Ophioluxin, a Convulxin-like C-type Lectin from *Ophiophagus hannah* (King Cobra) Is a Powerful Platelet Activator via Glycoprotein VI*

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Ophioluxin, a potent platelet agonist, was purified from the venom of *Ophiophagus hannah* (King cobra). Under nonreducing conditions it has a mass of 85 kDa, similar to convulxin, and on reduction gives two subunits with masses of 16 and 17 kDa, slightly larger than those of convulxin. The N-terminal sequences of both subunits are very similar to those of convulxin and other C-type lectins. Ophioluxin induces a pattern of tyrosine-phosphorylated proteins in platelets like that caused by convulxin, when using appropriate concentrations based on aggregation response, because it is about 2–4 times more powerful as agonist than the latter. Ophioluxin and convulxin induce [Ca\(^{2+}\)]\(_e\) elevation both in platelets and in Dami megakaryocytic cells, and each of these C-type lectins desensitizes responses to the other. Convulxin agglutinates fixed platelets at 2 μg/ml, whereas ophioluxin does not, even at 80 μg/ml. Ophioluxin resembles convulxin more than echicetin or alloagregin B because polyclonal anti-ophioluxin antibodies recognize both ophioluxin and convulxin, but not echicetin, and platelets adhere to and spread on ophioluxin- or convulxin-precoated surfaces in the same way that is clearly different from their behavior on an alloagregin B surface. Immobilized ophioluxin was used to isolate the glycoprotein VI-Fcγ complex from resting platelets, which also contained Fyn, Lyn, Syk, LAT, and SLP76. Ophioluxin is the first multiheterodimeric, convulxin-like snake C-type lectin, as well as the first platelet agonist, to be described from the Elapidae snake family.

Collagens in the vascular subendothelium and vessel wall are important platelet activators that have a critical role in hemostasis. A large number of different receptors have been proposed for collagen on platelets, including CD36 (glycoprotein (GP)\(^1\) IV, GPIIIib) (1), GP Ib-V-IX complex as an indirect collagen receptor acting via von Willebrand factor (2), a 65-kDa collagen type I specific receptor (3), and a 68–72-kDa doublet collagen type III-specific receptor (4), but the major ones have been identified as the integrin α\(_2\)β\(_1\) and Ig superfamily member GPVI. Integrin α\(_2\)β\(_1\) belongs to the class containing an I-domain, which has been well characterized and is considered to be responsible for platelet adhesion to collagen rather than platelet activation (5, 6) for which GPVI is largely responsible. The evidence that GPVI is the other major, established receptor essential for signaling and platelet activation was based on studies on patients with mild bleeding problems, whose platelets had an impaired response to collagen but not to other agonists and were GPVI-deficient (7, 8). This was substantiated by studies with GPVI-specific agonists including collagen-related peptide (9), convulxin, a snake C-type lectin (10, 11), and a rat monoclonal antibody JAQ1 (12), all of which stimulate a tyrosine phosphorylation pattern resembling that induced by collagen in platelets. GPVI is a member of the Ig superfamily and has a sequence and structure very similar to FcαR and to some of the natural killer receptor family (13). It forms a complex with the Fc receptor γ chain (Fcγ), which is critical not only for the signaling process, but also for the normal expression of this receptor in mice (14). The mechanism of signal transduction via platelet GPVI has been intensively investigated over the last few years using various GPVI ligands (9–12).

Snake C-type lectins have been widely used as tools to investigate platelet receptors (15–18). Some inhibit the function of platelets by preventing ligand binding, others activate platelets either directly via receptors or indirectly using plasma proteins. Examples of these are echicetin, which uses IgM (19), or botrocetin and bitiscetin that bind von Willebrand factor (20, 21). Earlier, these proteins were thought to activate or inhibit platelets via only one receptor. However, more detailed investigation, including the use of transfected cells expressing specific receptors and mice with genes for receptors “knocked out” have indicated that the situation may often be more complex. Alloagregin A was previously reported to target GPIb but more recently has been shown to act principally by GPVI (22–24). Aggrein has been shown to interact with both GP Ib and the integrin α\(_2\)β\(_1\) (25, 26). Obviously, these proteins have evolved in snakes as powerful agonists by interacting with several platelet receptors simultaneously. These multireceptor ligands are more difficult to use to study platelet signal pathways via single receptors because of cross-talk between the pathways although they may simulate some physiological ligands, such as von Willebrand factor, that also act via several receptors. Among the dozens purified so far only three snake C-type lectins have been characterized that target GPVI. Among them, alloagregin A (23, 24) and alboluxin (27) act via...
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both GPIb and GPVI. Convulxin was the first protein identified that acts via GPVI-binding and has been widely used for the isolation, characterization, and investigation of signaling mechanisms of this important receptor. However, the binding sites on GPV for convulxin or collagen and how this binding activates platelets are still unknown, as well as whether convulxin has only one type of binding site on GPV. 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 investigating the mechanism of activation of platelets by collagen through GPVI. Additional GPVI ligands from snake venoms may help to elucidate these mechanisms as well as casting light on snake evolution. Here the isolation and characterization of ophioluxin, the first convulxin-like protein and the first snake C-type lectin to be identified from the Elapidae family, is described. Evidence for its specificity for GPVI is presented and its properties are compared with convulxin. Some applications in the investigation of GPVI-associated molecules and signaling are also shown.

EXPERIMENTAL PROCEDURES

Materials—Lyophilized Ophiophagus hannah venom was purchased from CV Herpaufan, Indonesia. Protein A-Sepharose, peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies, fura 2-AM, fluorescein isothiocyanate (FITC), bovine serum albumin (BSA), 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP), streptavidin-alkaline phosphatase, bixinamidocaproate-N-hydroxysuccinimide ester (biotin-NHS), p-nitro blue tetrazolium chloride (NBT), Triton X-100, wortmannin, and U73122 were from Sigma. Polyvinyliden difluoride membranes were PolyScreen from PerkinElmer Life Sciences. Octanoyl-N-methylglucamide was from Oxychemie (Böbingen, Germany). FITC-coupled chicken anti-P-selectin (CD62P) was from WAK-Chemie Medical (Bad Soden, Germany). The SuperSignal chemiluminescence detection kit was from Pierce. Autoradiography (Fuji RX) films were from Fujifilm (Dielsdorf AG, Switzerland). Sepharose 4B was from Amersham Biosciences. Anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-Syk and anti-Fyn monoclonal antibodies, anti-LAT, anti-PLCγ2, anti-FAK, anti-Cbl, anti-Lyn, anti-phosphatidylinositol 3-kinase and anti-SLP76 polyclonal antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Fcγ polyclonal antibodies were a kind gift from Dr. J. P. Kinet. Methylated type I collagen was a kind gift from Dr. B. Kehrel. EMD 132338, a GPIIb/IIIa inhibitor, was a kind gift from Merck (Darmstadt, Germany).

Anti-GP Ib, anti-GPVI, anti-GP IIb, and anti-CD36, anti-I domain, and anti-GPVI (against the whole protein or the N-terminal 17-amino acid residues) polyclonal antibodies were prepared as previously described or using similar methods (13). Echis carinatus, aggreton, rhodocetin, and alboaggregin A were isolated from Trimeresurus albolabris (different concentrations were used depending on application) were collected and washed, and bound proteins were released by boiling for 10 min and resuspending in TBS. Biotinylated platelets were solubilized in Triton X-100 lysis buffer containing 2 m M N-ethylmaleimide, 4 m M EDTA, and 1 m M phenylmethylsulfonyl fluoride. After centrifugation, the supernatants were incubated with specific antibodies before adding 20 m M of Protein A-Sepharose and shaking overnight at 4 °C. The beads were collected and washed, and bound proteins were released by boiling for 3 min with 2% SDS, 10 m M Tris-HCl, pH 7.4.

Flow Cytometric Analysis of FITC-Ophioluxin or FITC-Convulxin Binding to Fixed Platelets—Freshly isolated platelets prepared as described before (33). Washed platelets were incubated with an equal volume of 1% formaldehyde in TBS at room temperature for 0.5 h. Then platelets were washed twice by centrifuging and then resuspended in the same buffer. Fixed platelets were diluted to 5 × 107 platelets/ml with TBS. Different inhibitors or antibodies were added to 100 μl of platelets, then shaken gently for 10 min at room temperature. Then FITC-ophioluxin

Sequence Analysis of Ophioluxin Subunits and Computer Analysis of the Sequence Data—Ophioluxin was separated by SDS-PAGE under reducing conditions, and then transferred to a polyvinyliden difluoride membrane. After Coomassie Blue staining, bands corresponding to the subunits were separately cut out and their N-terminal sequences were determined on an Applied Biosystem model 477A pulsed liquid-phase protein sequencer with a model 120A on-line phenylthydroxantin-derivative analyzer. The sequence data were analyzed using BLAST (PubMed).

Determination of Washed Platelets, Platelet Aggregation, and Immunoprecipitation—Fresh blood was obtained from the Swiss Red Cross Blood Transfusion Service. Washed platelets were prepared as previously described (10, 13). After successive washes and centrifugations, platelets were resuspended in 20 m M Hepes, 140 m M NaCl, 4 m M KCl, 5.5 m M glucose, pH 7.4, and adjusted to 5 × 109 platelets/ml. Platelet aggregation was performed using an aggregometer (Lumitec, France) with continuous stirring at 1100 rpm at 37 °C or in the case of small aggregations using a narrow beam laser aggregometer (Bioda, Moscow, Russia). For immunoprecipitation, resting as well as activated platelets (500 μl, 5 × 1010 platelets/ml) were lysed in RIPA buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) containing 1 m M N-ethylmaleimide, 2 m M sodium o-vanadate, 2 m M EDTA, and 1 m M phenylmethylsulfonyl fluoride. After centrifugation, the supernatants were incubated with specific antibodies before adding 20 m M of Protein A-Sepharose and shaking overnight at 4 °C. The beads were collected and washed, and bound proteins were released by boiling for 3 min with 2% SDS, 10 m M Tris-HCl, pH 7.4.

Anti-GPIb, anti-GPVI, anti-GPV, and anti-CD36, anti-I domain, and anti-GPVI (against the whole protein or the N-terminal 17-amino acid residues) polyclonal antibodies were prepared as previously described or using similar methods (13). Echis carinatus, aggreton, rhodocetin, and alboaggregin A were isolated from Trimeresurus albolabris (different concentrations were used depending on application) were collected and washed, and bound proteins were released by boiling for 10 min and resuspending in TBS. Biotinylated platelets were solubilized in Triton X-100 lysis buffer containing 2 m M N-ethylmaleimide, 4 m M EDTA, and 1 m M phenylmethylsulfonyl fluoride and centrifuged at 11000 rpm at 4 °C. The supernatant was applied to the column and eluted successively with TBS containing 0.2% octanoyl-N-methylglucamide, 0.1 or 0.5% SDS. The eluted fractions were analyzed by SDS-PAGE/silver stain and Western blotting. When surface proteins were investigated, platelets were labeled with biotin-NHS as described above, namely, washed platelets were incubated with biotin-NHS at 107 platelets/100 μg of biotin-NHS for 1 h at room temperature. They were then washed twice by centrifuging at 1000 × g for 10 min and resuspending in TBS. Biotinylated proteins were solubilized in the same way as normal platelets. Biotin-labeled platelet proteins on Western blots were detected with phenylthoselabeled streptavidin followed by NBT/BCIP. Color development was stopped by replacing the reagents and washing with distilled water to remove excess reagents.

Preparation of Ophioluxin-Sepharose 4B Affinity Chromatography and Separation of Platelet Proteins—Ophioluxin-Sepharose 4B was prepared at 1 m g of ophioluxin/ml of Sepharose 4B. Washed platelets (different concentrations were used depending on application) were solubilized in Triton X-100 lysis buffer containing 2 m M N-ethylmaleimide, 4 m M EDTA, and 1 m M phenylmethylsulfonyl fluoride and centrifuged at 11000 rpm at 4 °C. The supernatant was applied to the column and eluted successively with TBS containing 0.2% octanoyl-N-methylglucamide, 0.1 or 0.5% SDS. The eluted fractions were analyzed by SDS-PAGE/silver stain and Western blotting. When surface proteins were investigated, platelets were labeled with biotin-NHS as described above, namely, washed platelets were incubated with biotin-NHS at 107 platelets/100 μg of biotin-NHS for 1 h at room temperature. They were then washed twice by centrifuging at 1000 × g for 10 min and resuspending in TBS. Biotinylated proteins were solubilized in the same way as normal platelets. Biotin-labeled platelet proteins on Western blots were detected with phenylthoselabeled streptavidin followed by NBT/BCIP.

Measurement of Cytoplasmic (Ca2+), Responses to Agonists in Dami Cells or Platelets—Dami cells were harvested and washed with 20 m M Hepes, pH 7.4, 4.8 m M KCl, 136 m M NaCl, 5 m M glucose, and 4 m M CaCl2, and 105 cells or 4 × 107 washed platelets were resuspended in 400 μl of the same buffer and incubated with 2.5 μ M fura-2AM for 20 min at 37 °C. Agonists were added to 2.5 × 1010 cells or 1 × 1010 platelets, and fura-2- Ca2+ complex fluorescence was measured.

FITC Cytometric Analysis of FITC-Ophioluxin or FITC-Convulxin Binding to Fixed Platelets—Freshly isolated platelets prepared as described before (33). Washed platelets were incubated with an equal volume of 1% formaldehyde in TBS at room temperature for 0.5 h. Then platelets were washed twice by centrifuging and then resuspended in the same buffer. Fixed platelets were diluted to 5 × 107 platelets/ml with TBS. Different inhibitors or antibodies were added to 100 μl of platelets, then shaken gently for 10 min at room temperature. Then FITC-ophioluxin...
or FITC-convulxin were added and, while protected against light, shaken for another 10 min at room temperature. The platelets were washed once with TBS, and samples were analyzed by flow cytometry.

Examination of Platelets by Light Microscope—Washed or fixed platelets were stirred with agonists as for aggregometry. Aliquots were placed on glass slides, which had been precoated with BSA in the case of washed platelets. For adhesion assays, gel-filtered platelets were added to BSA-, alboagregin B-, convulxin- or ophioluxin-precoated coverslips at a density of $10^7$ platelets/ml. After incubation, non-adherent platelets were washed away, and FITC-labeled anti-P-selectin antibodies were added. After washing, the platelets were observed under ultraviolet or visible light. Fields were observed under $1000$ magnification on a Nikon Eclipse E600 microscope and images were captured using a Hamamatsu 3 CCD color video camera.

RESULTS

Ophioluxin Is a Snake C-type Lectin—Ophioluxin was purified by two-stage chromatography as described under “Experimental Procedures” (Fig. 1, A and B). It showed one band at a molecular mass of around 85 kDa under nonreducing conditions and two bands at 16 and 17 kDa under reducing conditions when separated by SDS-PAGE and detected with silver staining. Ophioluxin is about the same size as convulxin under nonreducing conditions, but the subunits are slightly larger (Fig. 1C). The N-terminal sequences of the subunits are GLCCPMRWSSSEG for the larger, and DFKCPSEWYAYDQHNCYRIIN for the smaller. Based on size these would normally be assigned to $\alpha$ and $\beta$, respectively. However, in this case there are two arguments against this interpretation. One is the unusual size of the larger subunit and the other is the sequence similarity with other members of this family, in particular those that form multimers of heterodimers. Based on these considerations it is most likely that these are the $\beta$ and $\alpha$ subunits, respectively, and that the unusually large size of the subunits and, particularly the $\beta$ subunit, is because of post-translational modification, probably, glycosylation. We have given this protein the name ophioluxin because the sequences of the subunits are highly similar to convulxin and some other snake C-type lectins (Fig. 1D). In addition, ophioluxin has similar properties to convulxin and is also likely to have a similar multiheterodimeric structure when the mass of the whole protein and the individual subunits are compared. Ophioluxin is the first snake C-type lectin (with heterodimeric structure) reported from King cobra.

Ophioluxin Activates Platelets via GPVI—Ophioluxin is a powerful platelet agonist both in PRP and washed platelets. Its platelet activating ability measured in terms of the aggregation response is about 2–4 times higher than convulxin on a mass basis. Amounts of each C-type lectin, which gave the same rate of aggregation (maximum slope), were chosen to investigate the tyrosine-phosphorylation signaling patterns. They resembled each other and that induced by collagen (Fig. 2A). Many tyrosine-phosphorylated pro-
teins were detected by the anti-phosphotyrosine monoclonal antibody 4G10, including Fcγ/H9253 and LAT that were confirmed by immunoprecipitation (Fig. 2B). Phosphorylated SLP76, phosphatidylinositol 3-kinase, and PLCγ/H9253 were also detected in anti-LAT immunoprecipitates, agreeing with the results in previous publications (34, 35) (data not shown).

**Ophioluxin Is a GPVI-binding Protein**—Platelets were surface-labeled with biotin-NHS, lysed in detergent, and after centrifugation the supernatant was loaded on an ophioluxin-Sepharose 4B column. Biotin-labeled platelet proteins in the bound fractions were detected on Western blots with phosphatase-labeled streptavidin followed by NBT/BCIP. As shown in Fig. 3A, a broad band around 60–65 kDa was stained and this surface protein was detected by anti-GPVI polyclonal antibodies (Fig. 3B). With longer exposures, two additional bands appeared, one larger than GPVI, the other smaller. The higher band is recognized better by the anti-GPVI N-terminal peptide antibodies than by the anti-whole GPVI antibodies, suggesting that both bands represent GPVI-related proteins with the same or similar N-terminal sequence (Fig. 3C). Meanwhile, the identification of GPVI was confirmed by the ability of biotynlated ophioluxin as well as polyclonal anti-GPVI antibodies to recognize a major band around 60–65 kDa on blots of platelet lysates separated under nonreducing conditions (data not shown).

In addition, a weakly biotin-labeled surface protein of about 80 kDa was also stained (see Fig. 3A). The membrane was stained using both polyclonal anti-GPVI and anti-CD36 antibodies but this band was negative with both (data not shown). Anti-GPⅠbα and anti-I domain antibodies were also tested against the bound proteins eluted from the column, but neither GPIbα nor αⅡb subunit was detected.

**Flow Cytometric Analysis of FITC-Ophioluxin Binding to Platelets**—FITC-ophioluxin binds to platelets to saturation (Fig. 4A). The binding was strongly inhibited by convulxin and polyclonal anti-GPVI antibodies, but not by echicetin, monoclonal antibody anti-GPIb VM16d, or anti-I domain polyclonal antibodies (representative results are shown in Fig. 4B). Rhodocetin, which is thought to inhibit collagen-stimulated platelet aggregation by binding to GPIbα2/H92521, also had no effect on FITC-ophioluxin binding to platelets (data not shown). These results suggest that GPVI is the main, and probably the only platelet receptor for ophioluxin.

**Ophioluxin and Convulxin Induce Release of Ca2+ in Both Platelets and Dami Cells**—Ophioluxin fully activates platelets and cross-talks with ADP and thromboxane A2 pathways to cause platelet aggregation (based on the inhibitory effect of aspirin and apyrase), which resembles the activating mechanism of convulxin. A strong calcium signal was measured from platelets activated by either ophioluxin or convulxin (data not shown). Prostaglandin E1 and sodium nitroprusside inhibit nearly completely platelet aggregation caused by ophioluxin showing that cAMP- and cGMP-dependent kinases are important negative feedback pathways.
In addition, ophioluxin and convulxin both activate Dami cells and induce Ca\(^{2+}\)/H\(_{1001}\) release. It was already known that Dami cells express amounts of GPVI adequate to respond to convulxin (19). Ophioluxin gave a strong calcium signal compared with convulxin when the same amounts were used. When convulxin was added first, ophioluxin added later induced less Ca\(^{2+}\)/H\(_{1001}\) release and vice versa. This mutual desensitization demonstrates again that they both act on GPVI and down-regulate its responses (see Fig. 5).

Platelets Adhere to Surfaces Coated with Ophioluxin—Adhesion assays showed that platelets adhere to and spread in a similar way on ophioluxin- or convulxin-covered surfaces, but platelets behave differently on alboaggregin B-coated surfaces. Platelets also adhered to alboaggregin, but formed fewer pseudopods and appeared less activated (echicetin has the same effect as alboaggregin B, data not shown) (Fig. 6b). Expression of P-selectin on the platelet surface after adhesion to different agonists also showed that ophioluxin and convulxin are alike, and slightly stronger than alboaggregin B. Phosphatidylinositol 3-kinase inhibitor (wortmannin) and PLC\(^{2}\) inhibitor (U73122) both reduce platelet spreading, as well as P-selectin release on ophioluxin surfaces (Fig. 6, e and f). Thus, GPIb and GPVI ligands on surfaces modulate platelet adhesion and release differently, which again suggests that platelet activation via GPIb is weaker than via GPVI. The results also suggest that phosphatidylinositol 3-kinase and PLC\(^{2}\) are key signaling molecules in platelet activation via GPVI.

Convulxin, but Not Ophioluxin, Agglutinates Fixed Platelets—Despite the many similarities characterized above, a major difference was found between these proteins concerning their ability to agglutinate fixed platelets. Convulxin at concentrations above 2 \(\mu\)g/ml agglutinates fixed platelets, although ophioluxin does not, even at 80 \(\mu\)g/ml. The agglutinates seen by light microscope are shown in Fig. 7A. Agglutination of fixed platelets induced by convulxin was inhibited by ophioluxin and also by echicetin. Compared with echicetin, alboaggregin B did not inhibit at the low concentration that could be used without itself inducing agglutination (alboaggregin B agglutinates platelets via GPIb). Unlike convulxin, alboaggregin A induced agglutination was not inhibited by ophioluxin, but by alboaggregin B even at low concentrations (Fig. 7B). This clearly indicates that alboaggregin A is more GPIb-dependent,
although convulxin mainly depends on GPVI. However, flow cytometry studies indicated that neither echicetin nor VM16d, a monoclonal antibody against GPIb, blocked FITC-convulxin binding to fixed platelets (data not shown).

Anti-ophioluxin Antibodies Recognize Convulxin but Not Echicetin—Aliquots of whole *E. carinatus*, *C. durissus terrificus*, and *O. hannah* venoms were separated by SDS-PAGE and blotted to polyvinylene difluoride membranes. Anti-echicetin and anti-ophioluxin antibodies, followed by phosphatase-coupled second antibodies and NBT/BCIP were used to detect antigens. Anti-echicetin antibodies clearly recognize echicetin (Fig. 8, right panel, lane 1), but cross-react only very weakly with ophioluxin and convulxin (lanes 2 and 3), suggesting that ophioluxin and convulxin share only partial structural similarities with echicetin. On the other hand, anti-ophioluxin antibodies do not detect echicetin at all, but recognize ophioluxin and convulxin equally well, which again demonstrates that ophioluxin and convulxin are structurally very similar (Fig. 8).

Isolation of a GPVI Complex Containing Fcγ and Signaling Molecules by Affinity Chromatography on Ophioluxin—Platelet from resting, aggretin-, or ophioluxin-activated platelets were lysed in buffer containing Triton X-100 and the supernatants were applied to ophioluxin-Sepharose 4B columns. After washing, bound proteins were eluted with SDS and analyzed by gel electrophoresis and blotting. As well as GPVI, the constitutively associated subunit, Fcγ, was detected as expected. At the same time, several tyrosine-phosphorylated bands were detected by 4G10 (Fig. 9A) and these were further analyzed with antibodies to other key signal transduction molecules. Fyn, Lyn, Syk, LAT, and SLP76 were detected in the GPVI complex.
both before and after platelet activation, whereas FAK and Cbl were not (Fig. 9B). More Lyn and Syk associate with GPVI after activation (Fig. 9B). To check the specificity of the affinity chromatography, some markers (SLP-76 and Fcγ/H9253) were compared in the major fraction eluted from other snake C-type lectins coupled to Sepharose 4B (rhodocetin and aggretin) as well as to control Sepharose 4B. These showed clear differences in the eluted protein patterns, which are presumably because of differences in signaling molecules associated with receptors binding to these proteins. These results indicate a putative extended GPVI complex that can be isolated using affinity methods, and some of these protein-protein interactions were already shown by immunoprecipitation, but it is the first time that these molecules have been shown to associate directly with the GPVI/Fcγ complex.

**DISCUSSION**

This study reports the isolation and characterization of ophioluxin, a novel snake C-type lectin from the venom of *O. hannah* (King cobra). Ophioluxin is the first C-type lectin reported from the Elapidae family and also the first platelet agonist from this family. There are five main families of venomous snakes: the Hydrophilidae, which are mainly myotoxic; Elapidae, mainly neurotoxic; Viperidae and Crotalidae, which have mainly hemorrhagic effects; and Colubridae, which include venomous and nonvenomous species and are poorly characterized but the venomous ones are known to contain several toxic activities. The typical clinical feature of Elapidae bites is the rapid onset of severe neurotoxicity and, perhaps because of that, their hemorrhagic effects have not been closely examined. Ophioluxin is a powerful platelet agonist, indicating that after King cobra bites a massive activation of platelets would be expected in the prey, leading to thrombocytopenia. Because ophioluxin appears to be specific for GPVI, which is only expressed on platelets and megakaryocytes, it is unlikely that other cells in the prey would be directly affected. A possible explanation for the presence of ophioluxin is that it is carried over from an earlier period when snake ancestors needed hemorrhagic toxins. However, under evolutionary pressure, it is more likely to have been retained and to be currently important, for example, by helping the neurotoxins to subdue the prey or in maintaining blood fluidity, which might promote the digestion of the prey. The presence of this snake C-type lectin and its effects on platelets and hemostasis needs to be taken into account when treating patients suffering from envenomation by King cobra.

Convulxin was the first snake C-type lectin shown to bind specifically to GPVI (11, 12) and along with collagen-related peptides and antibodies to GPVI it became a very useful tool to study the structure and function of GPVI and, in particular,
platelet activation mechanisms through GPVI. 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. For example, bilinexin only agglutinates platelets using GP Ib and αβγδ, as receptors (36), whereas these same glycoproteins are also employed by aggratin to stimulate full platelet activation (25). This may indicate that they have different binding sites for/on each receptor and/or that aggratin may bind to (an) additional receptor(s). Ophioluxin activates platelets more strongly than convulxin on a per mass basis. This can be because of different binding constants for GPVI. In addition, ophioluxin binds to two more proteins that may be GPVI isoforms. The detection of differential binding to GPVI isoforms may provide new insight into GPVI structure and function. Human GPVII with 339 amino acids (13) and murine GPVI with 319 amino acids (37) and a shorter cytosolic tail are 64% homologous. Two splice junction variants of human GPVI were reported in a cell-line (38), one of which, if expressed, would lack 18 amino acids in the mucin-like stalk domain of GPVI and might therefore represent the lower band we detected. However, proteolytic degradation is also a possible explanation for this band. In addition, the higher band that is recognized better by the anti-GPVI N-terminal peptide antibodies than by the anti-GPVI antibodies will require further investigation because it must be GPVI-related.

A significant difference between ophioluxin and convulxin is that the former does not agglutinate fixed platelets even at concentrations as high as 80 μg/ml, whereas convulxin at 2 μg/ml agglutinates fixed platelets. Ophioluxin completely inhibits platelet agglutination induced by convulxin, which indicates that the agglutination caused by convulxin must involve GPVI as receptor. Convulxin has the capacity to bind to GPVI molecules on different platelets but it can also cluster other convulxin molecules on the same platelet. It is interesting that echecitin, which is a snake C-type lectin that only binds GP Ib, can also inhibit convulxin-induced platelet agglutination. Surprisingly, flow cytometry studies show that ophioluxin, but not echecitin, can block FITC-convulxin binding to the surface of fixed platelets. Thus, echecitin inhibits platelet agglutination caused by convulxin, but not FITC-convulxin binding to platelets. There are no reliable reports about convulxin binding to GPIb and it is not easy to obtain other evidence. It should also be mentioned that alboaggregin B, but not ophioluxin, inhibits platelet agglutination by alboaggregin A. This result supports a concept that agglutination is GPIb-dependent and suggests a model where convulxin, bound to GPVI on the platelet surface, can interact further with GP Ib on other platelets. Whether the same binding site is involved or the interaction with GP Ib uses an additional charged patch interaction, as postulated for botrocetin, will require further investigation.

Based upon the molecular mass under nonreduced conditions and the mass of the subunits, it is likely that ophioluxin has a structure similar to convulxin. Convulxin has never been formally shown to be a trimer heterodimer but is generally assumed to be so based upon molecular mass considerations (39). A crystal structure will be necessary to resolve this problem.

Signal transduction via platelet GPVI has been of major interest because it was realized that platelet GPVI is also the principal signaling receptor for collagen. Particular problems that remain are the relative importance of αβγδ and GPVI and clarification of their contributions to the signal mechanism in the overall response to collagen, although GPVI is certainly regarded by many researchers as the major activation inducing receptor for collagen. GPVI is related to the immunoreceptor family, its counterparts in B- and T-cell are well studied; so GPVI signaling pathways can be compared with those of immune receptors in B- and T-cells (40). GPVI associates directly with Fcγ by a salt bridge connecting the arginine in the GPVI transmembrane domain and an aspartic acid in the Fcγ transmembrane domain (41). Fcγ contains immunoreceptor tyrosine-based activation motifs, which can be phosphorylated by Src family kinases such as Fyn and Lyn, associated with the GPVI cytoplasmic domain to induce activation of a kinase cascade (42, 43). Many aspects are still unclear although several versions of the GPVI signaling cascade have been proposed. Lipid raft (also known as glycolipid-enriched membrane domains) methodology starts to be applied to the study of platelet GPVI signaling and it is known that LAT, Fyn, and Lyn are enriched in lipid rafts (40). We have used an affinity chromatography approach to isolate GPVI and GPVI-associated molecules (GPVI complex). As expected the tightly associated Fcγ subunit is found in fractions of resting and activated platelet lysates eluted from ophioluxin-Sepharose 4B and Fcγ is tyrosine-phosphorylated in ophioluxin-activating platelets but not in resting or those activated by aggratin. Lyn, Fyn, LAT, Syk, and SLP76, but not FAK, phosphatidylinositol 3-kinase, or Cbl are also found associated with GPVI/Fcγ in resting, ophioluxin-, or aggratin-activated platelets. Immunoprecipitation has been widely used to investigate these signaling molecules and protein-protein interactions. Falati et al. (44) reported that Fcγ is physically associated with GP Ib and members of the Src family kinases Lyn and Fyn in platelets stimulated through GP Ib when GST-Syk-SH2 precipitates were investigated. Syk was reported to be a kinase downstream of Fcγ and upstream of LAT, associating with Fcγ or LAT by Gross et al. (45). Although this is the first report to investigate platelet signaling molecules by an affinity chromatography approach, many other results support this overall picture. Lyn, Fyn, and LAT are palmitoylated proteins enriched in glycolipid-enriched membrane domains, and the appearance of the cytosolic adaptor SLP76 in the GPVI complex can be explained in terms of the LAT-SLP76 signalsome proposed to regulate the activation of PLCγ2 (40, 46).

The New World Viperidae, including the Crotalidae, may have diverged from the Old World Viperidae and Elapidae at the point when the Atlantic opened, splitting Laurasia and separating North America from Eurasia. This divergence is supported by phylogenetic biochemical data on the sequences of the Lys-49 phospholipase A2s (47). Because we show that venom from an Old World Elapid (O. hannah, ophioluxin) and a New World Crotalid (C. durissus terrificus, convulxin) contain C-type lectins with the same overall structure as well as the same ligand specificity, there is a strong implication that both characteristics existed in the precursor of these snakes and have been conserved over at least the past 50 million years. This would therefore imply that GPVI also existed as a platelet receptor in the mammals established at that time. These results also suggest that similar C-type lectins are likely to exist in other snake species and await characterization. Ophioluxin, as a newly characterized GPVI-binding snake C-type lectin, is expected to be a useful tool for studies of GPVI and signaling mechanisms via platelet GPVI as well as in comparison with convulxin.

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