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ORIGINAL ARTICLE

Occurrence of O-linked Xyl-GlcNAc and Xyl-Glc disaccharides in trocarin, a factor Xa homolog from snake venom

J. S. JOSEPH,*† M. VALIYAVEETTIL,‡ D. C. GOWDA‡ and R. M. KINI§¶

*Departments of *Biochemistry, Faculty of Medicine, and §Biological Sciences, Faculty of Science, National University of Singapore, Singapore; †Genome Institute of Singapore, Singapore; ‡Department of Biochemistry & Molecular Biology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania; and ¶Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia, USA*

Summary. Trocarin is a 46515-Da group D prothrombin-activating glycoprotein from the venom of the Australian elapid, *Tropidechis carinatus*. Amino acid sequencing and functional characterization of trocarin demonstrated that it is a structural and functional homolog of mammalian blood coagulation factor (F)Xa. In this study we show that, in contrast to mammalian Xa, which is not glycosylated, trocarin contains an O-linked carbohydrate moiety in its light chain and an N-linked carbohydrate oligosaccharide in its heavy chain. Mass spectrometry and sugar compositional analysis indicate that the O-linked carbohydrate moiety is a mixture of Xyl-GlcNAc-, GlcNAc-, Xyl-Glc- and Glc- structures linked to Ser 52. The N-linked carbohydrate on Asn 45 of the heavy chain is a sialylated, diantennary oligosaccharide that is located at the lip of the active site of the prothrombin activator.

Keywords: blood coagulation, N-linked sialylated diantennary oligosaccharides, O-linked Xyl-GlcNAc, prothrombin activator glycoprotein.

Trocarin is a group D prothrombin activator [1] isolated from the venom of *Tropidechis carinatus*, the Australian rough-scaled snake [2]. Consistent with the characteristics of this unique family of prothrombin activators, trocarin proteolytically activates prothrombin to mature thrombin by the cleavage of two peptide bonds. This activity is markedly enhanced by the addition of Ca^{2+} ions, phospholipids and coagulation factor (F)Va [2]. Therefore, trocarin functionally resembles mammalian blood coagulation FXa. To understand the structural basis underlying this functional similarity between trocarin and FXa, we recently determined the complete amino acid sequence of the prothrombin activator. The data showed that trocarin shares high (~70%) homology and an identical domain structure to mammalian FXa [2]. Like FXa, the light chain of trocarin consists of an N-terminal domain rich in γ-carboxylated glutamate (Gla) residues, followed by two tandem EGF domains; the heavy chain is homologous to serine proteinases and contains the catalytic site. All residues in mammalian FXa essential for prothrombin activation are completely conserved in trocarin [2]. Thus, trocarin is a true structural and functional homolog of coagulation FXa. Interestingly however, trocarin is a glycoprotein, in contrast to mammalian FXa which is not glycosylated. Although the zymogen FX contains both N- and O-linked carbohydrate moieties [3], they are found on the peptide that is proteolytically cleaved off during its activation to FXa [4,5]. Based on the results of amino acid sequencing and mass spectrometry of peptides generated by enzymatic digestion, we had predicted earlier that both light and heavy chains of trocarin are glycosylated. The light chain was found to be modified with an O-linked carbohydrate moiety at Ser52 and the heavy chain with an N-linked oligosaccharide at Asn45 [2]. An O-linked glycosylation at a corresponding location in the light chain of coagulation FVIIa has been shown to have functional relevance (6,7; for details, see Discussion). The large N-linked oligosaccharide in trocarin is intriguingly located at the lip of the active site pocket, and suggests a role in the modulation of its enzymatic activity. Therefore, as a first step to understanding the role of glycosylation in trocarin, we studied the nature of the carbohydrate moieties on its light and heavy chains. We show that trocarin contains O-linked *N*-acetylglucosamine moieties. Modification of Ser/Thr residues in proteins with single *N*-acetylglucosamine moieties have thus far been found almost exclusively in cytoplasmic or nuclear proteins; to date, only in *Trypanosoma cruzi* cell surface glycoproteins has the single O-linked *N*-acetylglucosamine post-translational modification been described [8–13]. The N-acetylglucosaminyltransferase responsible for this modification in *T. cruzi* was recently isolated [14]. Here we report, in trocarin, the only other known occurrence of a secretory glycoprotein glycosylated in the Golgi with O-linked *N*-acetylglucosamine.

Correspondence: R. Manjunatha Kini, Department of Biological Sciences, Faculty of Science, National University of Singapore, Singapore 119260. Tel.: +65 68745235; fax: +65 67792486; e-mail: dbskinim@nus.edu.sg

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Experimental procedures

Materials

T. carinatus venom was obtained from Venom Supplies Pty Ltd (Tanunda, South Australia). Lysyl endopeptidase was purcha-

sed from Wako Pure Chemicals (Osaka, Japan), and 2-mercaptoethanol from Nacalai Tesque Inc. (Kyoto, Japan). Trypsin, pronase and 4-vinylpyridine were bought from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals and reagents were of the highest quality available.

Purification of prothrombin activators

Trocarin was purified from *T. carinatus* venom as described earlier [2]. Crude venom was successively fractionated by gel filtration on a Superdex 75 column (1.6 cm × 60 cm; Pharmacia), anion exchange chromatography on a Uno Q1 column (column volume 1 mL; Bio-Rad) and reversed-phase HPLC on a Jupiter C18 column (250 mm × 10 mm; Phenomenex). Homogeneity of trocarin was tested by electrospray ionization-mass spectrometry.

Enzymatic cleavage with lysyl endopeptidase (Lys C) and trypsin

To obtain carbohydrate-containing peptides of the light chain, the protein subunits of trocarin were separated by reduction and pyridylethylation, and the light chain digested with the endoproteinases, Lys C and trypsin. Typically, ~200 μg of protein was dissolved in 200 μL of 50 mmol L^{-1} Tris-HCl buffer, 4 mol L^{-1} urea, 5 mmol L^{-1} EDTA, pH 7.5. Endoproteinase Lys C (5 μg) or trypsin (5 μg) was added (enzyme/substrate ratio of 1 : 40 w/w) and the digestion carried out overnight at 37 °C. The peptides generated by enzymatic cleavage were separated by RP-HPLC on a Sephasil C18 (100 mm × 2.1 mm) column, using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA).

Isolation of N-linked carbohydrate by pronase digestion

The N-linked carbohydrate on the heavy chain of trocarin was isolated by pronase digestion followed by paper chromatography. The heavy chain (4.83 mg) was dissolved in 10 mM Tris-HCl pH 7.5. Pronase was added (1 : 25 w/w), the mixture incubated at 37 °C for 24 h and then lyophilized to dryness. The digest was dissolved in a small volume of water, spotted on a sheet of Whatman 5 paper and subjected to paper chromatography for 2 days using 1-butanol/ethanol/water (4 : 1 : 1 by volume) as the mobile phase. Oligosaccharides larger than trisaccharides remained at the origin and all degradation products (amino acids) from the pronase digest moved a fair distance on the paper. An area 0–5 cm from the origin was cut out and the carbohydrate recovered by elution with water.

Carbohydrate composition analysis

The sugar composition of the carbohydrates on the light and heavy chains of trocarin was analyzed by HPLC after acid hydrolysis as described previously [15]. To a 200-μL solution of HPLC-purified glycopeptide or intact trocarin light and heavy chains in water, 200 μL of 5 mol L^{-1} TFA was added and the solutions heated at 100 °C for 5 h. The hydrolysates were evaporated to dryness in a Speed-Vac and the residue dissolved in water and analyzed for neutral sugars and hexosamines. For hexosamine analysis, the glycopeptides or the glycoprotein chains were hydrolyzed in 4 mol L^{-1} HCl at 100 °C for 6 h. For the identification of sialic acid, samples were hydrolyzed in 0.05 mol L^{-1} sulfuric acid at 80 °C for 1 h, and then neutralized with 0.2 mol L^{-1} NaOH. Analyzes of the hydrolysates were performed with Dionex BioLC HPLC coupled to pulsed amperometric detection using a CarboPak PA1 high-pH anion-exchange column (4 mm × 250 mm). The eluents used were 20 mmol L^{-1} NaOH (for neutral sugars and hexosamines) and 100 mmol L^{-1} NaOH, 150 mM NaOAc (for sialic acids). Response factors for the monosaccharides were determined using standard sugar solutions.

Results

Recently, we determined the complete amino acid sequence of trocarin, a group D prothrombin activator from *T. carinatus* venom, by Edman degradation of overlapping peptides obtained by enzymatic and nonenzymatic digestion of its purified chains [2]. During sequencing of the light chain, amino acids at positions 6, 7, 14, 16, 19, 20, 25, 26, 29, 32, 35 and 52 were found to be modified. Subsequently, we determined that all the residues (6, 7, 14, 16, 19, 20, 25, 26, 29, 32, 35) except residue 52 were γ-carboxyglutamic acid (Gla) residues [2], as is the case in mammalian FXa. During preliminary characterization, we identified the presence of carbohydrate moieties in both light and heavy chains using the method of Dubois *et al*. [16]. Thus, we predicted that position 52 was glycosylated. Based on homology with other FXas, which have a Ser or Thr in position 52, we postulated that this amino acid in trocarin was modified with an O-linked carbohydrate moiety [2]. Furthermore, during sequencing of the heavy chain, the amino acid at position 45 was also found to be modified. Based on the presence of carbohydrate in the heavy chain, we suggested that residue 45 was also glycosylated. Since this residue occurs within the consensus sequence N-X-T for N-glycosylation, we concluded that the amino acid at position 45 of the heavy chain was Asn and it was substituted with an N-linked oligosaccharide. Based on the difference between the mass of the native protein and the mass calculated from its sequence, we estimated the size of the carbohydrate moiety to be ~3 kDa.

Glycosylation at Ser52 of the light chain of trocarin

Carbohydrate compositional analysis of the light chain of trocarin showed that it consisted mainly of the monosaccharides glucose, xylose, *N*-acetylglucosamine in molar ratios of 5.2 : 3.3 : 0.8 (Table 1). To generate shorter glycopeptides, the light chain was subjected to individual enzymatic digestions with Lys C (Fig. 1a) and trypsin (Fig. 1c). The reconstructed ESI mass spectrum of Lys C-generated peptide Lc37–62 containing residue 52 indicated four peaks with masses 3664.5, 3620.9, 3532.8 and 3489.1 (Fig. 1b). The species corresponding to a mass of 3489.1 was accounted for by the peptide with residue 52

Table 1 Carbohydrate compositional analysis (mole proportions) of the light and heavy chains of trocarin

Sugars	Light chain	Heavy chain	
		Glycoprotein*	Intact chain
Glucose	5.2	–	–
Xylose	3.3	–	–
Galactose	0.3	2.0	1.9
N-Acetylglucosamine†	0.8	4.0	4.2
N-Acetylgalactosamine‡	0.3	–	–
Mannose	–	2.8	3.0
N-Acetylneuraminic acid§	–	1.8	1.9

*Glycopeptide obtained by pronase digestion of the heavy chain of trocarin. †Identified as glucosamine after hydrolysis separately with 2.5 mol L^{-1} trifluoroacetic acid and 4 mol L^{-1} hydrochloric acid. ‡Identified as galactosamine after hydrolysis separately with 2.5 mol L^{-1} trifluoroacetic acid and 4 mol L^{-1} hydrochloric acid. §Analyzed after hydrolysis with 0.05 mol L^{-1} sulfuric acid.

being a Ser substituted with a hexose moiety. The peak with mass 3620.9 corresponded to the above glycopeptide with an additional pentose, hence modified with a pentose-hexose-disaccharide. Masses of 3532.8 and 3664.5 corresponded to the peptide substituted with *N*-acetylhexosamine- and *N*-acetylhexosamine-pentose-, respectively (Fig. 1b). The proportion of ions of masses 3532.8 and 3489.1, formed from glycopeptides that lack pentose were significantly greater than that expected based on the carbohydrate composition. This therefore suggests that the ions of mass 3532.8 and 3489.1 may be partly formed by the removal of pentose during mass spectrometry from the pentose-hexose- and the pentose-*N*-acetylhexosamine-(Ser) peptides. Taken together, the above results suggest that Ser52 of the light chain of trocarin is substituted either with a pentose-hexose and partly with single residues of hexose, or with pentose-*N*-acetylhexosamine and partly with single residues of *N*-acetylhexosamine. Mass spectrometric analysis of a shorter glycopeptide (Lc48–58) obtained by the tryptic digest of the light chain (Fig. 1c) supported the above conclusions. The reconstructed ESI mass spectrum of this peptide also yielded four characteristic ions with masses 1856.5, 1813.5, 1724.3, and 1681.1 (Fig. 1d). The ions with masses 1681.1 and 1813.5 corresponded to the hexose-(Ser) peptide and pentose-hexose-(Ser) peptide, respectively. The ions with masses 1724.3 and 1856.1 were assigned to the *N*-acetylhexosamine-(Ser) peptide and the pentose-*N*-acetylhexosamine-(Ser) peptide, respectively. Thus the results of compositional analysis and mass spectrometry demonstrate that Ser 52 of the light chain of trocarin is substituted with either the xylose-glucose disaccharide and single residues of glucose, or the xylose-*N*-acetylglucosamine disaccharide and single residues of *N*-acetylglucosamine. Further, substitution with xylose-glucose and glucose moieties predominates over that with xylose-*N*-acetylglucosamine and *N*-acetylglucosamine. In addition, small quantities of galactose and *N*-acetylgalactosamine were observed in the carbohydrate compositional analysis of the light chain (Table 1), indicating that Ser 52 is also substituted with either single residues of galactose and *N*-acetylgalactosamine and/or galactose-xylose and *N*-acetylgalactosamine-xylose disaccharides, albeit in a minor fraction of trocarin molecules. This suggests that the snake venom glycosylase may use glucose, *N*-acetylglucosamine and even galactose rather indiscriminately, in contrast to the mammalian enzyme which uses glucose strictly [17–19].

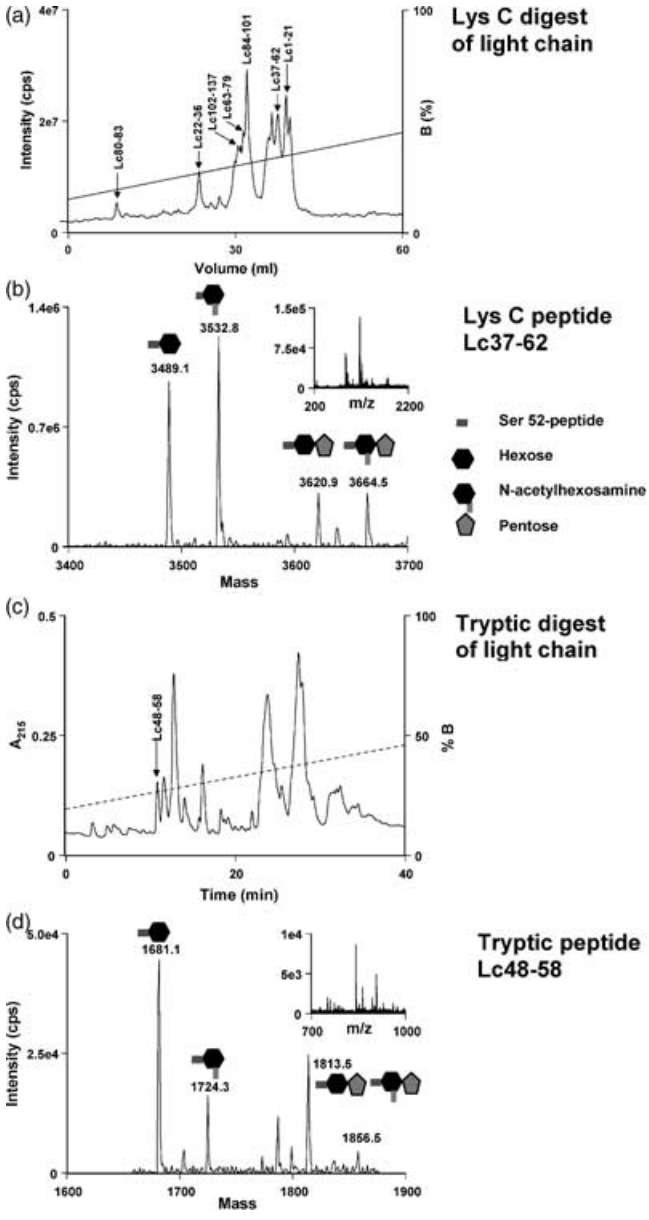

Fig. 1. (a) Endoproteinase Lys C digest of the light chain of trocarin. The glycopeptide is Lc37–62. (b) ESI-MS analysis of Lys C-generated glycopeptide Lc37–62. The reconstructed spectrum (main panel) of the raw spectrum (inset) of glycopeptide Lc37–62 shows peaks corresponding to peptides with Ser 52 O-glycosylated with either hexose-, pentose-hexose-, HexNAc- or HexNAc-pentose moieties. To obtain a shorter glycopeptide (Lc48–58), the light chain was digested with trypsin (c). ESI-MS of this peptide too showed a similar four-peak pattern corresponding to the above glycosylation variants (d).

No definite consensus sequences have been apparent for O-glycosylation of proteins. However, they are characteristically found in Pro/Ser/Thr-rich regions. Among all amino acids, Pro has the maximum preference around the O-glycosylation

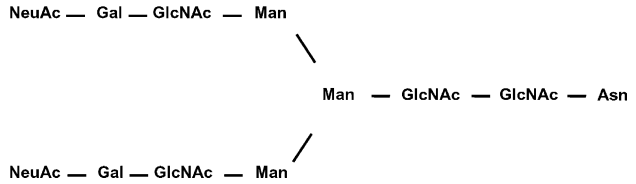

Fig. 2. Proposed structure of the di-sialylated biantennary structure N-linked to Asn 45 on the heavy chain of trocarin, based on the carbohydrate composition data (see text for details). This large carbohydrate (~2.2 kDa) sits at the lip of the catalytic site, just three residues away from active site His48.

site and Pro at +3 and/or −1 positions strongly favors glycosylation [20]. Interestingly, in trocarin, Ser 51 and Pro 54 (+3 position) are found in the immediate vicinity of Ser 52 (51-SSNP-54) [2]. Although Ser or Thr is found at position 52 in various mammalian FXas and its immediate sequence milieu in these proteins is similar to that of trocarin [2], the former are not glycosylated. Trocarin is the first FXa known to be glycosylated in its light chain.

Glycosylation at Asn45 of the trocarin heavy chain

As mentioned above, colorimetric analysis suggested the heavy chain of trocarin also contained carbohydrates. The carbohydrate moiety linked to a single amino acid or a short peptide was isolated by treatment of the trocarin heavy chain with pronase (which cleaves all or most peptide bonds), followed by paper chromatographic purification of the glycopeptide from the enzyme digest. This glycopeptide and the purified heavy chain of trocarin were separately subjected to sugar carbohydrate compositional analysis. In both cases, *N*-acetylneuraminic acid, galactose, *N*-acetylglucosamine and mannose were found in the ratios that corresponded to a commonly found diantennary complex N-linked oligosaccharide (Fig. 2).

Discussion

Trocarin, a group D prothrombin activator from *T. carinatus* snake venom, is structurally and functionally similar to mammalian coagulation FXa [2]. However, unlike mammalian FXa, trocarin is a glycoprotein; it has O-glycosylation at Ser52 of the light chain and N-glycosylation at Asn45 of the heavy chain.

O-linked N-*acetylglucosamine in secreted proteins*

We have shown that the carbohydrate moiety O-linked to Ser52 in the light chain of trocarin is a mixture of Xyl-GlcNAc-, GlcNAc-, Xyl-Glc- and Glc- structures (see above). Since trocarin is a secretory protein, it is likely that these carbohydrate moieties are the result of protein glycosylation in the Golgi, presumably by a novel *N*-acetylglucosaminyltransferase and a xylosyltransferase. The occurrence of single residues of O-linked *N*-acetylglucosamine in this secreted protein is of special interest. Thus far substitution of Ser/Thr of proteins with single residues of single of *N*-acetylglucosamine has been found only in cytoplasmic or nuclear proteins, and only in *T. cruzi*, on cell surface glycoproteins [8,9; for recent reviews, see 10–14]. In the case of cytoplasmic or nuclear proteins, single residues of O-linked *N*-acetylglucosamine have been found to occur exclusively in the cytoplasm through the action of a distinct cytoplasmic *N*-acetylglucosaminyltransferase [21–24]. Recently, a novel O-α-GlcNAc-transferase was isolated and studied in *T. cruzi*, which is capable of modifying Ser/Thr residues in cell surface glycoproteins with *N*-acetylglucosamine [14]. Apart from this known occurrence of extracellular proteins O-linked with *N*-acetylglucosamine in *T. cruzi*, trocarin is the only other known extracellular protein, and the first secreted protein with this modification. It is intriguing to note that most of the nuclear and cytoplasmic proteins modified by O-linked *N*-acetylglucosamine are present in macromolecular complexes whose assembly may be regulated [11]. Therefore, it will be interesting to study whether the O-linked *N*-acetylglucosamine in trocarin is involved in the assembly of the prothrombinase complex.

Glycosylation in vitamin K-dependent blood coagulation factors

Post-translational modification is well known in the vitamin K-dependent blood coagulation factors [25,26]. Coagulation FII, FVII, FIX and FX and proteins C, S and Z all have a domain rich in γ-carboxylated glutamate (Gla) residues. They also have one or more O- or N-linked glycosylation sites [for examples, see 4,17–19]. While the Gla residues are known to be involved in Ca^{2+}-dependent membrane binding [27], the role of the carbohydrate moieties in these proteins is largely unknown. Functional data is so far available only on the carbohydrate moieties in FX. These carbohydrates have been shown to be important for the activation of zymogen FX by both intrinsic and extrinsic tenase complexes [5,28]. However, though the zymogen FX is a glycoprotein (see [3], for structures of carbohydrate moieties on bovine FX), all carbohydrate-bearing peptides are only found on the peptide segment that is proteolytically cleaved off during activation [4,5].

The N- and O-linked carbohydrate moieties of many glycoproteins are known to perform as informational signals in cellular and molecular recognition and hence play crucial roles in embryogenesis, development, differentiation, immune regulation and cell–cell and cell–matrix interaction pathogenesis including atherosclerosis, rheumatoid arthritis and genetic disorders [for reviews, see 29,30]. The carbohydrate moieties of glycoproteins are also involved in the stabilization of protein conformation, the enhancement of their thermal stability, and protection from proteolysis [31–34]. The importance of glycosylation in trocarin with respect to protection from proteolysis and thermal stability has not yet been determined. However, the presence of a large *N*-glycan moiety in the heavy chain of trocarin is intriguing. Glycosylated Asn45 is only three residues away from active site His48, and the carbohydrate is very likely located at the lip of the active site. It would be interesting to determine the effect that the carbohydrate moiety at this position has on the catalytic activity of trocarin.

The presence of both O- and N-linked carbohydrates makes trocarin the first FXa protein known to be glycosylated. FXa

from chicken (isolated from embryo allantoic fluid) has a potential N-glycosylation site at a homologous position [35]; however, currently there is no evidence that this site is glycosylated. As mentioned earlier, although mammalian FX is glycosylated, its carbohydrate moieties are present exclusively in the activation peptide [4,5] and its light chain is not glycosylated. However, O-linked disaccharide (-Glc-Xyl) or trisaccharide [-Glc-$(Xyl)_2$] units are present in the corresponding Ser residues of the light chains of other coagulation factors such as human and bovine FVII and FIX and human protein Z [17–19]. Efforts by other investigators to understand the role of glycosylation at this position have not yielded conclusive results. Bjoern *et al*. [6] and Iino *et al*. [7] constructed the site-specific recombinant mutant of human FVII, in which Ser52 was replaced by Ala. The protein expressed by this construct exhibited lower clotting activity (∼60% that of wild-type FVIIa) but similar amidolytic activity to the wild-type FVII; however, the mechanistic basis for these observation is controversial. Based on kinetic studies, Bjoern *et al*. [6] reported that the carbohydrate moiety O-linked to Ser52 was not involved in the interaction of FVIIa with the tissue factor. Therefore, the lower activity of the mutant protein appears to be due to its enhanced ability to associate with tissue factor pathway inhibitor in plasma. On the other hand, the kinetic data by Iino *et al*. [7] indicate that the glycosyl moiety may provide unique structural elements required for the rapid association of FVII/VIIa with its cellular receptor and cofactor tissue factor. In any case, it appears that the O-linked carbohydrate moiety at Ser52 of the light chain plays a significant role in FVII/VIIa activity. It would be interesting to determine how the disaccharide moiety in trocarin modulates its enzymatic activity.

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