Identification and Recombinant Analysis of Botrocetin-2, a Snake Venom Cofactor for von Willebrand Factor-Induced Platelet Agglutination[+]

Yukiyo Yamamoto-Suzuki,[†] Yoshihiko Sakurai,[‡] Yoshihiro Fujimura,[§] Masanori Matsumoto,[§] Jiharu Hamako,[∥] Tetsuro Kokubo,[⊥] Hitoshi Kitagawa,[#] Sarkar M. A. Kawsar,[%] Yuki Fujii,[&] Yasuhiro Ozeki,[¶] Fumio Matsushita,[†] and Taei Matsui*,[†]

[†]Department of Biology, Faculty of Medical Technology, Fujita Health University School of Health Sciences, Toyoake, Aichi 470-1192, Japan

[‡]Department of Pediatrics, Nara Medical University, Kashihara, Nara 634-8522, Japan

[§]Department of Transfusion Medicine, Nara Medical University, Kashihara, Nara 634-8522, Japan

[∥]Department of Physiology, Faculty of Medical Management and Information Science, Fujita Health University School of Health Sciences, Toyoake, Aichi 470-1192, Japan

[⊥]Division of Molecular and Cellular Biology, Science of Supramolecular Biology, International Graduate School of Arts and Sciences, Yokohama City University, Yokohama, Kanagawa 230-0045, Japan

[#]Department of Veterinary Internal Medicine, Faculty of Applied Biological Sciences, Gifu University, Yanagido, Gifu 501-1193, Japan

[%]Laboratory of Carbohydrate and Protein Chemistry, Department of Chemistry, Faculty of Science, University of Chittagong, Chittagong-4331, Bangladesh

[&]Section of Functional Morphology, Faculty of Pharmaceutical and Sciences, Nagasaki International University, Sasebo, Nagasaki, 859-3298, Japan

[¶]Laboratory of Marine Biochemistry, Department of Environmental Biosciences, International Graduate School of Arts and Sciences, Yokohama City University, Yokohama, Kanagawa 236-0027, Japan

ABSTRACT: Botrocetin is a heterodimer snake venom protein that induces von Willebrand factor (VWF)- and platelet glycoprotein Ib (GPIb)-dependent platelet agglutination *in vitro*. We have cloned cDNAs for a botrocetin-2 from a cDNA library of the venom gland of *Bothrops jararaca* having a high similarity with botrocetin subunits. Recombinant botrocetin-2, expressed in 293T cells, showed cofactor activity comparable to natural botrocetin. In a single subunit expression experiment, a dimer of the β subunit was obtained, and it showed reduced, but apparent, platelet agglutination activity. Ala scanning mutagenesis showed that substitutions at Asp62, Asp70, Arg115, or Lys117 in the β subunit reduced platelet agglutination activity. The 3D homology modeling of botrocetin-2 complexed with the VWF A1 domain and GPIbα indicated that Asp62, Arg115, and Lys117 of the β subunit are located near Arg218 and Asp222 of GPIbα, respectively,

and that Aspβ70 is in proximity to Gln1391 of the A1 domain. Our results indicate that these charged amino acid residues in the β subunit have a preferential role in the activity of botrocetin-2. Since it has been time-consuming and difficult to obtain homogeneous botrocetin from natural venom, recombinant botrocetin-2 has potential benefits for clinical and basic investigations into hemostasis and thrombosis as a standard reagent.
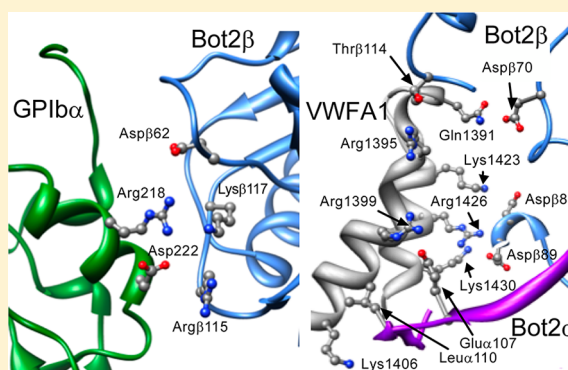
Hemostatic plug formation is initiated by the binding of plasma von Willebrand factor (VWF) onto subendothelial matrices (collagens and proteoglycans), followed by interaction between the immobilized VWF and platelet receptors GPIb and GPIIb/IIIa *in vivo* at the site of vascular injury.[1,2] VWF is a large plasma glycoprotein that circulates in multimeric forms consisting of 270 kDa subunits, and the multimer size is regulated by the plasma metalloproteinase ADAMTS13.[2] The

VWF subunit includes D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK domains, among which the A1 and the A3 domains have functional binding sites for GPIb and collagens, respectively.[3,4] In addition to physiological conditions, VWF- and GPIb-dependent platelet agglutination is inducible *in vitro* in the presence of cofactors such as ristocetin, botrocetin, and bitiscetin. These cofactor reagents are useful for clinical subdiagnosis of hemostatic disorders such as the Bernard-Soulier syndrome, which is caused by defects in GPIb[5] and VWD caused by quantitative or qualitative abnormalities in VWF.[6]

Ristocetin is a cationic low-molecular-weight antibiotic reagent,[7] whereas botrocetin and bitiscetin are snake venom proteins with a relative molecular mass of ~25 kDa.[6] Ristocetin has been widely used in clinical laboratories, but VWF from some animals, such as dogs and rabbits, does not respond to ristocetin.[8] Furthermore, ristocetin effectively agglutinates platelets between 0.5 and 2.0 mg/mL, but it flocculates fibrinogen at concentrations of more than 2 mg/mL.[9] To overcome these issues, Read et al.[10] screened snake venom for novel cofactors and found that some viperidae venom proteins induce VWF-dependent platelet agglutination in a wide range of animal species without precipitating plasma proteins. Among these snake venom-derived cofactors, only botrocetin[11] and bitiscetin[12] have been purified and characterized from *Bothrops jararaca* and *Bitis arietans* venom, respectively. These compounds are disulfide-linked heterodimers of two similar subunits (α and β) that have C-type lectin-like motifs.[11,13]

Snake venoms contain numerous structurally similar C-type lectin-like proteins with distinct activities,[14] acting as agonists or antagonists for platelet GPIb,[15,16] GPIa/IIa,[17] coagulation factors IX[18] and X,[19] and thrombin.[20] They commonly consist of heterodimers of two subunits, and concave aspects flanked by two subunits provide the binding sites for each individual ligand.[21] Ala scanning mutagenesis of the VWF A1 domain has found that botrocetin and bitiscetin bind to VWF in a distinct but overlapping area near the GPIb-binding site.[22,23] It has been speculated that the cofactor induces a conformational change in the A1 domain via an allosteric interaction. Recently, however, the 3D structure of the botrocetin-VWF-GPIbα complex was resolved, and it was found that the complex provides a high-affinity binding platform for platelet GPIb without inducing a conformational change in the A1 domain.[24]

Although botrocetin has potential for clinical investigations and basic studies into hemostasis, purification of botrocetin is time-consuming, and its contents in crude venom vary widely, possibly being affected by the living environments of individual snakes.[25,26] Furthermore, it has become more difficult to obtain crude venom samples from foreign countries since the adoption of the Washington Convention, which controls the international trade of endangered species.[27] To overcome these issues, we attempted to clone botrocetin cDNA and express recombinant proteins. In the present paper, we demonstrate the expression of botrocetin-like protein (botrocetin-2), which shows high similarity to botrocetin with regard to amino acid sequence, immunological features, and platelet agglutination activity. We also identified some specific amino acid residues important for eliciting its platelet agglutination activity via Ala scanning mutagenesis. Our results clearly advance the application of recombinant botrocetin-2 for use in clinical research as a subdiagnostic reagent and also in basic studies into thrombosis and hemostasis as a standard reagent.

■ EXPERIMENTAL PROCEDURES

Materials. Venom gland and crude venom of *B. jararaca* were kindly provided by Dr. Ida-Sano Marchin of the Butantan Institute in Brazil. Botrocetin was purified from crude venom as described previously.[28] Restriction enzymes and reagents of molecular biology grade, unless otherwise indicated, were purchased from New England BioLabs, Inc. (Beverly, MA), Toyobo Co., Ltd. (Osaka, Japan), and Wako Chemical (Osaka, Japan). Human 293T cells were purchased from the RIKEN Bioresource Center (Tsukuba, Japan).

cDNA Library Construction. RNA was isolated from a total of 3 g of tissue from four different *B. jararaca* venom glands using Isogen kits (Nippon Gene, Tokyo, Japan). Poly(A+) mRNA was purified using an oligo (dT)-cellulose column (Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. cDNA was synthesized using a cDNA synthesis kit (Pharmacia Biotech). This cDNA was ligated into a λgt10 vector using cDNA rapid cloning module-λgt10 (Amersham Life Science, Arlington Heights, IL) and was packaged using Gigapack II Gold packaging extract (Stratagene, La Jolla, CA). A total of 48 ng of cDNA was packaged. This primary library contained over 6.7×10^6 pfu/mL and was subsequently amplified.

Primer Design. Degenerate primers for α and β subunits were designed based on the amino acid sequences of botrocetin and were custom synthesized. For the α subunit, 5'-CACACACATATGGA(CT)TG(CT)CC(AGCT)(TA)(CG)-(ACGT)GG(ACGT)TGG-3' (forward primer with *Nde* I site) and 5'-ACAGAATTCTTA(ACGT)GG(ACGT)GG(ACGT)-GG(ACGT)GG(AG)CT(CT)TT(AG)CA-3' (reverse primer with *Eco*R I site), which were based on the N-terminal and C-terminal amino acid sequences of the mature protein, were used. As the signal sequences of snake venom proteins containing C-type lectin-like motifs show high similarity with regard to amino acid and nucleotide sequences (Table 1), 5'-ATGGGGCG-ATTC(AC)TCTTC(AGT)TGA-3' was designed as a forward primer for both subunits. For the β subunit, 5'-GG(AGT)AT-(AGT)ATCCACCA(TC)TT(GA)TT(GA)TT-3' was used as a screening reverse primer. The λgt10 sequence primer and poly T primer were used for rapid amplification of cDNA end (RACE).

Polymerase Chain Reaction (PCR) and DNA Sequencing. PCR was performed using Taq gold, GeneAmp High Fidelity (Applied Biosystems Japan Ltd., Tokyo, Japan) or Ex Taq (Takara, Otsu, Japan) DNA polymerase with temperature program of 94 °C (20 s), 40−55 °C (30 s), and 72 °C (30 s) for 25−35 cycles, or touchdown PCR. Amplified DNA bands monitored by agarose gel electrophoresis were isolated from gels, and DNA was extracted from gels with a QIA EXII DNA extraction kit (Qiagen, Tokyo, Japan). The extracted DNA was ligated into *p*GEM-T (Promega Co. Madison, WI) or *p*T7Blue-T (Takara) vectors and subjected to transformation into JM109 (TOYOBO) or NovaBlue (Takara) competent cells. Single colonies were picked up for mini-prep of plasmid DNA. DNA sequencing was performed using a Dye terminator cycle sequencing kit (Beckman Coulter, Inc., Fullerton, CA) and a CEQ2000 DNA sequencer (Beckman Coulter), in accordance with the manufacturer's instructions.

Expression of Recombinant Protein. Candidate cDNAs of botrocetin α and β subunits were ligated into the *p*Tandem-1 vector (Novagen, Madison, WI) using *Nco* I and *Not* I restriction enzyme sites for the α subunit and *Pme* I and *Blp* I for the β subunit. In some experiments, cDNAs were inserted into *p*CAGEN (Addgene, Cambridge, MA). Mammalian 293T cells

Table 1. Comparison of Signal Sequences of Snake Venom C-Type Lectin-like Proteins

C-type lectin-like proteins	Signal sequence
	*** * *** ** ** * *
Botrocetin-2 α & β	MGRFIFVSFG LLVGFLSLSG TAA
Bothrojaracin α	MGRFLFVSFG LLVVFLSLSG TAA
Bothrojaracin β	MGRFIFVSFG LLVVFLSLSG TAA
Agkisacutacin α & β	MGRFIFVSFG LLVVFLSLSG TAA
Mamushigin α & β	MGRFIFLSFG LLVVFVSLSG TGA
Halyxin A & B	MGRFIFLSFG LLVVFLSLSG TGA
Agglucetin α	MGRFIFVSFG LLVVFLSLSG TGA
Agglucetin β	MGRVIFVSFG LLVVFLSLSG TAA
FIX/X-BP A	MGRFIFMSFG LLVVAASLRG TGA
FIX/X-BP B	MGRFIFMSFG FLVVFLSLSG TAA
Convulxin α	MGRFIFVSFG LLVLFLSLSG TGA
Convulxin β	MGRFIFVSFG LLVVFLSLSG SEA
Forward primer**	5′-atggggcgattc(ac)tcttc(agt)tga-3′

*Conserved amino acid residues among 16 subunits (including botrocetin-2). **Forward primer was designed by referring cDNA sequences of conserved 7 amino acid residues of N-terminal signal sequence.

were transfected with expression vector using GeneJuice (Novagen), in accordance with the manufacturer's instructions. Cells were grown in D-MEM (Gibco Life Technologies Co. Tokyo, Japan) supplemented with 10% fetal calf serum for 48−72 h. Culture medium was collected and centrifuged at 3000 rpm for 10 min, and supernatant was frozen until use, after mixing with 0.5% protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan).

Purification of Recombinant Protein. Monoclonal antibody (mAb) BCT4-3 against botrocetin[29] was conjugated with BrCN-activated agarose (Sigma-Aldrich, St. Louis, MO). Culture medium was applied to antibotrocetin mAb column, and the column was washed thoroughly with Tris-buffered saline (TBS; 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) until the A280 of eluent decreased to baseline levels. The column-bound fraction was eluted with 100 mM Gly-HCl buffer (pH 2.7). After neutralizing immediately with 200 mM borate buffer (pH 11.0), peak fractions at A280 were collected and concentrated with Amicon Ultra (Millipore Co. Billerica MA), substituting medium with TBS. Protein concentration was determined by the BCA assay method (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Ala Scanning Site-Directed Mutagenesis. Several candidate residues in botrocetin subunits located at the interface between botrocetin and VWF or GPIb were selected for substitution with Ala residues. Point mutations were introduced to each corresponding codon using a Quick Change II site-directed mutagenesis kit (Stratagene) with primers containing mutated sequences. Conversion of the sequences was verified by DNA sequencing. Mutated cDNAs were amplified with a QIA filter plasmid maxi kit (Qiagen) and were subjected to transfection as described above. Culture medium was concen-

trated with Amicon Ultra (Millipore), and the concentrations of recombinant protein were estimated by enzyme-linked immunosorbent assay (ELISA) as described below.

Platelet Agglutination Assay. After obtaining informed consent, blood was drawn from healthy donors into a vacuum tube containing 1/10 volume of 3.7% sodium citrate. Citrated blood was centrifuged at 700 rpm for 10 min, and platelet-rich plasma (PRP) was collected. The remaining blood was further centrifuged at 2500 rpm for 15 min in order to collect platelet-poor plasma (PPP). PRP (250 μL) was stirred in a small cuvette at 37 °C for 2 min on an aggregometer (Mebanics model PT-2, Yokohama, Japan). Light transmission of PRP after addition of 5 μL of test solution (botrocetin, recombinant proteins, or ristocetin) was monitored using PPP as a reference. In canine platelet agglutination, PRP was prepared from healthy beagles in the same manner as described above and was used for assays. All experiments were performed within 3 h after blood collection.

ELISA. ELISA module plates (Nunc, Roskirde, Denmark) were coated with 50 mM bicarbonate buffer solution (pH 9.5) containing 10 μg/mL antibotrocetin mAb (BCT115-2) at 4 °C overnight. After washing with TBS, module plates were blocked with TBS containing 1% BSA (B-TBS) at 4 °C overnight. Concentrated culture medium containing recombinant protein or botrocetin was applied to ELISA plates for 1.5 h at 25 °C, followed by washing with TBS containing 0.1% BSA. Plates were incubated with B-TBS containing biotinylated antibotrocetin mAb (BCT107-3) followed by horseradish peroxidase (HRP)-conjugated streptavidin (Vector Laboratories, Inc., San Mateo, CA). HRP reaction was performed using o-phenylenediamine and H_2O_2 solution and was stopped by addition of 8 M H_2SO_4 solution. Absorbance at 490 nm was measured using the plate reader. Concentrations of recombinant protein were measured using purified botrocetin as a standard.

SDS−Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting. SDS-PAGE was performed using precast gels (e-PAGEL, ATTO Co. Tokyo, Japan) under reducing or nonreducing conditions. Gels were stained with Coomassie blue. Proteins on gels were electrically transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore), and the membranes were incubated with antibotrocetin mAbs, followed by detection with HRP-conjugated antimouse IgG (Zymed Lab. Inc., San Francisco, CA). In some experiments, protein bands on the blotted membranes were carved out and subjected to N-terminal amino acid sequencing analysis, as described elsewhere.[30]

Sequence Homology Search and 3D Modeling. Amino acid sequences of botrocetin-2 subunit were examined in the protein sequence database of UniProt using BLAST program.[31] The 3D structure of botrocetin-2 was predicted using a homology modeling technique based on the 3D structure of botrocetin with Protein Data Bank (PDB) entry as 1u0n[24] using the SWISS-MODEL program.[32,33] Molecular graphic images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081).[34]

■ **RESULTS**

cDNA Cloning of Botrocetin. In a preliminary experiment, we obtained cDNA of the α subunit lacking a signal sequence using degenerated primers; however, the recombinant protein was concentrated in an inclusion body, and refolding of the protein was unsuccessful, probably because botrocetin has three intramolecular disulfide bonds. Thus, we revised the method to

α subunit

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                                                                    1                         10
ATGGGGCGATTCATCTTCGTGAGCTTCGGCTTGCTGGTCGGGTTCCTCTCCCTGAGTGGAACTGCAGCTGATTGTCCCTCTGGTTGGTCCTCCTATGAAGGGAAT
Bot2  M  G  R  F  I  F  V  S  F  G  L  L  V  G  F  L  S  L  S  G  T  A  A  D  C  P  S  G  W  S  S  Y  E  G  N
Bot                                                               D  C  P  S  G  W  S  S  Y  E  G  N
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              20                            30                            40
TGCTACAAATTCTTCCAACAAAAGATGAACTGGGCCGATGCAGAGAGGTTCTGCTCGGAGCAGGCGAAGGGCGGCCATCTGGTCTCTATCAAAATTTATTCCAGA
Bot2  C  Y  K  F  F  Q  Q  K  M  N  W  A  D  A  E  R  F  C  S  E  Q  A  K  G  G  H  L  V  S  I  K  I  Y  S  R
Bot   C  Y  K  F  F  Q  Q  K  M  N  W  A  D  A  E  R  F  C  S  E  Q  A  K  G  G  H  L  V  S  I  K  I  Y  S  K
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        50                            60                            70                            80
GAAGTAGACTTTGTGGGCGACCTGGTCACTAAGAACATACAGAGCAGCGATTTGTATGCCTGGATCGGACTGAGGGTTCAAAACAAAGAAAAGCAATGCAGCTCG
Bot2  E  V  D  F  V  G  D  L  V  T  K  N  I  Q  S  S  D  L  Y  A  W  I  G  L  R  V  Q  N  K  E  K  Q  C  S  S
Bot   E  K  D  F  V  G  D  L  V  T  K  N  I  Q  S  S  D  L  Y  A  W  I  G  L  R  V  E  N  K  E  K  Q  C  S  S
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              90                            100                           110
---TGGAGCGATGGCTCCAGCGTCAGTTATGAGAACGTGGTTGAACGAACTGTGAAAAAGTGTTTTGCGCTGGAAAAAGACCTAGGGTTTGTTCTGTGGATCAGT
Bot2     W  S  D  G  S  S  V  S  Y  E  N  V  V  E  R  T  V  K  K  C  F  A  L  E  K  D  L  G  F  V  L  W  I  S
Bot   E  W  S  D  G  S  S  V  S  Y  E  N  V  V  E  R  T  V  K  K  C  F  A  L  E  K  D  L  G  F  V  L  W  I  N
```

```
        120                           130
CTTTACTGTGCACAAAAGAACCCTTTCGTCTGCAAGTCCCCACCACCGTGA
Bot2  L  Y  C  A  Q  K  N  P  F  V  C  K  S  P  P  P  *
Bot   L  Y  C  A  Q  K  N  P  F  V  C  K  S  P  P  P
```

β subunit

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                                                                    1                         10
ATGGGGCGATTCATCTTCGTGAGCTTCGGCTTGCTGGTCGGGTTCCTCTCCCTGAGTGGAACTGCAGCTGATTGTCCCCCTGATTGGTCCTCCTATGAAGGGAGT
Bot2  M  G  R  F  I  F  V  S  F  G  L  L  V  G  F  L  S  L  S  G  T  A  A  D  C  P  P  D  W  S  S  Y  E  G  S
Bot                                                               D  C  P  P  D  W  S  S  Y  E  G  H
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              20                            30                            40
TGCTACAGGGTCTTCGAACAAAAGATGGACTGGGACGATGCAGAGAAATTCTGCACAGAACAGCAGACAGGCGGACATCTGGTCTCCTTCCAGAGCAGGGAAGAA
Bot2  C  Y  R  V  F  E  Q  K  M  D  W  D  D  A  E  K  F  C  T  E  Q  Q  T  G  G  H  L  V  S  F  Q  S  R  E  E
Bot   C  Y  R  F  F  K  E  W  M  H  W  D  D  A  E  E  F  C  T  E  Q  Q  T  G  A  H  L  V  S  F  Q  S  K  E  E
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        50                            60                            70                            80
GCAGATTTTGTGAGTTCGCTCACCTCACCAATGTTGAAAGCAGATGTACTCTGGATCGGACTGAGCGATGTCTGGAATAAATGCAGGTTCGAGTGGACCGATGGC
Bot2  A  D  F  V  S  S  L  T  S  P  M  L  K  A  D  V  L  W  I  G  L  S  D  V  W  N  K  C  R  F  E  W  T  D  G
Bot   A  D  F  V  R  S  L  T  S  E  M  L  K  G  D  V  V  W  I  G  L  S  D  V  W  N  K  C  R  F  E  W  T  D  G
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```
              90                            100                           110
ATGGAGTTCGACTACGACGACTACTATTTCATTGCAGAATATGAGTGTGTCGCATCAAAGCCAACTAATAACAAATGGTGGATTATACCCTGCACAAGGCTCAAA
Bot2  M  E  F  D  Y  D  D  Y  Y  F  I  A  E  Y  E  C  V  A  S  K  P  T  N  N  K  W  W  I  I  P  C  T  R  L  K
Bot   M  E  F  D  Y  D  D  Y  Y  L  L  A  E  Y  E  C  V  A  S  K  P  T  N  N  K  W  W  I  I  P  C  T  R  F  K
```

```
        120
AATTTCGTCTGCGAGTTCCAGGCCTAG
Bot2  N  F  V  C  E  F  Q  A  *
Bot   N  F  V  C  E  F  Q  A
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Figure 1. Nucleotide sequence and deduced amino acid sequence of botrocetin-2 subunits. cDNAs (coding region) of botrocetin-2 α and β subunits were aligned with their deduced amino acid sequences and compared with botrocetin. The cDNA of the botrocetin-2 α subunit encodes a 23-residue signal peptide and a 132-residue mature protein containing four amino acid substitutions and one deletion, as compared with botrocetin. The cDNA of the botrocetin-2 β subunit contains an identical signal sequence as the α subunit and a 125-residue protein containing 15 amino acid substitutions (boxed). Degenerate primers designed for the first screening were underlined. Double underlines indicate primer sequences used for 5′- and 3′-RACE. Numbers shown in the upper sequence correspond to the amino acid residue numbers in botrocetin. Asterisk indicates stop codon.

obtain cDNAs with the signal sequence, in consideration of mammalian expression. cDNAs obtained by degenerate primers based on the conserved signal sequences from other snake venom C-type lectin-like proteins (Table 1) and C-terminal of botrocetin subunits showed amplified bands in the cDNA library of the *B. jararaca* venom gland. If DNA sequencing of the amplified band confirmed sequences corresponding to botrocetin subunits, matching DNA sequences were selected as subsequent PCR primers. Screening of the library was repeatedly carried out until the most similar cDNA sequences for botrocetin subunits were obtained. 5′-RACE and 3′-RACE were performed using λgt10, poly T, and the inner sequences of each subunit as PCR primers.

Both cDNA clones of α and β subunits thus obtained have an identical signal sequence of 23 residues, which are unique but very similar to other snake venom proteins (Table 1). The open

reading frames of both subunits are shown in Figure 1. The amino acid sequence deduced from cDNAs of α and β subunits after the signal sequence showed 96% and 88% identity to the reported botrocetin sequences, respectively (Figure 1). Among the clones screened to date, we could not find a cDNA clone showing higher similarity. The deduced amino acid sequences from cDNAs showed the highest similarity to botrocetin, as compared with bothrojaracin and GP-Ib binding protein (GPIb-BP) purified from the same *B. jararaca* venom (Figure 2). All Cys residues were conserved. In the candidate cDNA for the α subunit, four amino acids residues, Lys47, Lys49, Glu74, and Asn117, were substituted with Arg, Val, Gln, and Ser, respectively, with one amino acid deletion, Glu83, resulting in 132 amino acid residues (residue numbers refer to those of botrocetin). In the β subunit, 15 amino acid residues out of 125 residues were substituted in the cDNA as compared to

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                              10        20        30        40        50        60        70        80
Botrocetin-2 α    DCPSGWSSYEGNCYKFFQQKMNWAD-----AERFCSEQAKGGHLVSIKIYSREVDFVGDLVTKNIQSSDLYAWIGLRVQNKEKQC
Botrocetin α      DCPSGWSSYEGNCYKFFQQKMNWAD-----AERFCSEQAKGGHLVSIKIYSKEKDFVGDLVTKNIQSSDLYAWIGLRVENKEKQC
Bothrojaracin A   DCPSDWSSHEGHCYKFFQQKMNWAD-----AERFCSEQAKGGHLVSFQSDG-ETDFVVNLVTEKIQSTDLYAWIGLRVQNKEKQC
FIX-BP A          DCPSGWSSYEGHCYKPFKLYKTWDD-----AERFCTEQAKGGHLVSIESAG-EADFVAQLVTENIQNTKSYVWIGLRVQGKEKQC
jGPIb-BP α        DTPFECPSDWSTHRQYCYKFFQQKESWDDRSEYDAERFCSEQAKGGHLVSIESDE-EADFVAQLVAPNIGKSKYYVWIGLRIENKKQQC
Agglucetin α2     DFNCPPGWSAYDQYCYQVIKEPKNWDD-----AERFCTEQADGGHLVSIESKG-ERDFVAQLVSQNIESVEDHVWTGLRVQNKEKQC
Alboaggregin A-2  DFHCLPGWSAYDQYCYRVFNEPKNWED-----AERFCAKQADSGHLVSIETMG-EADFVAQLISENIQSEKHYVWIGLKVQNKEQQC

                              90       100       110       120       130
Botrocetin-2 α    SS-WSDGSSVSYENVVERTVKKCFALEKDLGFVLWISLYCAQKNPFVCKSPPP
Botrocetin α      SSEWSDGSSVSYENVVERTVKKCFALEKDLGFVLWINLYCAQKNPFVCKSPPP   (96%)
Bothrojaracin A   SSKWSDGSSVSYENVVGRTVKKCFALEKEQEFFVWINIYCGQQNPFVCKSPPP   (77%)
FIX-BP A          SSEWSDGSSVSYENWIEAESKTCLGLEKETGFRKWVNIYCGQQNPFVCEA      (62%)
jGPIb-BP α        SSKWSDYSSVSYENLVRGNVKKCFALEKKQGFRKWVNIDCVEGNPFVCKFIRPR  (57%)
Agglucetin α2     STEWSDGSSVSYENLLELYMRKCGALERETGFHKWINLGCIQLNPFVCKFPPQC  (56%)
Alboaggregin A-2  SSEWSDGSSVTYENLIKLYMRKCGALEQESGFRKWINLGCIQLNPFVCKFPPQ   (54%)


                              10        20        30        40        50        60        70        80
Botrocetin-2 β    DCPPDWSSYEGSCYRVFEQKMDWDDAEKFCTEQQTGGHLVSFQSREEADFVSSLTSPMLKADVLWIGLSDVWNKCRFEWTDGMEF
Botrocetin β      DCPPDWSSYEGHCYRFFKEWMHWDDAEEFCTEQQTGAHLVSFQSKEEADFVRSLTSEMLKGDVVWIGLSDVWNKCRFEWTDGMEF
Bothrojaracin B   DCPPDWSSYEGSCYRVFEQKMNWEDAEKFCTQQQTGGHLVSFQSSEEADFVVSLTSPILRDSFVWTGLSDVWKECSFEWSDGSDL
Purpureotin β     DCPSDWSSYEGHCYRVFNEPQNWADAEKFCRQQHTGSHLLSFHSSEEVDFVVSKTLPILKADFVWIGLTDVWSACRLQWSDGTEL
Alboaggregin A-4  DCPDWSSYDLYCYKVFQQRMNWEDAEKFCTQQHKGSHLVSFQSSEEADFVVQMTRPILNANLVWIGLSNLWNQCNSQWSDGTXL
Mamushigin β      DCPSDWSSYEGHCYRVFQKEMTWEDAEKFCTQQRKESHLVSFHSSEEVDFVVSMTWPILKYDFVWIGLNNIWNECMVEWTDGTRL
jGPIb-BP β        DCPSDWSPYGGHCYKLFKQRMNWADAENLCAQQRKESHLVSFHSSEEVDFLVLLTFPILGDLYWTGLSNIWNGCSFEWSDGTKV

                              90       100       110       120
Botrocetin-2 β    DYDD--YYFIAEYECVASKPTNNKWWIIPCTRLKNFVCEFQA
Botrocetin β      DYDD--YYLIAEYECVASKPTNNKWWIIPCTRFKNFVCEFQA   (88%)
Bothrojaracin B   SYKDNYQFVFSEYECVASKTKNNKWRIIPCTKLEYFVCEFQA   (73%)
Purpureotin β     KYNA----WTAESECIASKTTDNQWWTRSCSRTYPFVCKLEV   (55%)
Alboaggregin A-4  DYKX----WREQFECLVSRTTNNEWLSMDCSSTHSFVCEFQA   (54%)
Mamushigin β      SHNA----WITESECIAAKTTDNQWLSRPCSRTYNVVCKFQE   (54%)
jGPIb-BP β        NYNA----WASESECVASKTTDNQWWSFPCTRLQYFVCEFQA   (54%)
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Figure 2. Comparison of amino acid sequence of botrocetin-2 with those of similar snake venom proteins. Deduced amino acid sequences of botrocetin-2 subunits were compared to those of botrocetin, bothrojaracin, coagulation factor IX-binding protein (FIX-BP), jararaca GPIb-binding protein (jGPIb-BP), agglucetin, alboaggregin A, purpureotin, and mamushigin. Numbers shown in the upper sequence correspond to the residue numbers in botrocetin. Identical residues with botrocetin-2 are shaded. X indicates unknown residue. Numbers in parentheses indicate the percent similarity with the botrocetin-2 sequence.

botrocetin, but there were no deletions or insertions in the sequence. Alterations were particularly concentrated between Hisβ12 and Hisβ22 (6 of 11 residues), forming the first β strand of the β subunit of botrocetin.[35]

As these cDNA clones showed a high similarity to botrocetin, 3D homology modeling was performed using the SWISS-MODEL server, with reference to PDB data from botrocetin as a template. The predicted model was highly homologous with the main chain structure of botrocetin (Figure 3). Glu83 of the botrocetin α subunit, which was deleted in the botrocetin-2 α subunit, was located at the loop region that stretches and holds the subunits together, but its deletion had no significant effect on the overall shape of the loop. The side view in Figure 3 indicates that this loop region faces the opposite side as the tentative ligand-binding pocket.

Expression and Purification of Recombinant Botrocetin-2 (rBot2). As the deduced 3D model showed a similar conformation as botrocetin, we attempted to express the recombinant protein using mammalian culture cells. rBot2 produced by 293T cell was applied to an antibotrocetin mAb-conjugated agarose column. After washing the column, the column-bound fraction was obtained by elution with acidic buffer. The bound fraction showed single bands at approximately 24 and 14 kDa, which was consistent with the SDS-PAGE results for botrocetin under nonreducing and reducing conditions, respectively (Figure 4A). Although total amino acid residues differ among the α and β subunits, the relative molecular mass of

each subunit was too similar to allow separation by SDS-PAGE, as reported for botrocetin.[11] rBot2 was reacted with anti-botrocetin mAb under nonreducing conditions (Figure 4B). To date, eight different clones of antibotrocetin mAbs have been tested, and all mAbs reacted with rBot2 under nonreducing conditions (data not shown). The N-terminal amino acid sequence of the 24 kDa band showed a mixture of the N-termini of the α and β subunits, indicating that the signal peptide portions were processed normally and that the subunits formed a disulfide-linked dimer. Typically, 0.95 μg of rBot2 was recovered from 1 mL of culture medium.

Platelet Agglutination by rBot2. rBot2 was subjected to platelet agglutination assay using human PRP and was found to induce platelet agglutination in a concentration-dependent manner, similarly to botrocetin (Figure 5A). rBot2 showed almost the same activity as natural botrocetin; i.e., more than 0.5−1 μg/mL rBot2 was effective. When dog PRP was examined, both rBot2 and botrocetin induced platelet agglutination, in contrast to ristocetin, which showed no activity, even at 2 mg/mL (Figure 5B).

Site-Directed Mutagenesis of Botrocetin-2. In order to elucidate the essential amino acid residues for botrocetin-2 to elicit activity, we introduced point mutations in its subunits. The target amino acid residues were selected based on the ternary model of the botrocetin-VWF-GPIb complex.[24] Each amino acid residue of botrocetin proximal to either the GPIbα (Leuβ59, Lysβ60, Aspβ62, Argβ115, and Lysβ117), VWF A1 domain
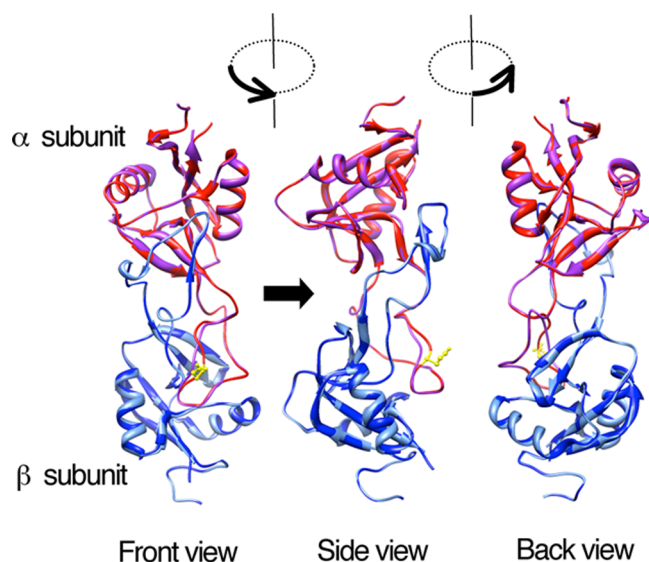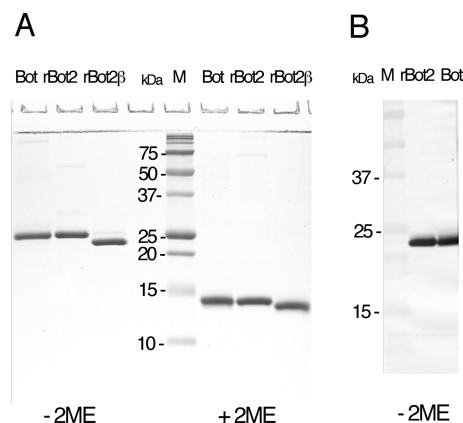
Figure 3. Homology modeling of botrocetin-2. The 3D structure of botrocetin-2 was predicted using a homology modeling technique based on the 3D structure of botrocetin with PDB entry 1u0n[24] using the SWISS-MODEL program.[32,33] Molecular graphics images were produced using the Chimera package[34] and are expressed in the ribbon model. Botrocetin α and β subunits are colored in red and blue, respectively. Botrocetin-2 α and β subunits are shown in purple and light blue, respectively. Both subunits overlap such that α helices and β sheets contain both colors. Front view shows each loop domain facing the front and is rotated at right angles to show the "side view" and "back view". Glu83 of botrocetin α subunit is shown as the ball and stick in yellow. Arrow in the "side view" indicates the concave side that functions as a tentative ligand-binding pocket.

Figure 4. SDS-PAGE and Western blotting of recombinant botrocetin-2. (A) Botrocetin (Bot), recombinant botrocetin-2 (rBot2), and recombinant botrocetin-2 β subunit (rBot2β) were subjected to SDS-PAGE in the presence (+) or absence (−) of 2-mercaptoethanol (2ME), followed by staining with Coomassie blue. Numbers indicate relative molecular mass (kDa) of standard marker protein (M). (B) Botrocetin and recombinant botrocetin-2 were transferred to PVDF membranes after SDS-PAGE under nonreducing conditions. Membranes were incubated with antibotrocetin mAb. Binding of mAb was detected using HRP-conjugated second antibody.

(Gluα48, Aspα50, Gluα107, Lysα108, Aspα109, Leuα110, Aspβ70, Aspβ88, and Aspβ89), or both GPIbα and the A1 (Thrβ114) were converted to an Ala residue. All targeted residues were commonly included in botrocetin-2. Mutated recombinant proteins produced by 293T cells were evaluated by platelet agglutination activity.

As shown in Figure 6A, Gluα48Ala, Aspα50Ala, Lysα108Ala, Aspα109Ala, Leuβ59Ala, and Lysβ60Ala mutations in rBot2 induced platelet agglutination at 0.5 μg/mL as wild-type rBot2 (with no mutation). Mutant rBot2 with Gluα107Ala, Leuα110-Ala, Aspβ88Ala, Aspβ89Ala, and Thrβ114Ala showed slower platelet agglutination even at 1 μg/mL (Figure 6B). Alterations in Aspβ62, Aspβ70, and further, Argβ115 or Lysβ117 to Ala on the protein decreased its platelet agglutination activity even at higher concentrations (Figure 6C,D).

Expression and Activity of Single Subunit. When the α or β subunit cDNA of botrocetin-2 was independently expressed in 293T cells, only the β subunit was recovered from the medium by immunopurification; we were unable to obtain recombinant α subunit. SDS-PAGE of the purified β product migrated to a slightly different position than the α-β heterodimer under nonreducing conditions, but no bands were observed at the monomer position until reduction with 2-mercaptoethanol, indicating that the protein is a disulfide-linked homodimer (Figure 4A). The N-terminal amino acid sequence of the product showed that the homodimer starts from the N-terminus of the β subunit, but not from the signal sequence. The β dimer protein showed the potential to induce the platelet agglutination, although the relative activity was weaker when compared with the heterodimer proteins (Figure 5A).

■ DISCUSSION

We have successfully cloned cDNAs for botrocetin-2, from a cDNA library of the venom gland of *B. jararaca*, with a high similarity to botrocetin subunits that have been registered in protein databases. Translation of these cDNAs by 293T cells was normal; i.e., cleavage of signal sequence and dimerization of α-β subunits was carried out properly. The translated botrocetin-2 showed the same electrophoretic mobility on SDS-PAGE as natural botrocetin as well as immunoreactivity against anti-botrocetin mAbs and similar cofactor activity for VWF as natural botrocetin. Although it does not fully match the cDNA for the botrocetin subunits, these results indicate that botrocetin-2 is an isoprotein of botrocetin. As Brinkhouse noted that "botrocetin" is widely used as a cofactor found in *Bothrops* species,[36] botrocetin-2 can be called "botrocetin" in the broad sense. Since venom contents are affected by factors such as living area, available prey, and physical condition, botrocetin probably exhibits microheterogeneity among individual snakes, even within the same species.[25,37] Microheterogeneity in the amino acid sequence of C-type lectin-like snake venom proteins has also been reported for alboaggregins,[38] agglucetin,[39] GPIb-BP,[15] and bitiscetin.[13,40]

Botrocetin-2 has several amino acid substitutions or deletions as compared with botrocetin, but these alterations had no significant effect on its overall conformation or on the platelet agglutination activity. In particular, the β subunit had numerous amino acid substitutions between Hisβ12 and Hisβ22. This region includes a first β strand located at the top of the β-subunit; Lys18 and Trp20 of botrocetin β contact with Pro260 and Gln221 of GPIbα, respectively, but Lysβ20 of botrocetin-2 is supposed to contact with Arg217 and Gln221 of GPIbα. Other substitutions were equivalent or located outside of the botrocetin-VWF-GPIb binding interface except for Lysα49 (Valα49 in botrocetin-2) that has a polar interaction with Gln1402 of the A1 domain, thus suggesting that these substitutions do not significantly affect the conformation of botrocetin or the association with the complex.
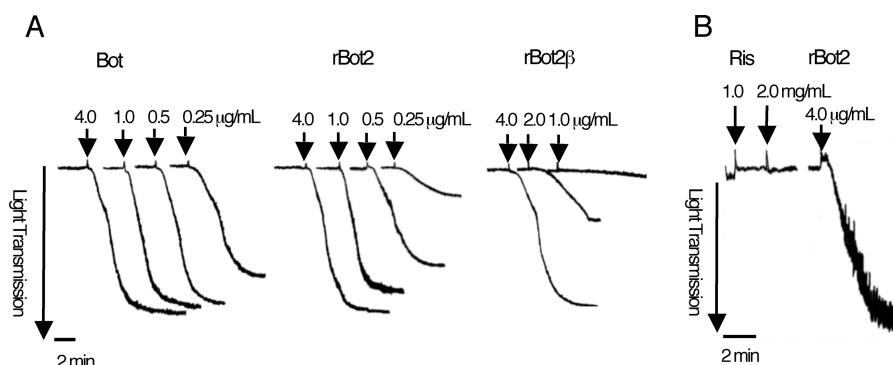
Figure 5. Platelet agglutination induced by botrocetin-2. (A) Human PRP was mixed with 0.25−4.0 μg/mL botrocetin (Bot), recombinant botrocetin-2 (rBot2), or recombinant botrocetin-2 β (rBot2β) at the point indicated (arrow). Platelet agglutination was monitored by light transmission using an aggregometer. (B) Dog PRP was tested for platelet agglutination induced by ristocetin (Ris) or rBot2. rBot2 showed similar platelet agglutination as botrocetin in dog PRP, but ristocetin had no activity.
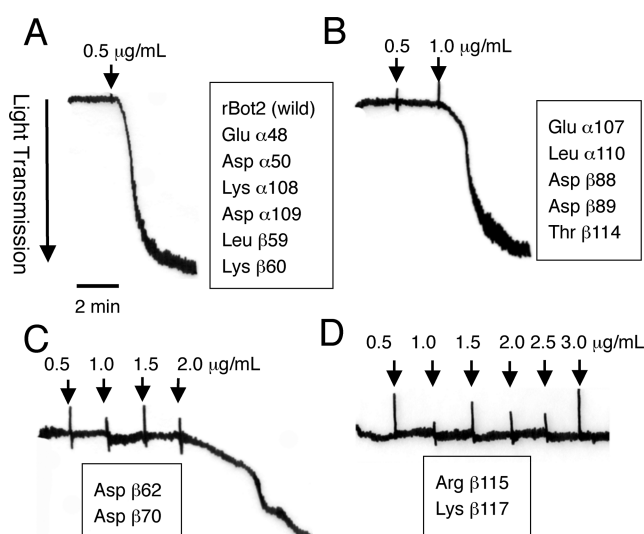

Figure 6. Effects of amino acid substitution on platelet agglutination activity of botrocetin-2. Mutant proteins were tested for platelet agglutination using human PRP as described for Figure 5A. Arrows indicate the addition of each mutant protein with final concentrations. Four typical agglutination patterns were obtained. (A) Mutant rBot2 with Ala substitutions at Gluα48, Aspα50, Lysα108, Aspα4109, Lueβ59, or Lysβ60-induced platelet agglutination as rBot2 with no mutation (rBot2 (wild)) at a concentration of 0.5 μg/mL. (B) Mutant rBot2 with Ala substitutions at Gluα107, Leuα110, Aspβ88, Aspβ89, or Thrβ114 showed no response at a concentration of 0.5 μg/mL and a slower response at 1.0 μg/mL. (C) Mutant rBot2 with Ala substitutions of β subunit at Asp62 or Asp70 showed a lower response even at high concentrations, and (D) those with Arg115 or Arg117 showed no response up to 3 μg/mL.

Conversely, substitutions of Aspβ62, Aspβ70, Argβ115, and Lysβ117 appeared to affect the platelet agglutination activity of botrocetin-2. All of these residues are conserved in botrocetin but are partially preserved in bothrojaracin or GPIb-binding proteins (Figure 2). Referring to the ternary model of the VWF A1 domain-GPIbα-botrocetin complex by Fukuda et al.[24] (PDB entry 1u0n), we have superimposed botrocetin-2 instead of botrocetin (Figure 7). In this model, Aspβ62, Argβ115, and Lysβ117 are located at the interface for GPIbα, and the opposite side of GPIbα appears to be Arg218 and Asp222 at a distance within 4.6 Å (Figure 7B). As the GPIbα chain contains sulfated Tyr residues at 276, 278, and 279,[41] and other charged amino acid

residues between residues 266 and 287[42] (Figure 7A only showing residues 1−265 of GPIbα), there must be further interactions between botrocetin-2 and GPIbα. Shimizu et al.[43] reported that mutant GPIbα containing Ala substitutions at Arg217 and Arg218, Asp274, and Asp277, and Glu285 and Asp288 showed decreased VWF-binding activity in the presence of botrocetin but that these mutations had a small effect on ristocetin-induced VWF binding, thus suggesting that these residues have a significant role in botrocetin binding to GPIbα. Other uncharged residues examined had no significant effect, despite being located near GPIbα. These electrostatic interactions may be important for the VWF-botrocetin complex to amplify or promote affinity for GPIb-binding.

The botrocetin-binding site on the VWF has been elucidated by Ala-scanning mutagenesis. Matsushita et al.[44,45] reported that mutant VWF with Ala substitution at Arg1392, Arg1395, Arg1399, or Lys1430 showed decreased binding activity with botrocetin, thus suggesting that these residues in the A1 domain play an essential role in botrocetin binding. Fukuda et al.[24] have shown 12 residues of the A1 domain (Gln1391, Arg1392, Arg1395, Val1398, Arg1399, Gln1402, Lys1406, Lys1423, Gln1424, Lue1427, Lys1430, and Gln1431) are in contact with botrocetin within the binary complex (only charged residues are depicted in Figure 7A). Further, they have shown, by comparing the 3D structures of binary (botrocetin-A1) and ternary (botrocetin-A1-GPIbα) complex, that botrocetin slides on the A1 domain to form a new interface for GPIbα. In their two-step process, two out of five salt bridges that occur within the A1-botrocetin interface[22] are disrupted when GPIbα binds, whereas four new polar interactions are formed (Gln1391-Aspβ70, Arg1395-Thrβ114, Lys1406-Leuα110, and Arg1426-Aspβ88). In our data, substitution of Aspβ70Ala effectively, and Gluα107Ala, Leuα110Ala, Aspβ88Ala, Aspβ89Ala, and Thrβ114Ala moderately reduced the platelet agglutination activity of botrocetin-2, suggesting that these residues are responsible for the VWF binding at the ternary complex. In the superimposed model, Aspβ70, Aspβ88, Aspβ89, Thrβ114, Gluα107, and Leuα110 are closely located to Gln1391, Lys1423 and Arg1426, Lys1430, Arg1395, Arg1399, and Arg1392 of VWF, respectively, within a distance of 4 Å (Figure 7C), showing a good alignment with those previously indicated by Fukuda et al.[24] The electrostatic and/or polar interactions among these residues may be responsible for the binding affinity between botrocetin and VWF. Further fine analysis for assignment of critical residues in botrocetin is necessary to elucidate the molecular interactions among the three components.
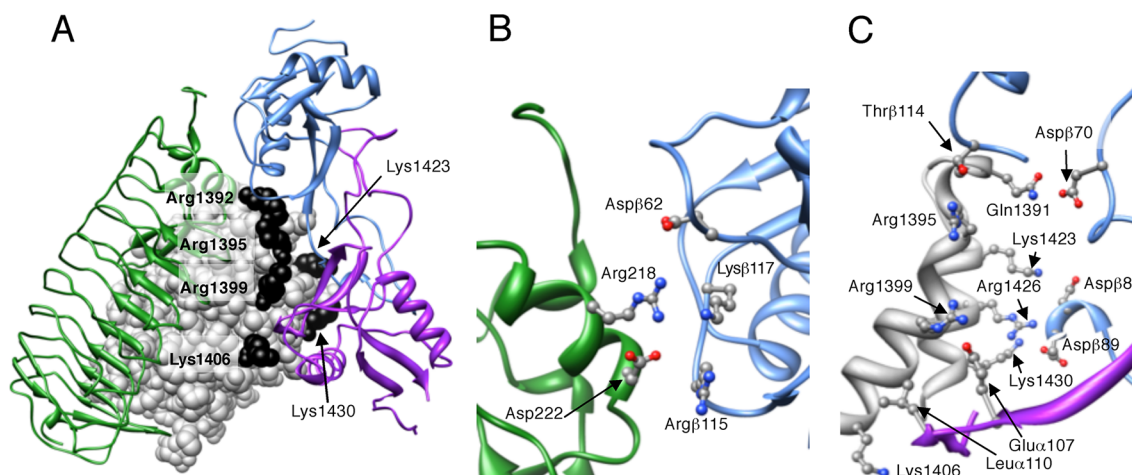
Figure 7. Important residues in botrocetin-2 for binding to VWF and GPIbα. (A) The 3D homology model of botrocetin-2 (ribbon model with α subunit in purple and β subunit in tint blue) was superimposed with VWF A1 domain (sphere model in gray) and GPIbα (ribbon model in green) using Chimera[34] with PDB entry 1u0n. Location of the charged residues in the A1 domain facing with botrocetin[24] is shown in black with residue number. (B) Interface between GPIbα and botrocetin-2β. As expressed in a ball and stick model (blue and red spheres indicate nitrogen and oxygen atom, respectively), Aspβ62 locates near Arg218 within 4 Å; Argβ115 and Lysβ117 locate near Asp222 within 4 and 4.6 Å, respectively. (C) Interface between VWF A1 domain and botrocetin-2 subunits. Two α-helices 4 and 5 of the A1 domain were depicted with the ribbon model in gray. Effective residues are shown in the ball and stick model with residue number. Gluα107-Arg1399, Lueα110-Lys1406, Aspβ70-Gln1391, Aspβ88-Lys1423 and Arg1426, Aspβ89-Lys1430, and Thrβ114-Arg1395 are close proximity within a distance of 4 Å, suggesting the electrostatic and/or polar interaction between them.

Monomer or homodimers of α and β subunits have not been detected, even when a single plasmid containing both subunit cDNAs was used, but a small amount of homodimers of β subunit, which migrated slightly faster than heterodimers, was observed when cells were cotransfected using independent plasmids containing single α and β cDNAs (data not shown). In a single subunit expression experiment, we have indicated that the β subunit plays a major role in the function of botrocetin-2. Further, critical residues for eliciting GPIb- and VWF-binding activity of botrocetin-2 are mainly localized in the β subunit. We were unable to detect a monomeric β subunit band by SDS-PAGE under nonreducing conditions (Figure 4A), thus suggesting that dimerization is a prerequisite for secretion from cells or stabilizing the molecule. The function of the α subunit is unclear, as we were not able to successfully express the α subunit monomer or dimer. It will be interesting to further examine the monomeric subunit and to determine whether it shows cofactor or inhibitory activity.

It is well-known that ristocetin has been widely used as a primary reagent for surveying VWF-induced platelet agglutination in clinical tests or the basic study of thrombosis especially for the subdiagnosis of VWD. Although ristocetin has been used traditionally, binding of ristocetin is not specific to VWF. Ristocetin interacts with the Pro-rich sequence in the A1 domain and/or negatively charged residues such as sialic acid residues, which reside in the A1 domain and GPIb.[9,46,47] Botrocetin has numerous advantages when compared with ristocetin, despite the presence of microheterogeneity, lot-to-lot variations, and difficulties in obtaining crude venom materials under the Washington Convention. In clinical laboratories, if homogeneous recombinant botrocetin of suitable quality and activity can be produced, it may be useful for clinical investigations or basic studies into hemostasis and thrombosis, acting as a standard reagent instead of ristocetin. As shown herein, botrocetin-2 also induced platelet agglutination in dog PRP. Dogs have been bred widely, and pedigree dogs are of commercial value to pet shops and breeders. Hereditary diseases, including hemostatic

disorders, are becoming increasingly common in dogs, partly due to extensive inbreeding. Dog VWD is a particularly common inherited bleeding disorder.[48,49] Thus, botrocetin-2 would be also applicable as a convenient tool for screening dog VWD that could not be detected with ristocetin.

It is quite interesting that C-type lectin-like proteins in snake venom have such a wide variety of activities. These proteins have basically a common dimer conformation but show individual specific functions as like antibodies. Determination of essential residues for eliciting each unique recognition mechanism would be necessary to elucidate the specific activities of each venom protein. These basic studies on VWF- or platelet-modulating proteins found in snake venoms would lead a valuable implication for drug design against antithrombotic reagent or *in vitro* assay reagents.

■ AUTHOR INFORMATION

Corresponding Author
*Phone (+81)-562-93-2954; fax (+81)-562-93-4595; e-mail tmatsui@fujita-hu.ac.jp.

■ ABBREVIATIONS

ELISA, enzyme-linked immunosorbent assay; GP, platelet glycoprotein; HRP, horseradish peroxidase; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PPP, platelet-poor plasma; PRP, platelet-rich plasma; PVDF, polyvinylidene difluoride; RACE, rapid amplification of cDNA end; rBot2, recombinant botrocetin-2; TBS, Tris-buffered saline; VWF, von Willebrand factor.

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