Introduction

Collagens in the vascular subendothelium and vessel wall are important platelet activators that have a critical role in hemostasis. The major receptors for collagens on platelets have been identified as the integrin \( \alpha_2\beta_1 \) and the immunoglobulin (Ig) super-family member GPVI. \( \alpha_2\beta_1 \) has been well characterized and is considered to be responsible for platelet adhesion to collagen rather than platelet activation for which GPVI is largely responsible (1-4). GPVI is a member of the Ig superfamily and structurally very similar to Fc\( \gamma \)R and to some of the natural killer receptor family (3). It forms a complex with the Fc receptor \( \gamma \) chain (Fc\( \gamma \)), which is critical not only for the signaling process, but also for the normal expression of this receptor in mice (4). The mechanism of signal transduction via platelet GPVI has been intensively investigated over the past few years using var-

Stejnulxin, a novel snake C-type lectin-like protein from Trimeresurus stejnegeri venom is a potent platelet agonist acting specifically via GPVI

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Summary

Stejnulxin, a novel snake C-type lectin-like protein with potent platelet activating activity, was purified and characterized from Trimeresurus stejnegeri venom. Under non-reducing conditions, it migrated on a SDS-polyacrylamide gel with an apparent molecular mass of 120 kDa. On reduction, it separated into three polypeptide subunits with apparent molecular masses of 16 kDa (\( \alpha \)), 20 kDa (\( \beta_1 \)) and 22 kDa (\( \beta_2 \)), respectively. The complete amino acid sequences of its subunits were deduced from cloned cDNAs. The N-terminal sequencing and cDNA cloning indicated that \( \beta_1 \) and \( \beta_2 \) subunits of stejnulxin have identical amino acid sequences and each contains two N-glycosylation sites. Accordingly, the molecular mass difference between \( \beta_1 \) and \( \beta_2 \) is caused by glycosylation heterogeneity. The subunit amino acid sequences of stejnulxin are similar to those of convulxin, with sequence identities of 52.6\% and 66.4\% for the \( \alpha \) and \( \beta \), respectively. Stejnulxin induced human platelet aggregation in a dose-dependent manner. Antibodies against \( \alpha_{IIb}\beta_3 \) inhibited the aggregation response to stejnulxin, indicating that activation of \( \alpha_{IIb}\beta_3 \) and binding of fibrinogen are involved in stejnulxin-induced platelet aggregation. Antibodies against GPIb\( \alpha \) or \( \alpha_2\beta_1 \) as well as echicetin or rhodocetin had no significant effect on stejnulxin-induced platelet aggregation. However, platelet activation induced by stejnulxin was blocked by anti-GPVI antibodies. In addition, stejnulxin induced a tyrosine phosphorylation profile in platelets that resembled that produced by convulxin. Biotinylated stejnulxin bound specifically to platelet membrane GPVI.

Keywords

Stejnulxin, platelet aggregation, GPVI, tyrosine phosphorylation

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ious GPVI ligands. All of these ligands stimulate a tyrosine phosphorylation pattern resembling that induced by collagen in platelets (5-8).

Snake C-type lectin-like proteins have been widely used as tools to investigate platelet receptors (9-12). Some inhibit the function of platelets by preventing ligand binding; others activate platelets either directly via receptors or indirectly using plasma proteins. Most of these proteins are simple αβ-heterodimers linked by a single interchain disulfide bond, with molecular masses around 30 kDa. Several higher molecular mass multimers of venom C-type lectin-like proteins have also been reported (13-18). These proteins have native molecular masses of 50-150 kDa, suggesting that they may represent the dimeric, trimeric or tetrameric forms, respectively, of common 30 kDa αβ-heterodimers. Convulxin, from the South American rattlesnake Crotalus durissus terrificus, is a 72 kDa protein that might have an (αβ)3 structure (15). On the other hand, flavocetin-A, from the venom of Trimeresurus flavoviridis, with a native molecular mass of 139 kDa, is a novel cyclic tetramer (αβ)4 stabilized by interchain disulfide bonds between heterodimers (19).

So far, several snake C-type lectin-like proteins that target GPVI have been characterized. Among them, alboaggregin-A (20, 21) and alboluxin (17), isolated from Trimeresurus albolabris venom, act via both GPIIb and GPVI. Convulxin was the first snake venom protein identified that acts via GPVI and has been widely used for the isolation, characterization, and investigation of signaling mechanisms of GPVI (6, 7). Ophioluxin, a convulxin-like platelet activator isolated from king cobra Ophiophagus hannah venom, probably only binds to GPVI and induces platelet aggregation (18). However, the binding sites on GPVI for collagen or the snake venom proteins such as convulxin are still unknown, as well as whether the convulxin class has only one type of binding site on GPVI. A better knowledge of these points will be important for understanding why convulxin and similar snake C-type lectins are such powerful platelet agonists as well as to investigate the mechanism of activation of platelets by collagen through GPVI. Additional GPVI ligands from snake venoms may help to elucidate these mechanisms (18).

Here, we describe the isolation, characterization, molecular cloning and interactions with platelet receptors of a novel platelet agonist called stejnulxin, which belongs to snake C-type lectin family, from Chinese green tree viper Trimeresurus stejnegeri. We present evidence for its specificity for GPVI and compare its properties with those of convulxin.

**Materials and methods**

Lyophilized T. stejnegeri venom was prepared by the Kunming Institute of Zoology, (Chinese Academy of Sciences). Protein A-Sepharose, Schiiff’s reagent, periodic acid, phosphatase-labelled streptavidin, peroxidase-conjugated goat anti-mouse, anti-rabbit antibodies, and bovine serum albumin (BSA) were from Sigma. Sephadex G-100 (superfine) and a preparative Mono-Q column (16/10) were from Amersham Biotech (Uppsala, Sweden). PVDF membranes were PolyScreen from DuPont NEN (Zaventem, Belgium). The SuperSignal chemiluminescence detection systems were from Pierce (Socochim, Lausanne, Switzerland). Anti-phosphotyrosine monoclonal antibody (mAb) 4G10 was from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-GPIIX, integrin α5 and β1 mAbs were from BD PharMingen (San Diego, CA). EMD 13233, an αGβ3 inhibitor, was a kind gift from Merck (Darmstadt, Germany). Anti-CD36 rabbit polyclonal antibodies were a kind gift from Dr. Beat Steiner. Anti-GPLIbα, anti-I domain polyclonal antibodies, anti-GPVI rabbit polyclonal antibodies and Fab fragments of anti-GPVI polyclonal antibodies were prepared as previously described (3, 6, 18). Echicetin, rhodocetin, convulxin and alboaggregin-A were purified from lyophilized Echis carinatus sochureki venom (Latoxan, Rosans, France), Calloselasma rhodostoma venom (CV Herpafauna, Indonesia), C. durissus terrificus venom (Sigma) and T. albolabris venom (Latoxan), respectively, as described previously (6, 13, 22, 23). The protein concentration of the final product was determined by a protein assay kit (Bio-Rad, Hercules, CA) with BSA as a standard.

**SDS-Polyacrylamide gel electrophoresis and Periodic acid-Schiff’s staining**

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (24). The gels were stained with Coomassie blue R-250 (Sigma). Reduced and non-reduced SDS-PAGE was carried out on 20% and 7.5% gels, respectively. Low molecular weight markers used were: bovine albumin, 66 kDa; egg albumin, 45 kDa; rabbit glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; bovine carbonic anhydrase, 29 kDa; bovine trypsinogen, 24 kDa; soybean trypsin inhibitor, was a kind gift from Merck (Darmstadt, Germany). Reduced and non-reduced SDS-PAGE was carried out on 20% and 7.5% gels, respectively. Low molecular weight markers used were: bovine albumin, 66 kDa; egg albumin, 45 kDa; rabbit glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; bovine carbonic anhydrase, 29 kDa; bovine trypsinogen, 24 KDa; soybean trypsin inhibitor, was a kind gift from Merck (Darmstadt, Germany). Reduced and non-reduced SDS-PAGE was carried out on 20% and 7.5% gels, respectively. Low molecular weight markers used were: bovine albumin, 66 kDa; egg albumin, 45 kDa; rabbit glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; bovine carbonic anhydrase, 29 kDa; bovine trypsinogen, 24 KDa; soybean trypsin inhibitor, was a kind gift from Merck (Darmstadt, Germany). Reduced and non-reduced SDS-PAGE was carried out on 20% and 7.5% gels, respectively. Low molecular weight markers used were: bovine albumin, 66 kDa; egg albumin, 45 kDa; rabbit glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; bovine carbonic anhydrase, 29 kDa; bovine trypsinogen, 24 KDa; soybean trypsin inhibitor, was a kind gift from Merck (Darmstadt, Germany). Reduced and non-reduced SDS-PAGE was carried out on 20% and 7.5% gels, respectively. Low molecular weight markers used were: bovine albumin, 66 kDa; egg albumin, 45 kDa; rabbit glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; bovine carbonic anhydrase, 29 kDa; bovine trypsinogen, 24 KDa; soybean trypsin inhibitor, was a kind gift from Merck (Darmstadt, Germany).
Preparation of washed platelets, platelet aggregation and inhibition
The methods used were mainly described previously (17). Briefly, human platelet rich plasma (PRP) and the platelet pellet were isolated by successive centrifugation steps. Platelets were resuspended in 113 mM NaCl, 4.3 mM K2HPO4, 24.4 mM NaH2PO4, 5.5 mM glucose, pH 6.5 (buffer B) and centrifuged at 250 × g for 5 min. The platelet rich supernatant was centrifuged at 1000 × g for 10 min, and the platelets were washed once more with buffer B. Washed platelets were resuspended in 20 mM Hepes, 140 mM NaCl, 4 mM KCl, 5.5 mM glucose, pH 7.4 (buffer C). The platelet count was adjusted to 5 × 10^8 platelets/ml for both PRP and washed platelets. Platelets were preincubated in buffer containing 2 mM CaCl2 and 2 mM MgCl2 at 37 °C for 2 min before starting the measurement by adding the samples for analysis. Platelet aggregation was monitored by light transmission in an aggregometer (Lumitec, France) with continuous stirring at 1100 rpm at 37 °C.

Measurement of time range of tyrosine phosphorylation
The methods were described previously (17). Platelets were treated as for aggregation. Aliquots (45 µl) were taken at fixed times and the platelet suspension was lysed by adding the aliquots to 5 µl of Hepes containing 10% SDS, 10 mM NEM, 20 mM Na2VO4, and 20 mM EDTA. After centrifugation, the supernatants were run on a 7-17% gradient SDS-PAGE gel and then electroblotted onto a PVDF membrane. The mAb 4G10 was used to detect tyrosine phosphorylated proteins followed by peroxidase-coupled rabbit-anti-mouse second antibody. Bound antibody was detected using chemiluminescence.

Isolation of platelet proteins with biotinylated stejnulxin and avidin-sepharose
The experiments were carried out using previously described methods (17) with minor modifications. Stejnulxin was dissolved in 0.9% NaCl and diluted to 125 µg/ml in 100 mM NaHCO3 solution (pH 8.5). Then 30 µg NHS-LC-biotin dissolved in DMSO was added to the stejnulxin solution. The mixture was incubated at 37 °C for 1 h, followed by dialysis against PBS (pH 7.4) at 4 °C for at least 4 h. Platelets (2.5 × 10^9) were lysed with 1% Triton X-100 and centrifuged for 20 min at 2000 × g; biotinylated-stejnulxin (25 µg) was added to the supernatant and incubated overnight. After two hours more incubation with avidin-Sepharose, the mixture was centrifuged and the beads were washed thoroughly with Tris-buffered saline. The avidin-Sepharose with bound biotinylated stejnulxin and platelet proteins was boiled for 2 min with Tris-buffered saline containing 2% SDS. Released proteins were separated by a 7-17% gradient SDS-PAGE gel and then electroblotted onto a PVDF membrane. Separated components were detected with specific antibodies, peroxidase-coupled second antibodies and chemiluminescence. Biotin (20 µg) and biotinylated alboaggre-gin-A (90 µg) were treated in the same way as the biotinylated stejnulxin.

Molecular cloning of stejnulxin
mRNAs were prepared from the total RNA of one T. stejnegeri venom gland by oligo(dt) cellulose chromatography. A directional cDNA library was constructed with a plasmid cloning kit (SuperScript plasmid system, GIBCO/BRL). Two oligonucleotide primers used to screen the library were designed according to the conserved regions of the cDNA

Figure 1: Purification of stejnulxin. (A) Gel filtration of T. stejnegeri venom (0.5 g) on a Sephadex G-100 column. The column (2.6 × 120 cm) was equilibrated with 50 mM Tris-HCl, pH 7.8 buffer, containing 0.1 M NaCl. Fractions (2 ml) were collected and protein concentration was estimated from the absorbance at 280 nm (●). The platelet aggregation inducing activity (△) was measured as described in "Methods". The fractions of 51-56, which contained platelet aggregation inducing activity, were pooled for further purification. (B) Ion-exchange chromatography on a Mono-Q (16/10) column at pH 7.8. The column was equilibrated with 20 mM Tris-HCl, pH 7.8 and eluted with a NaCl gradient as indicated (----). Platelet aggregation inducing activity was concentrated in the peak as indicated by an arrow. The insert shows the SDS-PAGE pattern of purified stejnulxin. Lanes 1 and 2 are from a 7.5% gel under non-reducing conditions; lane 3 and 4 are from a 20% gel under reducing conditions. Lanes 1 and 4 are high molecular mass and low molecular mass markers, respectively. Lanes 2 and 3, purified stejnulxin.
sequence of TSL, a galactose-binding C-type lectin from *T. stejnegeri* venom (25), and other related cDNA sequences of *T. flavoviridis* venom blood coagulation factor IX/X binding protein (26) and *Agkistrodon halys* blomhoffii venom glycoprotein Ib-binding protein (27). The two primers used were L5 (5′-AGGGAAGGAAGGAAGCATGG-3′, in the sense direction) and L3 (5′-GGGGGTTTCCTTGCTTCCAG-3′, in the anti-sense direction). L5 and L3 are located in the 5′- and 3′- noncoding regions of venom lectin cDNAs, respectively. All the oligonucleotide primers for PCR were prepared with a DNA synthesizer (Model 381A, Applied Biosystems).

A PCR-based method for high stringency screening of DNA libraries was used for screening and isolating the clones as previously described (28). L3 and L5 primers were used in PCR reactions under the following conditions: 2 min at 94 °C, followed by 35 cycles of 10 sec at 92 °C, 25 sec at 55 °C, 25 sec at 72 °C with a Peltier Thermal Cycler (model PTC-200, M.J. Research, USA).

Multiple sequence alignments were performed with Vector NTI suite 6.0 program (version 3.1, June 2000). The nucleotide sequence data reported in this paper are available from GeneBank database with accession numbers AF354924 for stejnulxin α subunit and AF354926 for stejnulxin β subunit.

### Results

#### Purification of stejnulxin

The purification was followed by determining the platelet aggregation inducing activity of each fraction. Gel filtration of *T. stejnegeri* venom by Sephadex G-100 gave seven protein peaks (Fig. 1A). The platelet aggregation inducing activity was predominantly associated with fraction I and was separated from thrombin-like activity and inhibitory activity of ristocetin-induced platelet agglutination. The latter two activities were concentrated in fractions II and IV, respectively.

The first half of fraction I, which showed strong platelet aggregation inducing activity, was diazylzed against 20 mM Tris-HCl, pH 7.8 buffer overnight, and further purified on an ÄKTA Explorer using a preparative Mono-Q (16/10) column equilibrated with the same buffer. Proteins were eluted with a linear NaCl gradient, yielding 5 protein peaks, and peak 4 contained homogeneous stejnulxin (indicated by an arrow, Fig. 1B). Approximately 1 mg of stejnulxin was obtained from 0.5 g of crude venom. Thus, stejnulxin is a minor component of the venom, about 0.2% of total venom proteins.

#### Characterization of stejnulxin

Purified stejnulxin gave a single 120 kDa band on SDS-PAGE, under non-reducing conditions in a 7.5% gel. On reduction, it separated into three polypeptide bands with apparent molecular masses of 16 kDa, 20 kDa and 22 kDa, respectively (Fig. 1B insert).

The N-terminal amino acid sequences of the purified protein were determined by Edman degradation. Reduced and alkylated stejnulxin was separated by SDS-PAGE. The N-terminal sequence of the 16 kDa band is DFDCPSGL120kDa protein (27). The two primers used were L5 (5′-AGGGAAGGAAGGAAGCATGG-3′, in the sense direction) and L3 (5′-GGGGGTTTCCTTGCTTCCAG-3′, in the anti-sense direction). L5 and L3 are located in the 5′- and 3′- noncoding regions of venom lectin cDNAs, respectively. All the oligonucleotide primers for PCR were prepared with a DNA synthesizer (Model 381A, Applied Biosystems).
with periodic acid-Schiff’s staining, showed that both bands with apparent molecular masses of 20 kDa and 22 kDa are glycosylated, while the band with apparent molecular mass of 16 kDa is not glycosylated.

**Stejnulxin does not activate platelets through GPIb, GPIX or CD36**

Stejnulxin activates platelets similarly in both PRP and washed platelets. Therefore, all other experiments were carried out with washed platelets, which are thought to be free from surface fibrinogen. Stejnulxin induced platelet aggregation in a dose-dependent way with the maximum platelet aggregation rate at a stejnulxin dose of 150 ng/ml (Fig. 2A). Polyclonal antibodies against epitopes on the N-terminal domain of GPIbα only slightly inhibited platelet aggregation induced by stejnulxin (20% inhibition at 5 µg/ml anti-GPIbα polyclonal antibodies). In addition, echinatrin (6.6 µg/ml) did not inhibit its activity (Fig. 2B). Monoclonal antibodies against CD36 and GPIX also had no obvious influence on platelet aggregation induced by stejnulxin. Polyclonal anti-GPIbα, anti-CD36 and anti-α2 integrin antibodies were also tested against the proteins eluted from the avidin-Sepharose under the same conditions as those of in Figure 3B, but neither GPIbα (Fig. 3C) nor CD36, nor α2 integrin were detected. The results indicated that GPIbα, CD36, GPIX and α2 integrin are not involved in the platelet aggregation induced by stejnulxin.

**Stejnulxin activates platelets via GPVI**

Stejnulxin, like convulxin, is a potent platelet agonist. In the presence of anti-GPVI polyclonal antibodies, stejnulxin-induced platelet aggregation was blocked (Fig. 2C). The anti-GPVI antibodies caused slight platelet activation. This did not affect platelet activation by other non-GPVI-directed agonists (data not shown). Based on the aggregation slopes when platelet suspensions were treated with convulxin, 5 ng stejnulxin is equivalent to 1 ng convulxin. Comparison of the time ranges of tyrosine phosphorylation for platelets activated by convulxin and stejnulxin showed that stejnulxin induced strong and rapid phosphorylation of a range of proteins resembling those seen during platelet activation by convulxin (Fig. 3A). The visible bands co-migrated with the indicated molecules, including Fcγ, LAT, PI3K and phospholipase Cγ2 (PLCγ2), suggesting that the observed bands contain these proteins. Platelet membrane glycoproteins binding to biotinylated stejnulxin were isolated by avidin-Sepharose. The isolated proteins were separated by SDS-PAGE and electroblotted onto a PVDF membrane. Biotinylated stejnulxin bound strongly and specifically to a 60-kDa platelet membrane protein. Blotting this membrane with polyclonal anti-GPVI antibodies indicated that the 60-kDa band is GPVI (Fig. 3B).

Stejnulxin signaling thus resembles that induced by convulxin. Inhibiting another important collagen receptor α1β1
Inhibition of α<sub>IIb</sub>β<sub>3</sub> partly blocks tyrosine phosphorylation induced by stejnulxin

Inhibition of α<sub>IIb</sub>β<sub>3</sub> by EMD 132338 prevented platelet aggregation induced by stejnulxin to a considerable extent by blocking fibrinogen binding, but not completely, even when higher amounts of the inhibitor were used in washed platelets (Fig. 4A). In the presence of EMD 132338, inhibition of α<sub>IIb</sub>β<sub>3</sub> in platelets activated by stejnulxin prolonged the activation time of several signaling molecules, such as LAT, P26 and Fcγ (Fig. 4B).

Molecular cloning and sequence analysis of stejnulxin

A cDNA library constructed from the *T. stejnegeri* venom gland was screened at high stringency, as described under “Methods”, by an efficient and rapid PCR-based procedure. Twenty positive clones, which contain about 700-800 base pair inserts, were thus identified and isolated. Both strands of the isolated clones were sequenced. The results indicated that all these clones contain a full-length cDNA insert that encodes a C-type lectin. Combining the protein sequencing results, clone 10J and clone 29J were found to code stejnulxin α and β subunits, respectively. The complete nucleotide sequences of stejnulxin α and β subunit cDNAs and their deduced amino acid sequences are listed in Figure 5. The cDNA structures of both α and β subunits of stejnulxin are similar to those of convulxin (15).

The encoded protein sequences correspond to polypeptides of 158 and 148 amino acid residues for stejnulxin α and β subunits, respectively (Fig. 5). A comparison of the deduced N-terminal sequences of these polypeptides with those of determined by Edman degradation of the isolated protein subunits shows that the sequences deduced from cDNAs include a signal peptide of 23 amino acid residues for both subunits, which is removed in the mature protein. The N-terminal sequences of the stejnulxin subunits determined by chemical methods are in good agreement with the polypeptide sequences deduced from the cloned cDNAs, therefore confirming the identity of isolated clones. The sequences of the mature subunits give a molecular mass of 15,713 Da for stejnulxin α subunit and 14,626 Da for stejnulxin β subunit. In the case of stejnulxin α subunit, the predicted molecular mass is in good agreement with the apparent molecular mass determined by SDS-PAGE. In the case of stejnulxin β subunit, the difference in the apparent molecular masses (20 kDa and 22 kDa) is consistent with the presence of sugars, since the β subunit is glycosylated. There are two potential N-glycosylation sites, Asn-X-Thr, at amino acid residues 24 and 55.

The deduced mature protein amino acid sequences of the stejnulxin subunits are 44.4% identical. The polypeptide sequences of stejnulxin α and β subunits compared with those of other snake venom C-type lectins: have 52.6%/66.4% identity with convulxin (15); 57%/76% with flavocetin-A (29); 44.5%/52.8% with echicetin (30, 31); and 43.7%/29.8% with rhodocetin (23), respectively. Unlike echicetin, which contains 7 cysteine residues in each subunit, and like convulxin and flavocetin-A, stejnulxin contains two additional cysteine residues (Cys-135 in α subunit, and Cys-3 in β subunit) (Fig. 6).
Discussion

Snake venoms contain many C-type lectins that act via platelet membrane receptors, either inducing or inhibiting platelet activation (10, 11). Many snake C-type lectins that activate platelets to various extents have been reported that either have different receptors or have different affinities for the same receptors, even though they share a high degree of sequence similarities. In this report, a novel snake C-type lectin was purified and characterized from the venom of *T. stejnegeri* (Chinese green tree viper). Sequence comparison established that it belongs to group VII of animal C-type lectins (32). This protein was named stejnulxin because of its similarities both in sequence and in signal transduction pattern induced in platelets by convulxin (Fig. 3 and 6).

From the known patterns of disulfide bridges in C-type lectin-like proteins of snake venoms such as flavocetin-A (19) and by homology, it can be seen in Figure 6 that cysteine residues forming intramolecular disulfide bridges are well conserved in stejnulxin. In the crystal structure of flavocetin-A, the αβ4 structure is mediated by an interchain disulfide bridge between cysteine residues at the C-terminal part of the α-subunit and at the N-terminal part of the β-subunit in the neighbouring αβ units. Its high affinity for GPIbα is thought to come from the...
cooperative action of the multiple binding sites in this tetramer. Like convulxin and flavocetin-A, stejnulxin α and β subunits each contains eight cysteine residues. According to the structural model of flavocetin-A, six of these cysteines are involved in the intracatenary disulfide bridges (Cys4-Cys15, Cys32-Cys129 and Cys104-Cys121 for the α subunit and Cys4-Cys15, Cys32-Cys121 and Cys98-Cys113 for the β subunit). Compared with flavocetin-A, besides these six intracatenary disulfide bridges, two disulfide bridges between the α and β subunits may exist in stejnulxin between Cys-81 of the α subunit and Cys-77 of the β subunit, and Cys135 in the α subunit and Cys-3 in the β subunit, which stabilize the 3-dimensional structure of stejnulxin. Based upon the apparent molecular mass under non-reducing conditions (120 kDa) and the apparent molecular masses of each subunit under reducing conditions (16 kDa, 20 kDa, 22 kDa), it is likely that stejnulxin has a trimeric (αβ)3 structure similar to convulxin, but a tetrameric structure remains a possibility. Convulxin has never been formally shown to be a triheterodimer but this is generally assumed to be so based upon the apparent molecular mass (15). A crystal structure will be necessary to resolve this problem.

Like convulxin, stejnulxin is a powerful platelet activator. It shows a distinct, dose-dependent, lag phase platelet aggregation inducing activity. Echicetin, a 26 kDa heterodimeric protein from the venom of saw-scaled viper, E. carinatus, binds to GPIbα and inhibits vWF-dependent platelet agglutination but has no effect on ADP-induced platelet aggregation (22). Rhodocetin, a 35 kDa noncovalent heterodimeric C-type lectin from C. rhodostoma venom, inhibits collagen induced platelet aggregation by interacting with α2β1 (23). Both of these two C-type lectin-like proteins have no inhibitory effects on the platelet aggregation induced by stejnulxin. These results, together with antibody studies and binding assay of biotinylated stejnulxin on platelet membrane lysates, indicate that stejnulxin does not activate platelet via GPIbα, CD36, GPIX, or α2β1.

As shown in Figure 2C, anti-GPVI polyclonal antibodies strongly inhibited stejnulxin-induced platelet aggregation, providing direct evidence that stejnulxin activates platelets through GPIIb/IIIa.

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**Figure 6:** Alignment of stejnulxin subunit sequences with those of other snake venom C-type lectins. The numbering starts at the N-terminal amino acid of stejnulxin. Gaps have been introduced to optimize the sequence homology. Residues identical in all sequences are shaded. Percentage identity between stejnulxin and the other sequences is shown on the right. Sequences were from the following sources: convulxin (15), flavocetin-A (19), echicetin (30, 31), and rhodocetin (23).
GPVI. In addition, the experimental data further demonstrate that stejnulxin binds specifically to GPVI and activates the $\alpha_{IIb}\beta_3$ receptor, resulting in activation of platelets and inducing phosphorylation of tyrosine in signaling proteins. This is shown by the observation that EMD 132338, an inhibitor of $\alpha_{IIb}\beta_3$, greatly inhibits platelet aggregation induced by stejnulxin. The result shows that the platelet aggregation induced by stejnulxin is via $\alpha_{IIb}\beta_3$ and not simply agglutination. At the same time, the phosphorylation on tyrosine of many signal molecules is not inhibited by EMD 132338, indicating that $\alpha_{IIb}\beta_3$ is not the direct platelet activating receptor for stejnulxin.

Stejnulxin is distinct from other venom C-type lectin GPVI agonists, such as convulxin, alboaggregin-A, alboluxin, and ophioluxin, in size, sequence and/or precise platelet activating mechanism. GPVI is the main, and probably the only platelet receptor for stejnulxin, activating platelets to cause aggregation via $\alpha_{IIb}\beta_3$ and fibrinogen. On the other hand, alboaggregin-A and alboluxin act on both GPIb and GPVI (20, 17). Stejnulxin appears most similar to ophioluxin, which probably only acts on GPVI, resulting in platelet activation (18). All these proteins use sites on GPVI as one of their binding targets, however, the proteins might share similar, but not necessarily identical, binding sites on GPVI. Obviously, it is important to understand the structure-function relationship of these molecules in terms of their platelet GPVI recognition and action mechanisms.

Carbohydrates attached to the conserved glycosylation site on the Fc portion of an antibody are critical to the recognition of immunoglobulins by the low affinity Fc$\gamma$ receptor. They are likely to function as a substitution for the hydrophobic core to preserve an optimal lower hinge conformation on the receptor binding (33, 34). To our knowledge, stejnulxin is the first snake venom C-type lectin-like protein that has been demonstrated to be N-glycosylated. In addition, different amounts of carbohydrates are present on its $\beta$ subunit, since distinct $\beta_1$ and $\beta_2$ bands were seen on SDS-PAGE. This might be due to glycosylation at single and double sites. The effect of glycosylation on the specificity of stejnulxin on GPVI needs to be further investigated. Any function of the glycosylation is still unclear.

In conclusion, stejnulxin, as a newly identified and specific GPVI-binding snake C-type lectin, should be a useful tool for studies of GPVI and signaling mechanisms via platelet GPVI as well as in comparison with other venom C-type lectin GPVI agonists, like convulxin. Its cloning offers a possibility for the site-specific mutation studies related to the structure-function of these venom GPVI agonists, as well as providing useful insights into the molecular basis of GPVI receptor function and cell signaling pathways of platelets after activation, via this receptor.

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