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ORIGINAL ARTICLE

Effect of multimer size and a natural dimorphism on the binding of convulxin to platelet glycoprotein (GP)VI

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Summary. *Background:* Convulxin (CVX), a C-type lectin from the venom of *Crotalus durissus terrificus*, is a potent activator of human platelets, binding predominantly to glycoprotein (GP)VI. Native CVX is an octamer composed of four $\alpha\beta$-heterodimers $[(\alpha\beta)_4]$. Two different native sequences have been reported, one bearing lysine (K), the other glutamic acid (E), at β chain residue 89, but the physiological relevance of this difference is unknown. *Objective:* We used the *Drosophila* S2 system to express recombinant CVX (rCVX) heterodimers ($\alpha\beta$) and site-directed mutagenesis to evaluate the influence of multimer size and the substitution βK89E on CVX function. *Methods:* By flow cytometry, native CVX and both recombinant forms bind to human platelets in whole blood. By surface plasmon resonance (BIAcore, Piscataway, NJ, USA), the calculated equilibrium dissociation constants (K_D) were: rCVX $\alpha\beta$89K, 11.3×10^{-8} M; rCVX $\alpha\beta$89E, 9×10^{-8} M; and native CVX, 2.8×10^{-8} M. *Results:* Thus, the affinities of the two rCVX forms for human, recombinant GPVI are essentially the same, but the relative affinity of native CVX is about 3-fold higher. The minimum concentration of native CVX that induces maximal human platelet aggregation (70 pM) is roughly 400-fold lower than that of either rCVX (29 nM). *Conclusions:* These results are consistent with the hypothesis that the ability of the native CVX octamer to cluster mobile GPVI molecules within the platelet membrane may be the single most important factor that contributes to the efficiency with which CVX is able to induce platelet activation.

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Introduction

Convulxin (CVX), a glycosylated C-type lectin, was first isolated from the venom of *Crotalus durissus cascavella* [1] as a potent inducer of platelet aggregation. It was later shown that it is also produced in the venom of another related subspecies, *C. durissus terrificus* [2]. Because of its high affinity for glycoprotein (GP)VI, CVX has been used as a powerful tool to investigate the function of this important collagen receptor that is instrumental in the initiation of platelet activation and thrombus formation.

Previous findings are consistent with the notion that clustering of GPVI is necessary to induce platelet aggregation. For example, polypeptides composed of repeat Glycine–Proline–Hydroxyproline sequences, one of the fundamental collagen-binding motifs, do not activate human platelets unless they are cross-linked [3]. Moreover, purified F(ab′)2 fragments of anti-GPVI antibodies can induce platelet aggregation, whereas Fab fragments of the same do not [4,5]. Likewise, rat anti-GPVI antibody (JAQ1) induces mouse platelet aggregation only when the antibodies are cross-linked by polyclonal rabbit antirat immunoglobulin G (IgG) antibodies [6]. Thus, it is reasonable to argue that GPVI-specific ligands, whether antibodies, collagens or CVX, will initiate signal transduction and platelet activation most efficiently when they are able to cluster GPVI on the membrane surface.

Studies of the structure of CVX have a direct bearing on this hypothesis. The initial reports, based on hydrodynamic studies, suggested that CVX has a hexameric structure $(\alpha\beta)_3$, involving two distinct subunits, as is the case with alboluxin [2,7]. However, two independent studies employing crystallographic analysis have since shown conclusively that native CVX exists as a tetra-heterodimer composed of four $\alpha\beta$-heterodimers $(\alpha\beta)_4$ [8–10]. The $\alpha\beta$ heterodimers are linked by interchain disulfide bonds between Cys79 of the α-subunit and Cys76 of the β-subunit. The heterodimers are then linked one to another by intersubunit disulfide bonds between Cys135 of the α-subunit and Cys3 of the β-subunit. This distinctive, cyclic

$(\alpha\beta)_4$ structure of CVX has also been observed for another snake venom toxin, flavocetin-A [11]. It is reasonable that multimerization of CVX is essential for efficient platelet activation, but proof of this hypothesis has awaited the availability of monomeric CVX.

Two different native gene sequences for CVX have been reported (GenBank Accession Numbers Y16349 and AF541881) differing by a single amino acid residue, lysine (K) vs. glutamic acid (E) at β89. The possibility exists that this single amino acid substitution might influence CVX function, as these amino acids have very different properties: glutamic acid is negatively charged, while lysine is positively charged. In CVX, amino acid residue 89 is located in the swapped loop of the β chain [11].

Because of this location is adjacent to the amino acid residues involved in the putative-binding site for GPVI [8], we were compelled to analyze the effect of this substitution on the interaction between GPVI and CVX. Our study is the first report of the synthesis of recombinant CVX (rCVX) and the first analysis of the relevance of this putative natural dimorphism to CVX function.

Experimental procedures

Convulxin cDNA constructs

A cDNA library of the venomous gland of *C. durissus terrificus* was developed as previously reported [12]. Full length CVX α- and β-subunit cDNAs were amplified by KOD Hot Start DNA Polymerase (Novagen, Madison, WI, USA) from the cDNA library using the primer pairs: CVX-AF: 5 ′-TCTCTCTGC-AGGGAAGGAAG -3′ and CVX-AR: 5′-TCCTTGCTT-CTCCAGACTTCA-3′ for the α-subunit; and CVX-BF: 5′-TCTCTCTGCAGGGAAGGAAG-3′ and CVX-BR: 5′-ACTTCACACAGCCGGATCTT-3′ for the β-subunit. The polymerase chain reaction (PCR) products were subcloned into the TA cloning vector PCR2.1 (Invitrogen, Carlsbad, CA, USA) and sequenced. The result showed an A418G substitution in the β-chain cDNA (sequence numbered according to the GenBank Y16349).

Using full length cDNA as template, both the α- and β-subunit cDNA fragments, each encoding the mature polypeptide, were again amplified by PCR using the following primer pairs: CVXA-F1 (5′-ACTAGATCTGGT TTACATTGTCCCTCTGA-3′) and CVXA-R1 (5′-CTC-GAGTTAACACTGTGGCGGGAACTTGC-3′) for the α-subunit; and CVXB-F1 (5′-AGTAGATCTTGGTTT-CTGTTGTCCCTCCC-3′) and CVXB-R1 (5′-CTCGA-GTGCCTCGAACTTGCAGACGA-3′) for the β-subunit. The cDNA fragments were individually subcloned into the *Drosophila* expression vector pMT/BiP/V5-His (Invitrogen). Sequencing verified that the final α-subunit cDNA construct (pMT/CVX-A) encodes a BiP signal peptide upstream from the mature CVX α-subunit, while the β-subunit cDNA construct (pMT/CVX-BE), from which the original termination codon was removed, encodes, in order, the BiP signal peptide, the mature CVX β-subunit, a V5 epitope, and a hexa-Histidine tag. The β-subunit sequence was identical to that of GenBank accession number AF541881.

The CVX β-subunit having the same sequence as Y16349 was then created by replacing G with A at 418. For this single base change, site-directed mutagenesis was performed in two stages. In the first stage, the following primers were used: MTF: 5′-CATCTCAGTGCAACTAAA-3′, EK1: 5′-TTCTTCATG-CCATTCTTT-3′; BGHR: 5′-TAGAAGGCACAGTC-GAGG-3′, and EK2: 5′-ACCAAGCCTGAGTACAAA-3′. The MTF/EK1 primer pair amplified a 382 bp PCR product; the BGHR/EK2 pair, a 256 bp product. A mixture of PCR products from the first stage was used as template for the second stage PCR. In the second stage, a 605 bp product was created using the primer pair of MTF/BGHR and subcloned into the same vector, pMT/BiP/V5-His (Invitrogen). Sequencing verified that this construct (pMT/CVX-BK) is identical to the first construct (pMT/CVX-BE) except for the substitution G418A.

Expression of rCVX

Drosophila S2 cells (ATCC, Manassas, VA, USA) were grown in Schneider's Modified Drosophila Medium supplemented with 10% fetal bovine serum (complete medium). 4×10^6 S2 cells in 2 mL complete media were transfected with 1 μg each of pMT/CVX-A and pMT/CVX-BE or pMT/CVX-BK together with 50 ng of pPac-HygB [13], a selection vector, using FuGENE6 (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer's instructions. The cells were cultured for 2 days in presence of the DNA mixture before they were exposed to 300 μg mL^{-1} of Hygromycin B. Stable cell lines were obtained after 4 weeks of culture in the selection medium, and then secretion of rCVX was induced by addition of 500 μM $CuSO_4$. Culture supernatants were harvested 4–6 days after the addition of $CuSO_4$ and screened for rCVX by Western blotting using the commercial antihexa-Histidine antibody His Probe (Santa Cruz Biochemistry, Santa Cruz, CA, USA). Positive cell lines were transferred to Insect-Xpress serum free medium (BioWhittaker, Inc., Walkersville, MD, USA), and then secretion of CVX was induced by addition of 750 μM $CuSO_4$ and 1% DMSO. Culture supernatants were harvested 4–6 days after induction, and secreted rCVX with its C terminal hexa-Histidine tag was directly purified by the method of Lehr [14] using a HiTrap chelating HP column (Amersham Biosciences, Piscataway, NJ, USA). The harvested rCVX was further purified on a Ni-NTA column (Qiagen Inc., Valencia, CA, USA). Purified rCVX was extensively dialyzed against phosphate-buffered saline (PBS), pH 7.4, to remove imidazole.

Fast protein liquid chromatography (FPLC)

An rCVX was analyzed on a Superdex 200 10/300 GL column (Amersham Biosciences) with a bed volume of 24 mL. The column was pre-equilibrated in PBS (pH 7.4) containing 5 mM *N*-ethylmaleimide (NEM) (Sigma-Aldrich, St Louis, MO, USA) to prevent disulfide exchange. 0.2 mg of rCVX was

loaded in 0.2 mL of PBS with 5 mM NEM and run at 0.5 mL min^{-1}. Elution of the compounds was established by monitoring absorbance at 280 nm.

Binding of rCVX to recombinant soluble GPVI

rCVX was tested for its reactivity with recombinant soluble human GPVI (rhGPVI) by a ligand-blotting assay. The hrGPVI was produced by a *Drosophila* S2 expression system as previously reported. One microgram rCVX was first subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% milk in Tris-buffered saline, pH7.4, containing 0.05% Tween 20 (TBST), the membrane was incubated at ambient temperature for 1 h in TBST buffer containing biotinylated, soluble rhGPVI [13] at a concentration of 1 μg mL^{-1}. After thorough rinsing, the membrane was further incubated with Peroxidase-conjugated Streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at ambient temperature for 1 h. After subsequent rinsing, bound biotinylated rhGPVI was visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) entailing exposure to X-ray film.

Specificity of rCVX for GPVI

Human platelets were isolated from platelet-rich plasma (PRP) and solubilized in 1% Triton X-100 or 1% SDS as described [15]. Forty micrograms of platelet proteins were subjected to SDS–PAGE and then transferred to a PVDF membrane. The membrane was blocked in 5% milk/TBST and then incubated at ambient temperature for 1 h in TBST containing 1 μg mL^{-1} rCVX. Bound rCVX was visualized with His Probe followed by AP-conjugated antirabbit IgG, followed by NBT and BCIP.

Flow cytometry

To prepare biotin-conjugated rCVX (biotin-rCVX), purified rCVX in PBS, pH 7.4, at a concentration of 1 mg mL^{-1} was mixed with 1/100 volume of Sulfo-NHS-LC biotin (Molecular Probes, Eugene, OR, USA) dissolved in DMSO at a concentration of 10 mg mL^{-1} to give a final concentration of 0.1 mg mL^{-1}. After 2 h incubation at ambient temperature, the mixture was extensively dialyzed against PBS, pH 7.4, containing 0.05% sodium azide to remove free biotin.

Three microlitres of whole blood were mixed with 50 μL of PBS containing 1 mg mL^{-1} bovine serum albumin (PBS-BSA) and 0.1 μg of biotin-rCVX. The mixture was gently agitated and incubated for 15 min at ambient temperature. Two microlitres of streptavidin-phycoerythrin (PE; PharMingen, San Diego, CA, USA) were added, and the mixture was gently agitated and incubated for an additional 15 min at ambient temperature. One microlitre of PBS was added to each mixture just prior to flow cytometry assay. Measurements were obtained using a Becton Dickinson FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) and the data were reported as geometric mean fluorescence intensity (GMFI). Data analysis was performed using WinMDI 2.8 (http://facs.scripps.edu/software.html).

Surface plasmon resonance studies

Evaluation of kinetic-binding parameters was performed using an surface plasmon resonance (SPR)-based Biacore 3000 biosensor. The following reagents were obtained from Biacore, Inc.: carboxymethyl dextran chips (CM5; research grade); *N*-hydroxysuccimide (NHS); 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC); ethanolamine–HCl; sodium acetate buffer, pH 4.5, and pH 3 or 2.5 glycine buffers. A quantity of 0.01 M Hepes buffer, pH 7.4, 0.15 M NaCl containing 2 mM *N*-ethylmaleimide (HBS-NEM) was added to all solutions. Native CVX $(\alpha\beta)_4$ and both recombinant forms of CVX ($\alpha\beta$89K and $\alpha\beta$89E) were immobilized on CM5 chips by an amine coupling procedure [16]. Briefly, after two surfaces were activated with NHS/EDC for 5 min, CVX was injected at a concentration of 15 μg mL^{-1} in sodium acetate buffer pH 4.5 in one flow cell at a flow rate of 10 μL min^{-1}. The other flow cell was perfused with HBS-NEM buffer as a blank. Any remaining activated groups were then blocked by a 5 min injection of 1 M ethanolamine. Binding kinetics were established over a concentration range of 1.5×10^{-6} to 0.13×10^{-6} M for hrGPVI, using an association phase of two min at 10 μL min^{-1} and a dissociation phase of 4 min. At the end of each cycle, the surface was regenerated at a flow rate of 50 μL min^{-1} with a 30 s injection of 10 mM glycine–HCl (pH 2.5) once for native CVX and a 6 s injection of 10 mM glycine–HCl (pH 3.0) twice for rCVX. The stabilization was performed for 1 min between each cycle. The kinetic analysis was conducted using BIA evaluation 3.2 software (Biacore AB). The analysis of reaction kinetics could be affected by the phenomenon of mass transport. To assess the influence of this phenomenon, the CVX-coated surface was perfused at different flow rate between 5 and 75 μL min^{-1} with hrGPVI (0.975×10^{-6} M). As no change was observed in the initial binding, any effect of mass transport was considered negligible. All binding curves were corrected for background by subtraction of results obtained with reference flow cells and injection of 0 M hrGPVI. Models were fitted globally using a single site-binding model.

Platelet aggregation assay

Platelet-rich plasma was isolated from 3.2% sodium citrate-anticoagulated whole blood, as described [15], and the platelet count was adjusted to 3.0×10^5 μL^{-1} with autologous platelet poor plasma (PPP). Four hundred microliters of PRP was dispensed into a glass cuvette and prewarmed at 37 °C. PPP was used as a blank. Native CVX purchased from Centerchem (Norwalk, CT, USA) and rCVX were added to PRP as agonists. Aggregation profiles were generated on a Chrono-Log aggregometer (Havertown, PA, USA).

Results

Expression of rCVX the Drosophila S2 system

After protein synthesis was induced by addition of $CuSO_4$, rCVX was detected by Western blot assay in the culture supernatant of transfected S2 cells (Fig. 1). The yield of rCVX $\alpha\beta$89K and rCVX $\alpha\beta$89E was similar, ranging from 3–5 μg mL^{-1}. Using immobilized metal-ion affinity chromatography, the relatively pure rCVX was eluted in fractions 3–5 (lane 2–4, Fig. 1A). After these, fractions were combined and diluted against PBS, the purity of rCVX was estimated to be > 95% as judged by sliver staining (Fig. 1B). The apparent molecular weight of the β-subunit, with the V5 and hexa-Histidine tags, was 18 kDa; that of the α-subunit was 14 kDa.

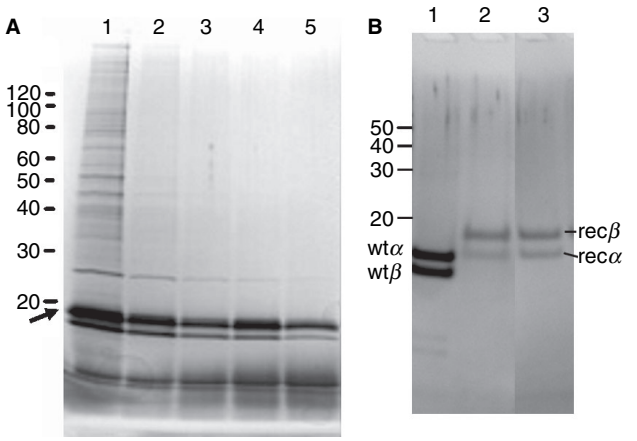

Fig. 1. Purification of recombinant CVX (rCVX). (A) *Drosophila* S2 cells (ATCC) were grown in Schneider's Modified Drosophila Medium supplemented with 10% fetal bovine serum. 4×10^6 S2 cells in 2 mL complete media were transfected with 1 μg each of pMT/CVX-A and pMT/CVXA-BK or -BE along with 50 ng of pPac-HygB, as selection vector, using FuGENE6 (Roche Applied Science). Stable cell lines were obtained after 4 weeks of culture in the Hygromycin B selection medium (300 μg mL^{-1}), and then protein secretion of rCVX was induced by addition of 500 μM $CuSO_4$. Culture supernatants were harvested 4–6 days after induction, and secreted rCVX with a β-chain C terminal hexa-Histidine tag was directly purified using a HiTrap chelating HP column (Amersham). The harvested rCVX was further purified on a Ni-NTA column (Qiagen) and then analyzed by SDS–PAGE under reduced conditions using 4–20% Tris-Glycine gels (Invitrogen). Proteins were visualized by silver staining. Lanes 1–5 correspond to fractions 2, 3, 5, 6, and 7 containing rCVX$\alpha\beta$89E eluted from the NTA column. Fraction 2 was eluted with 50 mM imidazole, while fractions 3, 5, 6, and 7 were eluted with 250 mM imidazole. A prominent band with an MW_{App} of 18 kDa, consistent with the electrophoretic mobility of the rCVX β-chain, was detected in each eluted fraction (arrowhead). Magic Mark (Invitrogen) was employed as protein molecular weight marker. (B) Fractions containing rCVX that had been eluted with 250 mM imidazole were combined, and the purity of the pooled rCVX preparation was estimated to be > 95%, as judged by SDS–PAGE and visualization with sliver stain. The following purified protein preparations were separated under the reduced conditions using a 12 % Bis-Tris polyacrylamide gels (Invitrogen): (Lane 1) native CVX; (Lane 2) rCVX$\alpha\beta$89K; (Lane 3) rCVX $\alpha\beta$89E. In the case of native CVX (lane 1), the expected protein bands corresponding to the 14 kDa wild-type α-subunit (wtα) and the 13 kDa wild-type β-subunit (wtβ) are indicated. In the case of either isoform of rCVX (lane 2 or 3), only two bands are also evident: the larger with an MW_{App} of 18 kDa corresponding to the recombinant β-subunit (recβ), larger than the native β-subunit because of the addition of the V5 and hexa-histidine tags, and the smaller with an MW_{App} of 14 kDa corresponding to the recombinant α-subunit (recα).

Under non-reduced conditions, a Western blot assay using His Probe showed that the recombinant protein has an apparent molecular weight of about 30 kDa, which corresponds to the size expected of an $\alpha\beta$-heterodimer (Fig. 2A, lane 1). The results for rCVX $\alpha\beta$89E are shown; identical findings were obtained for rCVX $\alpha\beta$89K (not shown). Under reduced conditions, the hexa-histidine tagged β-subunit was detected, with an apparent molecular weight equivalent to the expected size (18 kDa with theV5 and hexa-Histidine tags) (Fig. 2A, lane 3). The α-subunit, lacking, a hexa-histidine tag, is not visible by this approach.

Analysis of the peak fractions by size exclusion chromatography using a Superdex 200 10/300 GL column in the presence of NEM (non-reduced) yielded a single 30 kDa peak, a size of consistent with the presence of $\alpha\beta$-heterodimers (not shown). No larger-sized multimers were detected by FPLC.

Ligand blot assay of CVX to human GPVI

The binding of soluble, rhGPVI to the constituents of purified rCVX, separated by SDS–PAGE and transferred to nitrocellulose, was tested by a ligand blot assay (Fig. 2B,C). rhGPVI bound predominantly to a protein with an MW_{App} of 27 kDa in the rCVX preparation (Fig. 2B, lane 3). Binding to a minor constituent with an MW_{App} of 55 kDa was also observed. This larger band has an MW_{App} equivalent to that expected for rCVX tetramers $[(\alpha\beta)_2]$. On the other hand, in native CVX, the rhGPVI-binding capability lies predominantly in large molecular weight components, with an MW_{App} expected of CVX octamers $[(\alpha\beta)_4]$ (Fig. 2B, lanes 1, 2).

The specificity of rCVX was also tested by Western blot, using total proteins of human platelets lyzed in either 1% Triton X-100 or 1% SDS. Platelet proteins were separated by SDS–PAGE, transferred to a PVDF membrane, and probed with biotin-rCVX. As previously reported for native CVX [15], biotin-rCVX binds to two protein bands in the SDS-solubilized platelet lysates, but to a single band in Triton X-100 solubilized lysates (Fig. 2C).

Binding of rCVX to intact platelets

By flow cytometry, native CVX and both recombinant forms of CVX ($\alpha\beta$89K and $\alpha\beta$89E) bind to human platelets in whole blood in dose-dependent manner (data not shown). When an excess amount of biotin-labeled native CVX, rCVX $\alpha\beta$89K and rCVX $\alpha\beta$89E (each, 0.1 μg) was incubated with whole blood, binding was reflected by GMFI of 134.3, 71.47, and 68.22, respectively.

Kinetics of the interaction between hrGPVI and CVX

The interaction between rhGPVI and either native CVX or rCVX was directly measured by surface plasmon resonance. Native CVX, rCVX $\alpha\beta$89K or rCVX $\alpha\beta$89E were first

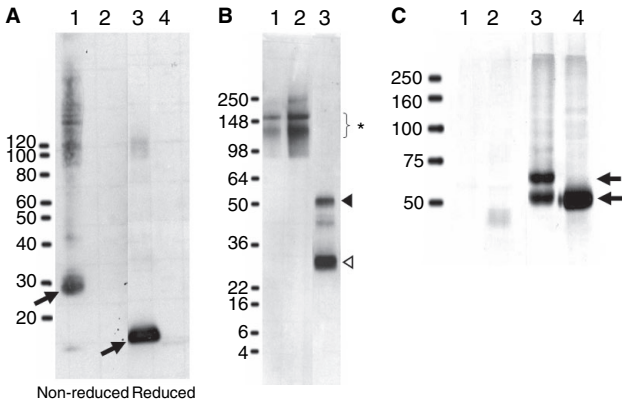

Fig. 2. Recombinant CVX binds specifically to human GPVI. (A) Western blot. Total proteins secreted into the media of *Drosophila* S2 cells cultured for 4 days were separated by SDS–PAGE and transferred to PVDF membranes. The presence of rCVX was determined by the binding of the antihexa-Histidine antibody His Probe (Santa Cruz Biochemistry) and visualization with ECL Western Blotting Detection Reagents (Amersham Biosciences). Magic Mark (Invitrogen) was used as the standard for protein molecular weight determinations. Proteins in media from *Drosophila* S2 cells transfected with pMT/CVX-A plus pMT/CVX-BK were analyzed without disulfide reduction (Non-reduced) in the presence of *N*-ethylmaleimide (NEM) (lane 1) or following disulfide reduction (reduced) with 2-ME (lane 3). For comparison, media from non-transfected (control) S2 cells were also analyzed under non-reduced (lane 2) or reduced (lane 4) conditions. Under non-reduced conditions, a major band with an MW_{App} of 28 kDa (diagonal arrow) is visible in media from S2 cells secreting rCVX (lane 1); after reduction (lane 3), a single band with an MW_{App} < 20 kDa (diagonal arrow), corresponding to the His(6)-tagged β chain, is visible. The electrophoretic mobility of molecular weight markers and their corresponding size (kDa) are indicated to the left of the gel. These results as well as those depicted in (B) and (C) below were derived from single experiments, in each case, that are representative of three separate experiments that generated essentially identical results. (B) Ligand blot. A ligand blot assay was employed to test the ability of biotin-rhGPVI to bind to (Lane 1) 3.8 μg of native CVX, (lane 2) 15.2 μg of native CVX or (lane 3) 3.5 μg of rCVX, which had been separated by SDS–PAGE under non-reduced conditions (in the presence of NEM) and transferred to a PVDF membrane. The presence of bound biotin-rhGPVI was visualized with HP-streptavidin and ECL. In the case of native CVX (lanes 1 and 2), biotin-rhGPVI binds exclusively to high molecular weight components with an MW_{app} between 120 and 160 kDa (*), which likely represent CVX hexamers and/or octamers. In the case of rCVX (lane 3), biotin-rhGPVI binds predominantly to components with an MW_{App} of 27 kDa (white arrowhead), corresponding to $\alpha\beta$-heterodimers, while binding to a minor band with an MW_{App} of about 55 kDa (black arrowhead) was also observed. This higher molecular weight band likely represents a minor proportion of tetramers. Relative molecular weights were determined using the protein molecular weight marker SeeBlue Puls2 (Invitrogen) and are indicated to the left of the gel. (C) The binding of rCVX to platelet proteins. Total protein (40 μg) from washed human platelets, solubilized in either 1% SDS (Lanes 1 and 3) or 1% Triton X-100 (Lanes 2 and 4), were separated by SDS–PAGE and transferred to PVDF membranes. The binding rCVX to separated proteins was then determined by ligand blot. Bound rCVX was visualized with His Probe followed by AP-conjugated antirabbit IgG, followed by NBT and BCIP. Full-range rainbow molecular weight markers (Amersham Biosciences) were employed to determine relative molecular weights and are indicated to the left of the gel. Lanes 1 and 2 were probed with culture media from non-transfected S2 cells (control); lanes 3 and 4 were probed with culture media containing rCVX. Protein bands with an MW_{app} expected for GPVI, solubilized in either SDS (lane 3) or Triton X-100 (lane 4), were observed and their positions are indicated by black arrows to the right of lane 4.

immobilized onto CM sensor chips. After the chip surface was activated, rhGPVI at concentrations ranging from 1.5×10^{-6} M to 0.13×10^{-6} M was perfused over the chip, and the molecular interactions were monitored in real time. Table 1 summarizes the kinetics of these protein–protein interactions, as association rate constants: K_a (M^{-1} s^{-1}), dissociation rate constants: K_d (s^{-1}), and equilibrium constants: K_A (M^{-1}), K_D (M). Based on relative equilibrium constants, the affinities of the two rCVX forms for hrGPVI were essentially the same, while that of native CVX was roughly 3-fold higher.

Relative activity of native CVX and rCVX

The relative ability of each CVX preparation to induce human platelet aggregation was investigated (Fig. 3). Native CVX, rCVX $\alpha\beta$89K and rCVX $\alpha\beta$89E were added to human PRP at a concentration ranging from 5 ng mL^{-1} to 40 μg mL^{-1}, and the minimum concentration required to induce maximal aggregation was determined. These were: 70 pM, in the case of native CVX, and 29 nM, in the case of either form of rCVX. Thus, on a molar basis, roughly a 400-fold higher concentration of rCVX is required, relative to that of native CVX.

Discussion

This is the first report of the successful synthesis of rCVX in any expression system. The V5 and hexa-histidine tags were added to the C-terminus of the β-subunit to facilitate purification of the assembled $\alpha\beta$ heterodimers by immobilized metal-ion affinity chromatography. With this approach, we were able to recover rCVX in culture supernatants at concentrations as high as 3–5 μg mL^{-1}. The recombinant protein has an MW_{App} of 30 kDa, which likely represents the $\alpha\beta$ heterodimer. In a ligand blot assay, rhGPVI strongly binds to the rCVX $\alpha\beta$ heterodimer, indicating that the $\alpha\beta$ heterodimer retains its ability to bind to human GPVI. Furthermore, rCVX $\alpha\beta$ heterodimers bind exclusively to a single protein in human platelet lysates with an MW_{App} of 60 KDa, the expected size of GPVI. These findings confirm that the synthesized rCVX retains its specificity for GPVI.

To date, two isoforms of the CVX β-subunit have been reported. The most probable explanation for this dimorphism is that the amino acid difference is a natural variation within this species. Geographically restricted polymorphisms in snake venom toxin genes have been recorded [17,18]. For example, crotamine, another toxin from *C. durissus terrificus*, has a number of such polymorphisms. [19] Each of four isoforms of crotamine contains a different amino acid substitution at the same position. Another example is acidic phospolipase A2 produced by *C. viridis viridis* [20]. Our cDNA library was constructed from the venomous gland of a single adult specimen captured near Sao Paulo, Brazil, but the geographical origin of other cDNA libraries have not been specified [8,10,21]. Moreover, additional studies of several additional members of *C. durissus terrificus* obtained at more than one

Table 1 Kinetics of the interaction between recombinant soluble human GPVI (hrGPVI) and convulxin (CVX)

	K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	K_A (M^{-1})	K_D (M)
Glycoprotein VI (GPVI) vs. recombinant CVX (rCVX)$\alpha\beta$89K	1.28×10^4	1.44×10^{-3}	0.890×10^7	11.3×10^{-8}
GPVI vs. rCVX$\alpha\beta$89E	1.33×10^4	1.19×10^{-3}	1.12×10^7	8.95×10^{-8}
GPVI vs. native CVX $(\alpha\beta)_4$	3.62×10^4	0.998×10^{-3}	3.63×10^7	2.75×10^{-8}

An evaluation of kinetic-binding parameters was performed using SPR-based Biacore 3000 biosensor by direct measurement of the interaction between rhGPVI and either native CVX or rCVX. Native CVX and both recombinant forms of CVX were immobilized on CM5 chips by an amine-coupling procedure. After the chip surface was activated, rhGPVI at concentrations ranging from 1.5 to 0.13×10^{-6} M was perfused over the chip, and the molecular interactions were monitored in real time. The kinetics of these protein–protein interactions, as association rate constants: K_a (M^{-1} s^{-1}), dissociation rate constants: K_d (s^{-1}), and equilibrium constants: K_A (M^{-1}) or K_D (M) is summarized here. Based on the relative equilibrium constants, the affinities of the two rCVX forms for hrGPVI were essentially the same, while that of native CVX as roughly 3-fold higher.

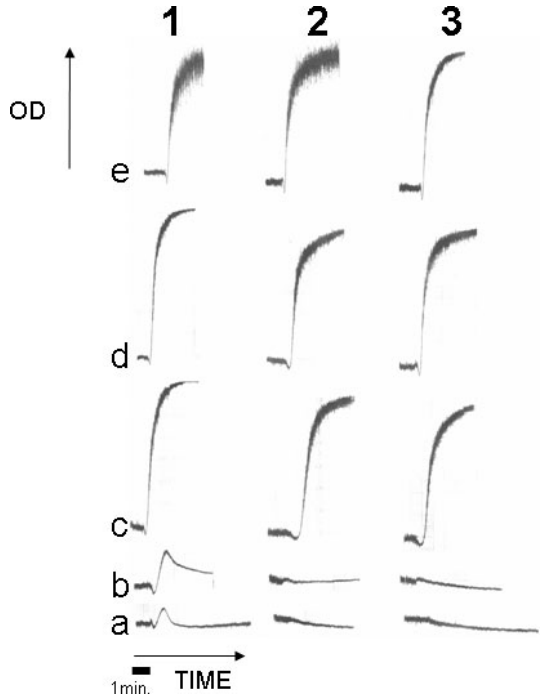

Fig. 3. A comparison of platelet aggregation induced by native CVX and rCVX. Platelet aggregation was induced by (i) native CVX, (ii) rCVX $\alpha\beta$89K or (iii) rCVX $\alpha\beta$89E. In each case, the increase in optical density (ordinate) was recorded as a function of time (abscissa). Native CVX (i) was added at the following final concentrations (ng mL^{-1}): (a) 5; (b) 7.5; (c) 10; (d) 12.5; and (e) 50; while rCVX $\alpha\beta$89K (ii) or $\alpha\beta$89E (iii) were each added at the following concentrations (μg mL^{-1}): (a) 0.5; (b) 0.75; (c) 1; (d) 7.5; and (e)10. By inspection, the minimum concentrations required to induce maximal aggregation are 10 ng mL^{-1} (70 pM) for native CVX, and 1 μg mL^{-1} (29 nM) for either rCVX. Thus, compared with native CVX, on a molar basis, roughly a 400-fold higher concentration of rCVX is required to induce aggregation. These results were obtained in one set of experiments that generated results essentially identical to those obtained in three independent sets of experiments.

geographical location would be required to conclusively prove that E89K is a naturally occurring polymorphism, but these are beyond the scope of this study.

The possibility that E89K would affect CVX function was formally tested in this report because this non-conservative amino acid substitution would be expected to markedly change side chain association in the localized region. Our result shows no substantial difference between these isoforms of rCVX, with respect to the kinetics of rCVX-GPVI binding or the ability to induce platelet aggregation. Thus, our results clearly demonstrate that β chain K89E has no effect. It is likely that this dimorphism has persisted because it is innocuous.

Prior reports have suggested that higher-ordered structure of certain platelet agonists is required for maximal platelet activation and signal transduction [22]. One such agonist is von Willebrand factor (VWF). The native VWF, multimer form of VWF, is much more effective than monomeric VWF although the binding of the native and monomer proteins is comparable. [23] Another is Echicetin, a heterodimeric snake C-type lectin, which specifically binds to GP Ibα [24]. A heterodimeric form of Echicetin activates platelets only when multimerized by IgM. It is likely that clustering of receptor molecules by polyvalent ligands results in a significant augmentation of the activation signal.

Therefore, it has been assumed that the multimerization of CVX contributes significantly to its full activity and the efficiency with which it induces platelet activation. Polgar [25] reported that partially denatured CVX could not induce platelet aggregation and even acted as an inhibitor of collagen-induced platelet aggregation. On the other hand, our results indicate that the purified monomeric rCVX retains the ability to induce platelet aggregation, although its activity is significantly attenuated. This observation indicates that the $\alpha\beta$-heterodimer retains sufficient structure necessary for the binding properties of CVX. However, the increased avidity of the octameric native CVX molecule is evident in our analyses and confirms the significant contribution of multimerization to the activity of the native molecule. As shown in real-time protein–protein-binding studies (Table 1), the octameric form $(\alpha\beta)_4$ of native CVX exhibits at least a threefold increase in affinity for GPVI. In addition, the results of platelet aggregation assays verify that octameric CVX has 400-fold higher ability to induce platelet aggregation relative to the recombinant heterodimer. This means that the increased agonist activity can be attributed to a greater extent to increased avidity, owing to multimer size, as opposed to an increase in affinity alone. These results are consistent with the hypothesis that the native CVX octamer is able to cluster mobile GPVI molecules within the platelet membrane and that clustering of the GPVI together with its coreceptor FcR γ chain facilitates signal transduction and platelet activation.

The development of rCVX will now enable us to analyze specific sequences that are involved in its binding to GPVI as well as GPIbα. As two different binding sites for GPVI have been proposed within CVX [7], site-directed mutagenesis will enable us to differentiate the relative contribution of each site. In addition, modified forms of rCVX heterodimers may prove to be efficient inhibitors of platelet activation by native CVX and other GPVI agonists.

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References

1 Vargaftig BB, Prado-Franceschi J, Chignard M, Lefort J, Marlas G. Activation of guinea-pig platelets induced by convulxin, a substance extracted from the venom of *Crotalus durissus cascavella*. *Eur J Pharmacol* 1980; **68**: 451–64.

2 Marlas G. Isolation and characterization of the alpha and beta subunits of the platelet-activating glycoprotein from the venom of *Crotalus durissus cascavella*. *Biochimie* 1985; **67**: 1231–9.

3 Farndale RW, Siljander PR, Onley DJ, Sundaresan P, Knight CG, Barnes MJ. Collagen-platelet interactions: recognition and signalling. *Biochem Soc Symp* 2003; **70**: 81–94.

4 Sugiyama T, Okuma M, Ushibuki F, Sensaki S, Kanaji K, Uchino H. A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. *Blood* 1987; **69**: 1712–20.

5 Clemetson JM, Polgar J, Magnenat E, Wells TNC, Clemetson KJ. The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcαR and the natural killer receptors. *J Biol Chem* 1999; **274**: 29019–24.

6 Nieswandt B, Bergmeier W, Schulte V, Rackebrandt K, Gessner JE, Zirngibl H. Expression and function of the mouse collagen receptor GPVI is strictly dependent on its association with the FcR gamma chain. *J Biol Chem* 2000; **275**: 23998–4002.

7 Nidergang F, Alcover A, Knight CG, Farndale RW, Barnes MJ, Francischetti IMB, Bon C, Leduc M. Convulxin binding to platelet receptor GPVI: competition with collagen related peptides. *Biochem Biophys Res Comm* 2000; **273**: 246–50.

8 Batuwangala T, Leduc M, Gibbins JM, Bon C, Jones EY. Structure of the snake-venom toxin convulxin. *Acta Crystallogr D Biol Crystallogr* 2004; **60**: 46–53.

9 Murakami MT, Watanabe L, Gava LM, Zela SP, Cintra AC, Arni RK. Initial structural analysis of an alpha4beta4 C-type lectin from the venom of *Crotalus durissus* terrificus. *Acta Crystallogr D Biol Crystallogr* 2003; **59**: 1813–5.

10 Murakami MT, Zela SP, Gava LM, Michelan-Duarte S, Cintra AC, Arni RK. Crystal structure of the platelet activator convulxin, a disulfide-linked alpha(4)beta(4) cyclic tetramer from the venom of *Crotalus durissus terrificus*. *Biochem Biophys Res Commun* 2003; **310**: 478–82.

11 Fukuda K, Mizuno H, Atoda H, Morita T. Crystal structure of flavocetin-A, a platelet glycoprotein Ib-binding protein, reveals a novel cyclic tetramer of C-type lectin-like heterodimers. *Biochemistry* 2000; **39**: 1915–23.

12 Radis-Baptista G, Oguiura N, Hayashi MA, Camargo ME, Grego KF, Oliveira EB, Yamane T. Nucleotide sequence of crotamine isoform precursors from a single South American rattlesnake (*Crotalus durissus terrificus*). *Toxicon* 1999; **37**: 973–84.

13 Kanaji S, Kanaji T, Furihata K, Kato K, Ware JL, Kunicki TJ. Convulxin binds to native, human glycoprotein Ib alpha. *J Biol Chem* 2003; **278**: 39452–60.

14 Lehr RV, Elefante LC, Kikly KK, O'Brien SP, Kirkpatrick RB. A modified metal-ion affinity chromatography procedure for the purification of histidine-tagged recombinant proteins expressed in *Drosophila* S2 cells. *Protein Expr Purif* 2000; **19**: 362–8.

15 Furihata K, Clemetson KJ, Deguchi H, Kunicki TJ. Variation in human platelet glycoprotein VI content modulates glycoprotein VI-specific prothrombinase activity. *Arterioscler Thromb Vasc Biol* 2001; **21**: 1857–63.

16 Johnsson B, Lofas S, Lindquist G. Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Anal Biochem* 1991; **198**: 268–77.

17 Chippaux JP, Williams V, White J. Snake venom variability: methods of study, results and interpretation. *Toxicon* 1991; **29**: 1279–303.

18 Daltry JC, Wuster W, Thorpe RS. Diet and snake venom evolution. *Nature* 1996; **379**: 537–40.

19 Smith LA, Schmidt JJ. Cloning and nucleotide sequences of crotamine genes. *Toxicon* 1990; **28**: 575–85.

20 Tsai IH, Wang YM, Chen YH, Tu AT. Geographic variations, cloning, and functional analyses of the venom acidic phospholipases A2 of *Crotalus viridis viridis*. *Arch Biochem Biophys* 2003; **411**: 289–96.

21 Leduc M, Bon C. Cloning of subunits of convulxin, a collagen-like platelet-aggregating protein from *Crotalus durissus terrificus* venom. *Biochem J* 1998; **333**: 389–93.

22 Andrews RK, Gardiner EE, Shen Y, Berndt MC. Structure-activity relationships of snake toxins targeting platelet receptors, glycoprotein Ib-IX-V and glycoprotein VI. *Curr Med Chem Cardiovasc Hematol Agents* 2003; **1**: 143–9.

23 Senogles SE, Nelsestuen GL. von Willebrand factor. A protein which binds at the cell surface interface between platelets. *J Biol Chem* 1983; **258**: 12327–33.

24 Navdaev A, Dormann D, Clemetson JM, Clemetson KJ. Echicetin, a GPIb-binding snake C-type lectin from *Echis carinatus*, also contains a binding site for IgMkappa responsible for platelet agglutination in plasma and inducing signal transduction. *Blood* 2001; **97**: 2333–41.

25 Polgar J, Clemetson JM, Kehrel BE, Wiedemann M, Magnenat EM, Wells TNC, Clemetson KJ. Platelet activation and signal transduction by convulxin, a C-type lectin from C*rotalus durissus terrificus* (Tropical Rattlesnake) venom via the p62/GPVI collagen receptor. *J Biol Chem* 1997; **272**: 13576–83.