and proteoglycans (Table V). In addition, the sequence of the human notch protein (*TAN-1*) has 12 such sites (11 threonines and 1 serine) within its 35 EGF domains (Ellisen et al., 1991). Analysis of these and any other potential sites should help define the sequence requirements for O-fucosidic modification as well as any possible biological function.

Studies on the biological function of the first (N-terminal) EGF domain of factor IX have shown that a high-affinity calcium binding site is contained within EGF-1 that involves Asp-47, Asp-49, and Asp-64 (or Hya-64) residues [reviewed by Stenflo (1991)]. The Ser-61 tetrasaccharide is not required for calcium binding since a synthetic peptide (that lacks this modification) comprising EGF-1 of factor IX continues to bind calcium (Huang et al., 1989). The possible role for the EGF regions of factor IX in mediating endothelial cell binding was first supported by experiments demonstrating that synthetic peptides spanning EGF-1 could weakly inhibit the binding of radiolabeled bovine factor IX (Ryan et al., 1989). More recent experiments with factor VII/factor IX hybrid molecules localized the factor IX endothelial cell binding site to the amino terminal residues (in the Gla domain) of factor IX, indicating an important role for the Gla domain but not EGF-1 in endothelial cell binding (Toomey et al., 1992).

The presence of a sialylated structure in EGF-1of human factor IX is particularly intriguing in light of a study that demonstrated a loss of activity of factor IX or factor IXaα (factor IX cleaved at Arg^{180}–Val^{181}) upon neuraminidase digestion (Chavin & Weidner, 1984), although it is possible that factor IXaα in this study may have been degraded by a contaminant in the venom preparation. Studies with a hybrid factor IX (in which EGF-1 was replaced by the corresponding domain of factor X) showed that this molecule had nearly normal factor IX clotting activity in a partial thromboplastin time assay (Lin et al., 1990) and that its binding to a receptor on thrombin-activated platelets was unaffected (Ahmad et al., 1992), suggesting that EGF-1 of factor IX does not contain any unique features that cannot be replaced by EGF-1 of factor X (which lacks the Ser-61 tetrasaccharide). Studies with EGF-1 of bovine factor IX showed that it may be involved in factor IXa–factor X interactions (Astermark et al., 1992).

Unfortunately, many studies on the biological role of the EGF domains of factor IX have been performed with bovine factor IX, which has methionine (Katayama et al., 1979) instead of serine (Kurachi & Davie, 1982) at position 61 and thus cannot have this novel tetrasaccharide. Such bovine factor IX studies include those performed by Astermark et al. (1992) that had been cited by Nishimura et al. (1992) as a basis for their proposal that the Ser-61 tetrasaccharide is a ligand for the lectin-type sequence in the N-terminal region of factor VIII. Perhaps, instead, differences between the activities of the first EGF domains of human and bovine factor IX will be observed and will shed light on the biological role of the Ser-61 tetrasaccharide. Ser-61 is also replaced in canine and murine factor IX by valine and isoleucine, respectively (Evans et al., 1989; Wu et al., 1990), but is conserved in rabbit factor IX (Pendurthi et al., 1992). Rabbit factor IX has not yet been examined for O-fucosidic modification at the corresponding serine.

Residues 47–64 of factor IX are identical to residues 46–63 of factor VII except in three positions. The two proteins have xylosylglucose glycans at a conserved serine residue, and each one has structural domains in the order Gla–EGF–EGF–protease. Despite these similarities, factor VII has O-linked fucose at Ser-60 (Bjoern et al., 1991), while factor IX has a novel tetrasaccharide involving an O-fucosidic linkage at the corresponding position (Ser-61). The basis for this difference in glycosylation merits investigation.

NMR-based structural analyses of synthetic peptides comprising all or part of EGF-1 of factor IX did not assign any major role to Ser-61 (Huang et al., 1991; Baron et al., 1992). Ser-61 is opposite a Trp residue (Trp-72) within an antiparallel β-sheet structure, with no involvement of the side chain. Trp-72 is replaced by Gln, Asn, Phe, and Leu in the O-fucosylated EGF domains of t-PA, UK, factor VII, and factor XII, respectively. The Tyr/Phe residue of the β-hydroxylation consensus sequence lies directly opposite the aspartate hydroxylation site and may flag the hydroxylation modification in some way (Cooke et al., 1987); perhaps the Trp-72 residue that is opposite the Ser-61 of factor IX has a similar role.

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