Activation Peptide of Human Factor IX Has Oligosaccharides *O*-Glycosidically Linked to Threonine Residues at 159 and 169†

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Received November 30, 1993; Revised Manuscript Received February 23, 1994⊛

ABSTRACT: *O*-Linked oligosaccharide chains were identified in the activation peptide (AP) of human blood coagulation factor IX. The peptide obtained from human factor IX was separated into three molecular species (APα, APβ, and APγ) by reversed-phase high-performance liquid chromatography. Amino acid analysis showed that APα, but not APβ and APγ, contained galactosamine in addition to glucosamine, thereby suggesting the presence of an *O*-linked sugar chain(s) in the molecule of APα. A nonapeptide (APα-D4, residues 157–165) and an undecapeptide (APα-D5, 166–176) derived from APα contained Thr-159 and Thr-169, neither of which could be identified using a gas-phase protein sequencer. All other serine and threonine residues present in APα were identified by peptide sequencing. Component sugar and sialic acid analyses of APα-D4 and APα-D5 revealed that they contained 1 mol each of *N*-acetyl-D-galactosamine (GalNAc), D-galactose (Gal), and sialic acid. Fast atom bombardment tandem mass spectrometric analysis of APα-D4 suggested the existence of Gal-GalNAc-Thr, NeuNAc-(Gal-)GalNAc-Thr, and NeuNAc-Gal-GalNAc-Thr structures. On the basis of amino acid analysis after the isolation of APα, it accounted for approximately 35% of the total activation peptide obtained. From these results, it was concluded that a part of the activation peptide of human factor IX in circulating blood has tri- and tetrasaccharides *O*-glycosidically linked to the threonine residues at 159 and 169.

Factor IX is a vitamin K-dependent plasma glycoprotein that participates in the middle phase of the intrinsic pathway of the blood coagulation cascade (Davie et al., 1991). It is a single-chain serine protease zymogen with a molecular weight of 56 000. This protein is synthesized in the liver and undergoes several posttranslational modifications prior to secretion. These modifications include *N*-glycosylation, vitamin K-dependent γ-carboxylation of the NH_2-terminal glutamic acid residues (Furie & Furie, 1990), and β-hydroxylation of an aspartic acid residue in the first epidermal growth factor (EGF[1])-like domain (Stenflo, 1991). Human factor IX contained the activation peptide from positions Ala-146 to Arg-180. Human factor IX is activated by either factor XIa or the factor VIIa/tissue factor/phospholipid complex. The initial cleavage between Arg-145–Ala-146 yields the intermediate IXα, which is subsequently converted to the fully active form IXaβ followed by a second cleavage between Arg-180–Val-181. These two cleavage reactions result in the release of an activation peptide with a molecular weight of 10 000. The activation peptide contains two *N*-linked sugar chains at positions 157 and 167 (Kurachi & Davie, 1982; Suehiro et al., 1989).

We earlier identified a trisaccharide, $(Xyl)_2$-Glc, linked to a serine residue in the first EGF-like domain of factors VII (Ser-52) and IX (Ser-53) (Hase et al., 1988). The same trisaccharide was also found in protein Z (Nishimura et al., 1989) and platelet glycoprotein thrombospondin (Nishimura et al., 1992a). The complete structure of this trisaccharide found in human factor IX was determined to be Xyl*p*-α1–3-Xyl*p*-α1–3-Glc*p*-β1-*O*-Ser-53 (Hase et al., 1990). Bjoern et al. (1991) reported that human plasma factor VII, as well as recombinant human factor VII produced in baby hamster kidney cells, contains glucose *O*-linked to Ser-52 and fucose *O*-linked to Ser-60. We also reported another modification in the first EGF-like domain of human factor IX, which is a tetrasaccharide *O*-fucosidically linked to Ser-61 (Nishimura et al.,1992b), and the complete covalent structure has been determined (Harris et al., 1993; Kuraya et al., 1993).

During these studies, we also detected oligosaccharides *O*-glycosidically linked to threonine residues in the activation peptide released from human factor IX. This report describes the identification of these *O*-linked sugar chains.

MATERIALS AND METHODS

Materials. Human factor IX purified by immunoaffinity chromatography (Sugo et al., 1990) was kindly provided by the Chemosero-therapeutic Research Institute (Kumamoto, Japan). Bovine factor VII and tissue factor were purified as described elsewhere (Higashi et al., 1992). Endoproteinase Asp-N from the *Pseudomonas fragi* mutant and peptide-*N*-glycosidase F from *Flavobacterium meningoseptium* were obtained from Boehringer Mannheim. Neuraminidase from

† This work was supported by Grant-in-Aid for Scientific Research on Priority Areas No. 05274101 (S.I.) and Grant-in-Aid for Developmental Scientific Research No. 04558022 (Y.S.) from the Ministry of Education, Science and Culture of Japan.

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⊛ Abstract published in *Advance ACS Abstracts,* April 1, 1994.

[1] Abbreviations: EGF, epidermal growth factor; RP-HPLC, reversed-phase high-performance liquid chromatography; FAB, fast atom bombardment; MS/MS, tandem mass spectrometry; GalNAc, *N*-acetyl-D-galactose; Gal, D-galactose; NeuNAc, *N*-acetyl-D-neuraminic acid; Sia, sialic acid; Hex, hexose; HexNAc, *N*-acetylhexosamine; AP, activation peptide.

0006-2960/94/0433-5167$04.50/0

Streptococcus 6646K was obtained from Seikagaku Kogyo Co., Ltd. (Tokyo). β-Galactosidase from *Streptococcus 6646K* was kindly provided by Seikagaku Kogyo Co., Ltd. Columns (Cosmosil) for reversed-phase high-performance liquid chromatography (RP-HPLC) were obtained from Nakalai Tesque, Inc. (Kyoto). All other chemicals were analytical grade or of the highest quality commercially available.

Purification of the Activation Peptide. In the presence of a 10 molar excess of bovine tissue factor, purified human factor IX (14 mg) was activated with bovine factor VIIa (E/S = 1/200, w/w) in 50 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl and 25 mM $CaCl_2$ at 37 °C for 2 h. The resulting activation peptide and factor IXa were separated on a column of antifactor IX monoclonal antibody (Sugo et al., 1990) coupled to Sepharose CL-6B (5 mL of gel) equilibrated with 50 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl and 5 mM $CaCl_2$. Activation peptide in the flow-through fractions was lyophilized and then subjected to RP-HPLC using a Cosmosil 5C4-300 column (4.6 × 150 mm). Peptides were eluted with a linear gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid for 60 min at a flow rate of 0.5 mL/min. The effluent was monitored at 230 or 214 nm. Each peptide peak was further purified using a Cosmosil 5C18 column (8 × 250 mm), under the same conditions.

Treatment of Activation Peptide with N-Glycosidase F and Subdigestion with Endoproteinase Asp-N. One of the activation peptides, APα (24 nmol), was dissolved in 50 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl and digested with *N*-glycosidase F (0.5 unit/nmol) at 37 °C for 42 h. The resulting *N*-linked sugar-free peptide was separated by RP-HPLC on the Cosmosil 5C18 column. The peptide obtained was then digested with endoproteinase Asp-N (E/S = 1/400, mol/mol) at 37 °C for 17 h in 50 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl. The resulting peptides were further purified by RP-HPLC on the same column used above.

Amino Acid Analysis and Sequence Determination. The isolated peptides were lyophilized, hydrolyzed in 6 M HCl in tubes sealed under reduced pressure for 20 h at 110 °C, and then converted to phenylthiocarbamoyl derivatives. Amino acid analysis was performed by RP-HPLC using the Pico Tag system (Waters) (Heinrikson & Meredith, 1984). Amino acid sequence analysis of the purified peptides was performed with gas-phase protein sequencers (Models 473A and 477A, Applied Biosystems), with the chemicals and program supplied by the manufacturer.

Component Sugar and Sialic Acid Analyses. Component sugar analysis with 2-aminopyridine and quantification of sialic acid were performed as described elsewhere (Nishimura et al., 1992b).

Exoglycosidase Digestion. APα-D4 (2 nmol) was incubated with 50 munits of neuraminidase in sodium acetate (pH 6.5) containing 0.1 M $CaCl_2$ and separated by RP-HPLC using a Cosmosil 3C18 column. The asialoglycopeptide was further incubated with 10 munits of β-galactosidase in 0.05 M sodium acetate (pH 5.5) at 37 °C for 12 h and separated by RP-HPLC on the same column.

Fast Atom Bombardment Mass Spectrometry (FAB-MS) and Tandem Mass Spectrometry (FAB-MS/MS). Positive-ion FAB-MS and FAB-MS/MS spectra were obtained on a JMS-HX/HX 110A four-sector MS/MS spectrometer (Jeol) equipped with a FAB ion gun, collision cell, and array detector. Samples were placed on the tip of a stainless steel probe, mixed with a liquid matrix [a mixture of dithiothreitol and dithioerythritol, 5/1 (w/w)] and measured, as described previously (Takao et al., 1993). The MS/MS product ion spectra were acquired on an array detector with 10% mass dispersion by stepping up electric and magnetic fields and keeping the ratio of B2/E2 constant in MS2.

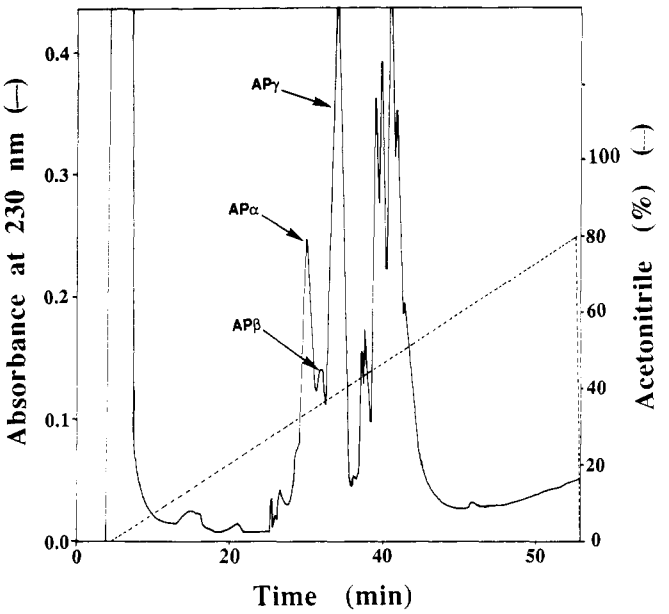

FIGURE 1: RP-HPLC of the activation peptide obtained from human factor IX. Activation peptide isolated from human factor IX was applied to a Cosmosil 5C4-300 column (4.6 × 150 mm), as described under Materials and Methods. Peaks indicated by an arrow were collected.

Table 1: Amino Acid Compositions of the Activation Peptides Derived from Human Factor IX

	residues/molecule[a]		
	APα	APβ	APγ
Asp	6.9 (7)	6.8 (7)	7.1 (7)
Glu	5.0 (5)	5.0 (5)	5.2 (5)
Ser	2.5 (3)	2.6 (3)	2.5 (3)
Arg	0.9 (1)		0.8 (1)
Thr	5.1 (6)	4.9 (5)	5.1 (6)
Ala	2.0 (2)	2.0 (2)	2.0 (2)
Pro	1.1 (1)	1.0 (1)	1.0 (1)
Tyr	0.9 (1)	0.8 (1)	0.9 (1)
Val	3.1 (3)	3.1 (3)	2.9 (3)
Ile	1.9 (2)	1.8 (2)	1.8 (2)
Leu	1.0 (1)	1.1 (1)	1.1 (1)
Phe	2.8 (3)	1.8 (2)	3.1 (3)
$GalNH_2$	+		
$GlcNH_2$	+	+	+
positions	146–180	146–177	146–180

[a] Values in parentheses and peptide positions in factor IX are referenced with the cDNA sequence data (McGraw et al., 1985; Yoshitake et al., 1985).

RESULTS

Isolation of the Activation Peptide. The activation peptide prepared by using an antifactor IX monoclonal antibody column, as described under Materials and Methods, was first applied to a Cosmosil 5C4-300 column. The activation peptide was separated into three peaks eluting at 31 (APα), 33 (APβ), and 34 min (APγ) on the column (Figure 1). A large cluster of several peaks that eluted after APγ contained no amino acids. The three peaks were then rechromatographed on a Cosmosil 5C18 column (data not shown). Amino acid (Table 1) and NH_2-terminal sequence (data not shown) analyses indicated that APα and APγ exactly corresponded to the

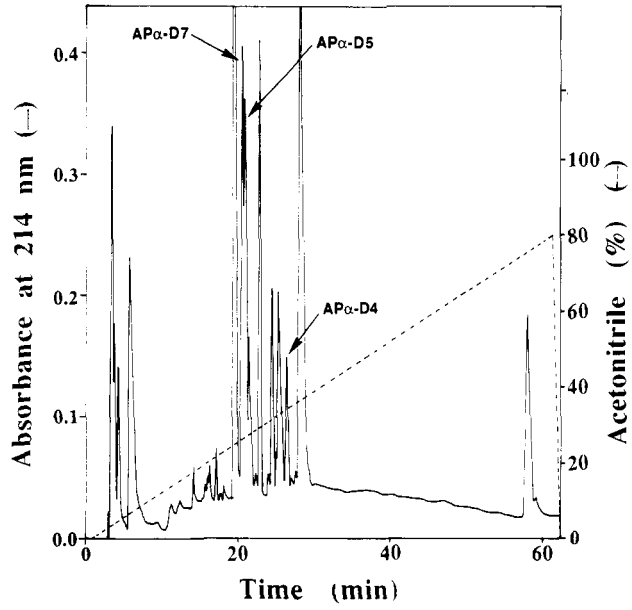

FIGURE 2: RP-HPLC of the endoproteinase Asp-N digest of APα. The digest with endoproteinase Asp-N was applied to a Cosmosil 3C18 column (4.6 × 100 mm) and eluted as described under Materials and Methods.

Table 2: Amino Acid Compositions of Glycopeptides Isolated from the Endoproteinase Asp-N Digest of APα

	residues/molecule[a]		
	APα-D4	APα-D5	APα-D7
Asp	1.1 (1)	3.0 (3)	2.1 (2)
Glu	2.1 (2)	2.0 (2)	2.0 (2)
Ser	0.9 (1)	1.8 (2)	1.8 (2)
Thr	2.2 (2)	2.0 (2)	2.1 (2)
Ala	1.0 (1)		
Ile	1.0 (1)	1.0 (1)	1.0 (1)
Leu	1.1 (1)		
Phe		1.1 (1)	1.1 (1)
positions	157–165	166–176	167–176

[a] Values in parentheses and peptide positions in factor IX are referenced with the cDNA sequence data (McGraw et al., 1985; Yoshitake et al., 1985).

Table 3: Sugar Compositions of APα-D4, APα-D5, and APα-D7

	residues/molecule[a]		
	APα-D4	APα-D5	APα-D7
GalNAc	0.8	0.9	0.9
Gal	1.1	1.2	1.1
sialic acid	1.0	1.0	1.0

[a] Sugar compositions were calculated from the data of component sugar and sialic acid analyses according to the methods of Takemoto et al. (1985) and Hara et al. (1987), respectively.

activation peptide of human factor IX (residues 146–180), while APβ lacked a few amino acid residues located in the COOH-terminal portion, probably Phe-Thr-Arg (178–180). On the basis of amino acid analysis, APα, APβ, and APγ, respectively, accounted for 35.4%, 14.4%, and 50.2% of the total activation peptide obtained. The amino acid analysis also indicated that APα contained galactosamine in addition to glucosamine; however, APβ and APγ contained only glucosamine (Table 1). These results suggested the presence of an O-linked sugar chain(s) in a molecular species of the activation peptide, APα.

Identification of the O-Linked Sugar Chain in APα. N-Linked sugar chains of APα were removed by peptide-N-glycosidase F and separated by RP-HPLC on a Cosmosil 5C18 column (data not shown). The removal of N-linked sugar chains was confirmed by component sugar analysis (data not shown). N-Linked sugar-free APα was further digested with endoproteinase Asp-N, and the resulting peptides were purified by RP-HPLC on a Cosmosil 3C18 column (Figure 2). Since the two asparagine residues (Asn-157 and Asn-167) with N-linked sugar chains, after the N-glycosidase F treatment, were converted to aspartic acids, both residues were now sensitive to endoproteinase Asp-N. From this digest, peptides APα-D1–APα-D8, which cover the entire sequence of the activation peptide, were all obtained (data not shown except for APα-D4, APα-D5, and APα-D7 in Table 2). Component sugar and sialic acid analyses indicated that three peptides, APα-D4 (residues 157–165), APα-D5 (166–176), and APα-D7 (167–176), contained 1 mol each of GalNAc, Gal, and sialic acid (Table 3). Amino acid sequence analyses for APα-D4 and APα-D5 indicated that no phenylthiohydantoin derivatives were extracted at sequencing cycles corresponding to the Thr-159 and Thr-169 positions (Table 4). On the other hand, the other serine and threonine residues contained in the activation peptide were all confirmed by sequence analysis of intact APα and peptides derived from the Asp-N digest (data not shown). These findings strongly suggested the existence of O-glycosidically linked oligosaccharides consisting of GalNAc, Gal, and sialic acid at the two threonine residues of 159 and 169.

Exoglycosidase Analysis. When APα-D4 was treated with neuraminidase, 95% sialic acid was removed. The asialoglycopeptide was further digested with β-galactosidase and purified by RP-HPLC. Component sugar analysis of the resulting peptide indicated that galactose was totally removed

Table 4: Amino Acid Sequence Analyses of APα-D4 and APα-D5

	PTH amino acid		yield (pmol)	
cycle no.	APα-D4	APα-D5	APα-D4	APα-D5
1	Asp	Asp	39	18
2	Ser	Asp	36	39
3	Thr	Ile	0	87
4	Glu	Thr	12	0
5	Ala	Gln	60	57
6	Glu	Ser	21	18
7	Thr	Thr	21	24
8	Ile	Gln	15	39
9	Leu	Ser	9	21
10		Phe		36
11		Asp		27
positions	157–165	166–176		
amount of peptide used (pmol)	104	110		

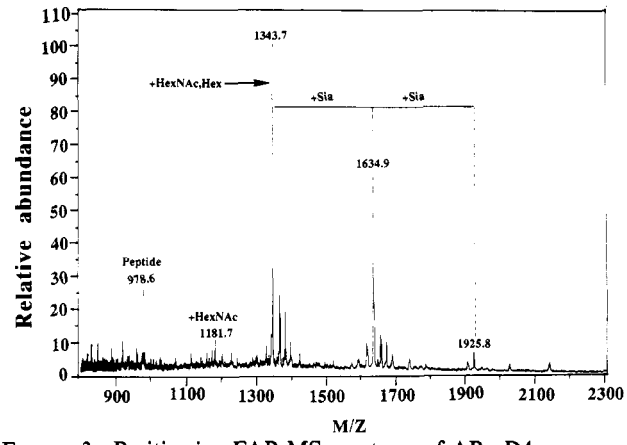

FIGURE 3: Positive-ion FAB-MS spectrum of APα-D4.

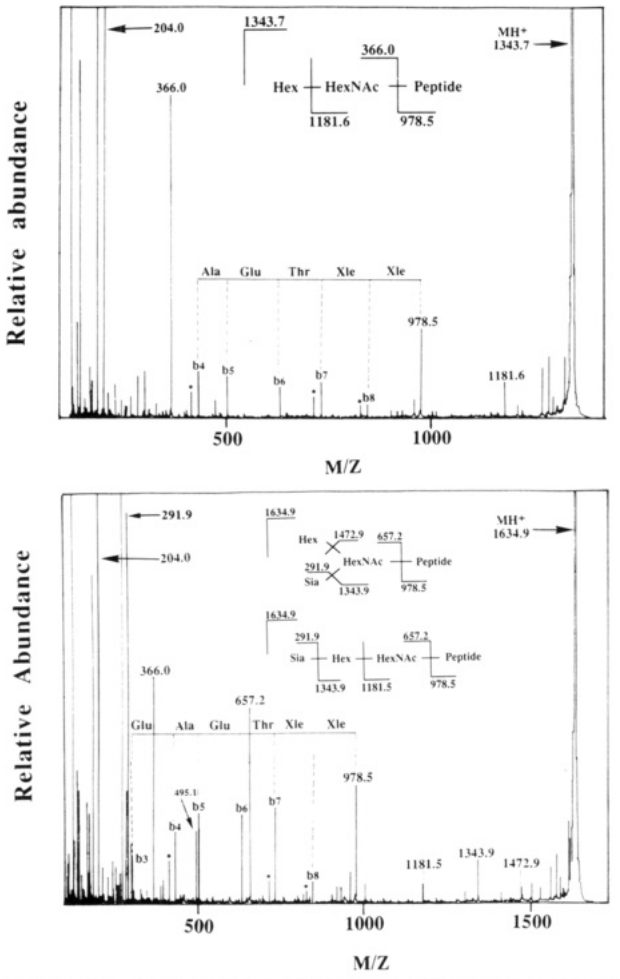

FIGURE 4: Positive-ion FAB-MS/MS spectra from m/z 1362.3 (A, top) and 1632.3 (B, bottom) observed for APα-D4 (Asp-Ser-Thr-Glu-Ala-Glu-Thr-Ile-Leu, 157–165) in Figure 3. The signals at 204.0 (A) and at 204.0, 366.0, and 495.1 (B) represent internal carbohydrate oxonium ions resulting from the consecutive fragmentation of two glycosidic bonds. The nomenclature of sequence ions for the peptide portion is as described elsewhere (Roepstorff & Fohlman, 1984). The asterisks correspond to the b18 ion fragmentation series (Johnson et al., 1987).

and 1.0 mol of GalNAc was quantified; hence, the reducing end of the oligosaccharide was likely to be GalNAc.

Mass Spectrometric Analysis. Figure 3 shows an FAB mass spectrum of APα-D4 (157–165). The mass spectrum is dominated by a series of ion clusters at m/z 1343.7, 1634.9, and 1925.8 with a consecutive mass difference of 291 Da, strongly suggesting of the presence of sialic acid (Sia). The residual mass (365.1 Da) obtained by subtracting the mass (978.6) of the peptide portion from the most abundant ion at 1343.7 is identical with the sum of the mass of *N*-acetylhexosamine (HexNAc) and hexose (Hex). In addition, the ions at m/z 1181.7 and 978.6 correspond to the subsequent losses of a Hex and a HexNAc plus Hex from m/z 1343.7, respectively, the latter of which is the MH^+ ion for the peptide. The results indicate the presence of the heterogeneous oligosaccharide structure at Thr-159 and are compatible with the sugar composition analysis of APα-D4 (Table 3); however, it is difficult to absolutely assess whether the apparent heterogeneity observed in the mass spectrum is due to the individual components or is derived from fragmentation during the ionization process.

The two major signals at m/z 1343.7 and 1634.9 were further subjected to FAB-MS/MS. Upon high-energy collisional activation decomposition of these MH^+ ions, abundant fragment ions due to the loss of the carbohydrate residues by glycosidic bond cleavage were observed, thereby suggesting sequential losses of carbohydrate from the glycopeptides: Hex-HexNAc-peptide for 1343.7 (Figure 4A) and Sia-(Hex-)-HexNAc-peptide or Sia-Hex-HexNAc-peptide for 1634.9 (Figure 4B). Moreover, carbohydrate oxonium ions were evident at low mass in both spectra, findings also indicative of the above oligosaccharide structure. In addition, the ions observed at 978.5 represent the deglycosylated peptide ion resulting from the loss of carbohydrate moieties, and the sequence ions formed by cleavage along the peptide backbone provided conformation of the partial amino acid sequence of the peptide portion.

As FAB mass spectrometric analyses of the other glycopeptides, APα-D5 (166–176) and APα-D7 (167–176), gave similar results (data not shown), it was suggested that the same oligosaccharide structure of Gal-GalNAc-Thr partially carrying one or two *N*-acetylneuraminic acids is attached at Thr-169

DISCUSSION

The activation peptide of factor IX prepared from the digest with factor VIIa in the presence of tissue factor was separated into three molecular species on RP-HPLC: APα, APβ, and APγ. APα accounted for one-third of the total activation peptide obtained, and it contained *O*-linked sugars. The component sugar analysis, including exoglycosidase digestion and FAB mass spectrometry of APα and APα-derived peptides, and its amino acid sequence analysis indicated that an oligosaccharide, Gal-GalNAc-, NeuNAc-(Gal-)GalNAc-, or NeuNAc-Gal-GalNAc-, is linked to Thr-159 and -169. On the other hand, APβ (14% of the total) and APγ (50%) had *N*-linked sugar chains, but not *O*-linked ones; however, APβ lacked the COOH-terminal portion. Therefore, the *O*-linked sugars found in the activation peptide are partial modifications. It could not be determined whether the threonine residues at 159 and 169 are partially *O*-glycosylated in the step of biosynthesis or whether the sugar chains are partially degraded (or released) during circulation in the blood.

Figure 5 shows an alignment of activation peptides derived from human, bovine, rabbit, canine, and mouse. The threonine residues at 159 and 169 are all conserved, except for the Ser-159 in bovine, although these modifications at the threonine residues have been confirmed only for human factor IX. The *O*-linked oligosaccharides of glycoproteins are usually clustered

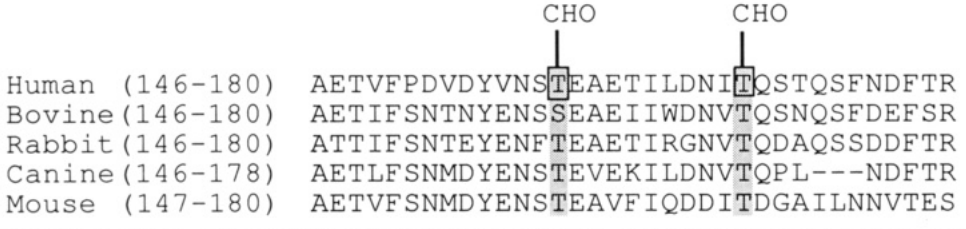

FIGURE 5: Alignment of activation peptides derived from human (Yoshitake et al., 1985), bovine (Katayama et al., 1979), rabbit (Pendurthi et al., 1992), canine (Evans et al., 1989), and mouse (Wu et al., 1990).

within heavily glycosylated regions of the peptide chain (Jentoft, 1990). Thus, it appears to be so additionally for the O-linked oligosaccharides of the activation peptide of factor IX. Jentoft also demonstrated that a major function of the O-glycosylation of glycoproteins is to induce a specific conformation, a stiff and extended conformation of the peptide core. Therefore, the O-linked sugar chains in the activation peptide may have a pertinent role in the induction of a specific conformation required for zymogen activation or for yet-to-be-determined biological activities of the peptide.

ACKNOWLEDGMENT

We are grateful to Chemosero-therapeutic Research Institute (Kumamoto) and Seikagaku Kogyo Co., Ltd., for providing human factor IX and β-galactosidase. We also thank Hiroko Hashimoto and Chika Yano for excellent technical assistance in amino acid and sequence analysis, Satomi Matsumura for secretarial assistance, and M. Ohara for helpful comments on the manuscript.

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