Binding Site on Human von Willebrand Factor of Bitiscetin, a Snake Venom-Derived Platelet Aggregation Inducer†

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ABSTRACT: Bitiscetin, a C-type lectin-like heterodimeric snake venom protein purified from *Bitis arietans*, binds to human von Willebrand factor (VWF) and induces the platelet membrane glycoprotein (GP) Ib-dependent platelet agglutination in vitro similar to botrocetin. In contrast with botrocetin which binds to the A1 domain of VWF, the A3 domain, a major collagen-binding site of VWF, was proposed to be a bitiscetin-binding site. In the competitive binding assay, neither bitiscetin nor botrocetin had an inhibitory effect on the VWF binding to the immobilized type III collagen on a plastic plate. The anti-VWF monoclonal antibody NMC-4, which inhibits VWF-induced platelet aggregation by binding to α4 helix of the A1 domain, also inhibited bitiscetin binding to the VWF. Binding of VWF to the immobilized bitiscetin was competitively inhibited by a high concentration of botrocetin. A panel of recombinant VWF, in which alanine-scanning mutagenesis was introduced to the charged amino acid residues in the A1 domain, showed that the bitiscetin-binding activity was reduced in mutations at Arg632, Lys660, Glu666, and Lys673 of the A1 domain. Those substituted at Arg629, Arg636, and Lys667, which decreased the botrocetin binding, showed no effect on the bitiscetin binding. These results indicate that bitiscetin binds to a distinct site in the A1 domain of VWF spanning over α4a, α5 helices and the loop between α5 and β6 but close to the botrocetin- and NMC-4-binding sites. Monoclonal antibodies recognizing the α-subunit of bitiscetin specifically inhibited bitiscetin-induced platelet agglutination without affecting the binding between VWF and bitiscetin, suggesting that the α-subunit of bitiscetin is located on VWF closer to the GPIb-binding site than the β-subunit is. Bitiscetin and botrocetin might modulate VWF by binding to the homologous region of the A1 domain to induce a conformational change leading to an increased accessibility to platelet GPIb.

von Willebrand factor (VWF)[1] plays an essential role in the early process of hemostasis by mediating the interaction between the subendothelial matrix and platelet membrane glycoprotein (GP) Ib (*1*, *2*). Under physiological conditions, VWF interacts with a surface of subendothelial matrix proteins such as collagens at vascular injury leading to the exposure of the cryptic GPIb-binding site in the A1 loop domain (residues 497−716 of the mature VWF subunit) by stretching the molecule under high-shear stress (*3*, *4*). Under nonphysiological conditions, however, VWF becomes accessible to GPIb with the help of cofactors such as antibiotic ristocetin (*5*) or snake venom botrocetin (*6*) or by desialylation (*7*) even under static conditions. Although the reaction mechanisms of these in vitro modulators are not clear, ristocetin and botrocetin, which have a very basic and acidic nature, respectively, may interact with counter charge groups of the A1 domain, leading to a conformational change in the GPIb-binding site of VWF (*8*): ristocetin interacts with a hinge region of the loop which is rich in Pro and sugar chains containing sialic acid residues (*9*), whereas botrocetin interacts with basic amino acid residues in the inner-loop region (*10*). Matsushita et al. (*11*, *12*) have indicated that Arg629, Arg632, Arg636, and Lys667 localizing in the A1 domain of VWF have an essential role in the interaction with botrocetin. Furthermore, the crystal structure of botrocetin has been recently determined and has shown to have a central concave region formed by two subunits, and the negatively charged patch near the concave structure composed of several acidic amino acid residues might be a candidate for the VWF-binding site (*13*).

We have isolated and determined the complete amino acid sequence of a novel VWF-modulator protein termed bitiscetin from the venom of the viperid *Bitis arietans* (*14*, *15*). Like

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[1] Abbreviations: ABIS, anti-bitiscetin monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; GP, platelet membrane glycoprotein; HRP, horseradish peroxidase; mAb, monoclonal antibody; pAb, polyclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PVDF, poly(vinylidene difluoride); TBS, Tris-buffered saline; VWF, von Willebrand factor.

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botrocetin, bitiscetin is a heterodimer of α- and β-subunits and belongs to the C-type lectin-like protein superfamily. However, bitiscetin, unlike botrocetin, has an alkaline p*I*, and its amino acid sequence is only about 45% identical to that of botrocetin. This homology is almost as low as those with other botrocetin-like snake venom proteins such as coagulation factor IX/X- or the GPIb-binding proteins which do not modulate VWF activity (*6*, *16*). These structural and molecular features of bitiscetin suggest that bitiscetin interacts with VWF in a different manner than botrocetin does with VWF. Recently, Obert et al. (*17*) reported that bitiscetin binds to the A3 domain (residues 910−1111), a major collagen-binding domain of VWF, and suggested that it remotely induces the conformational change of VWF resembling that induced when anchored by collagen in the flow (*18*). To elucidate the interaction of bitiscetin with VWF, we surveyed the bitiscetin-binding site on VWF by competition binding assays on immobilized type III collagen. We further determined several residues essential for bitiscetin binding by charged-to-alanine scanning mutagenesis of VWF, since charged amino acid residues are often employed for the molecular interaction and the molecular conformation (*19*). Finally, we determined which subunit of bitiscetin is closer to the GPIb-binding site by using subunit-specific monoclonal antibodies (mAbs).

EXPERIMENTAL PROCEDURES

Materials. Bitiscetin and botrocetin were purified from crude venoms (Latoxan, Rosans, France, and Sigma, St. Louis, MO) as previously described (*14*, *20*). Biotinylation of bitiscetin was performed using biotin−NHS reagent (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Soluble human type III collagen was purchased from Seikagaku (Tokyo, Japan). VWF was purified from factor VIII concentrates as previously described (*21*). Anti-VWF mAb VW40-1 recognizing the C-terminal domain of the VWF subunit (*22*), anti-VWF mAb VW53-2 which inhibits VWF binding to type III collagen (*16*), and anti-botrocetin mAb BCT4-3 (*23*) were kindly donated by Takara-Shuzo (Otsu, Japan). Anti-VWF mAb NMC-4, specifically recognizing the A1 domain of VWF, was prepared as described (*24*). Anti-VWF polyclonal antibody (pAb) and horseradish peroxidase- (HRP-) conjugated anti-VWF pAb (P0226) were purchased from Medical and Biological Laboratories (Nagoya, Japan) and Dakopatts (Glostrup, Denmark), respectively. HRP-conjugated streptavidin and HRP-conjugated anti-mouse IgG were purchased from Vector Laboratories and Zymed Laboratories (San Francisco, CA), respectively. The mAbs against bitiscetin (ABIS) were prepared as described using the lyophilized crude venom of *B. arietans* as an antigen. All ABISs were classified as IgG_1 and purified from the ascites fluid by protein A−agarose column chromatography (*23*).

VWF-Binding Assay to the Immobilized Collagen and Bitiscetin. An enzyme-linked immunosorbent assay (ELISA) plate (Nunc, immunomodule, Kamstrup, Denmark) was coated with 50 μL of human type III collagen solution (10 μg/mL), diluted with Tris-buffered saline (TBS; 150 mM NaCl, 20 mM Tris-HCl, pH 7.5) or 50 μL of bitiscetin solution (10 μg/mL) in phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer, pH 7.2, 150 mM NaCl) overnight at 4 °C, and blocked with 200 μL of TBS containing 1% BSA (B/TBS). VWF solution (0−4 μg/mL) was mixed with botrocetin or bitiscetin (0−20 μg/mL), mAb NMC-4 (0−50 μg/mL), VW40-1, VW53-2 (0−50 μg/mL), or TBS for 10 min, and the mixture (50 μL) was introduced to the collagen- or bitiscetin-coated plate. After a 90 min incubation at room temperature, the plate was thoroughly washed with TBS containing 0.1% BSA followed by incubation with 50 μL of HRP-conjugated anti-VWF pAb (1:1000 diluted with B/TBS) for 60 min. After several washings, the HRP reaction was performed using *o*-phenylenediamine and H_2O_2. The binding of VWF was monitored by measuring the absorbance at 490 nm with a plate reader as described (*25*).

Preparation of Charged-to-Alanine Mutagenesis Recombinant VWF. The construction of a plasmid containing charged-to-alanine mutations of the VWF A1 domain was described previously (*11*). The polymerase chain reaction method was used to introduce a mutation into plasmid pGEM-4ZNK (*26*), and each mutation was confirmed by DNA sequencing. The mutated *Ngo*MI−*Kpn*I fragments were cloned into pSVHVWF1.1. Each plasmid was amplified by transformation into DH5α-competent cells (Toyobo, Osaka, Japan) and was highly purified by a QIA filter plasmid maxi kit (Qiagen, Hilden, Germany). Human 293T cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies). The cells were transfected by a calcium phosphate transfection system (Life Technologies), and the recombinant mutant VWF (Ala-rVWF) was secreted into serum-free media (OPTI-MEM, Life Technologies) as described (*11*). After addition of EGTA (final concentration of 6 mM), Tris-HCl buffer, pH 7.5 (final concentration of 40 mM), and diisopropyl fluorophosphate (final concentration of 0.5 mM), the Ala-rVWFs were concentrated by Centriplus 30 (Millipore, Bedford, MA) and were frozen at −80 °C in aliquots until use. VWF antigen was measured by a sandwich ELISA using mAb VW40-1 (10 μg/mL) as a capturing antibody and HRP-conjugated anti-VWF pAb (1:1000) as a detecting antibody with a purified VWF as a standard.

Binding of Bitiscetin to the Immobilized Ala-rVWF. The Ala-rVWFs (1 μg/mL in T/TBS containing 1 mM EDTA) were incubated in an ELISA plate coated with mAb VW40-1 (10 μg/mL) for 2 h. After several washings with T/TBS, the plate was incubated with biotinylated bitiscetin (1 μg/mL in B/TBS) for 1 h and then with HRP-conjugated streptavidin (1:1000 diluted with B/TBS) for 45 min. The HRP reaction was performed as described above. The binding of biotinylated bitiscetin to wild-type rVWF at 1 μg/mL was expressed as 100% binding.

SDS−Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting. Bitiscetin and botrocetin (1 μg) were subjected to SDS−PAGE under reduced (20% gel) and nonreduced (15% gel) conditions according to the method of Laemmli (*27*) and electrotransferred to a poly(vinylidene difluoride) (PVDF) membrane (Millipore) as described (*28*). The PVDF membrane was soaked in T/TBS at 4 °C overnight and incubated with each mAb of ABIS (5 μg/mL in T/TBS) for 90 min at room temperature. The membrane was washed with T/TBS and incubated with HRP-conjugated anti-mouse IgG (1:1000 diluted with T/TBS) for 60 min,

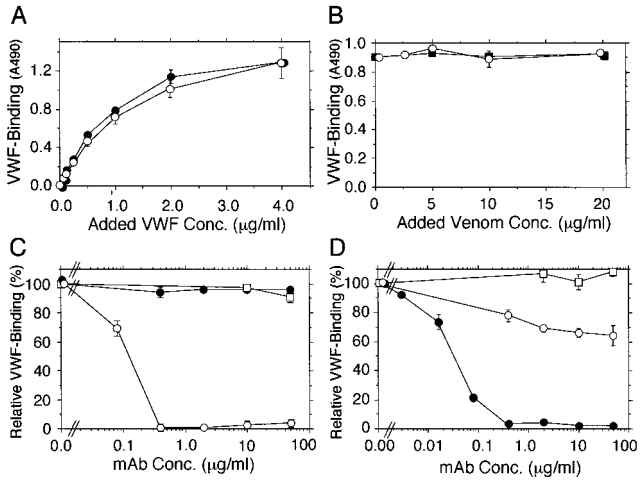

FIGURE 1: Effects of bitiscetin and anti-VWF mAbs on the binding between VWF and immobilized collagen or bitiscetin. (A) VWF (0−4 μg/mL) was incubated in an ELISA plate coated with type III collagen in the presence (○) or absence (●) of 5 μg/mL bitiscetin, and the binding of VWF was detected with HRP-conjugated anti-VWF pAb. (B) VWF at a fixed concentration (2 μg/mL) was incubated in an ELISA plate coated with type III collagen in the presence of various concentrations (0−20 μg/mL) of bitiscetin (○) or botrocetin (■). (C, D) VWF (2 μg/mL) was incubated in an ELISA plate coated with type III collagen (C) or bitiscetin (D) in the presence of 0−50 μg/mL anti-VWF mAb (○, VW53-2; □, VW40-1; ●, NMC-4), and the binding of VWF was detected by HRP-conjugated anti-VWF pAb. VWF binding in the absence of mAb was expressed as 100% binding. The data show the mean ± SE of three experiments.

followed by peroxidase reaction using 3,3′-diaminobenzidine tetrahydrochloride and H_2O_2 as substrates.

Bitiscetin Binding to the Immobilized VWF in the Presence of ABIS. An ELISA plate was coated with 50 μL of anti-VWF pAb solution (10 μg/mL in 100 mM bicarbonate buffer, pH 9.6) at 4 °C overnight and blocked with 200 μL of B/TBS at 4 °C overnight. The plate was incubated with 50 μL of VWF (2 μg/mL) solution for 90 min, biotinylated bitiscetin (1 μg/mL) with or without ABIS (up to 10 μg/mL) for 60 min, and then with HRP-conjugated streptavidin (1:1000 dilution) for 45 min. The binding of bitiscetin to the plate was monitored by HRP reaction as described above.

Platelet Agglutination Assay. Formalin-fixed platelets (300 μL, 3×10^5 platelets/μL in TBS containing 1 mM EDTA) were incubated at 37 °C in a small glass cell with gentle stirring. Bitiscetin (1 μg/mL) and ABIS (5 μg/mL) were added to the platelets, and VWF (5 μg/mL) was introduced after 1 min. After 2 min, ristocetin (1.3 mg/mL) and additional VWF (10 μg/mL) were subsequently added to the mixture. Platelet agglutination was monitored with a light transmittance aggregometer (Chrono-Log, Harvertown, PA).

RESULT

Effects of Bitiscetin and Anti-VWF mAbs on the VWF Binding to Immobilized Collagen. VWF bound to type III collagen immobilized on an ELISA plate in a concentration-dependent manner, but this binding was not significantly reduced in the presence of bitiscetin even at a higher concentration level (Figure 1A,B). The same results were obtained with botrocetin (Figure 1B). The binding between VWF and the immobilized collagen was specifically inhibited in the presence of anti-VWF mAb VW53-2 as previously reported (*16*) with IC_{50} = 0.12 μg/mL, but VW40-1 and NMC-4 having an epitope within the C-terminus and the A1 domain of VWF, respectively, had no inhibitory effect on the binding (Figure 1C).

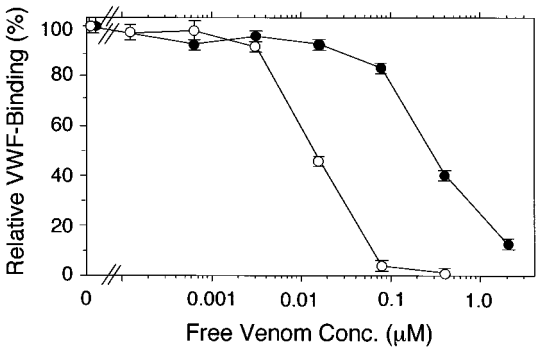

FIGURE 2: Competitive inhibition of free bitiscetin and botrocetin on the VWF binding to a bitiscetin-coated plate. The VWF binding to a bitiscetin-coated plate was investigated in the presence or absence of free bitiscetin (○) or botrocetin (●) during the incubation with VWF (2.5 μg/mL). The VWF binding detected by HRP-conjugated anti-VWF pAb in the absence of the competitor was expressed as 100%. The data show the mean ± SE of three experiments.

Effects of Botrocetin and Anti-VWF mAbs on the VWF Binding to Immobilized Bitiscetin. The VWF binding to immobilized bitiscetin on an ELISA plate was clearly inhibited with mAb NMC-4 with IC_{50} = 0.03 μg/mL (Figure 1D). VW40-1 up to 50 μg/mL showed no effect on the binding between bitiscetin and VWF. Although VW53-2 moderately reduced the VWF binding, it reached about a 40% inhibition level even at 50 μg/mL (Figure 1D).

To analyze the competition between botrocetin and bitiscetin on the binding to VWF, the effects of free botrocetin and bitiscetin on the VWF binding to immobilized bitiscetin were examined (Figure 2). Free bitiscetin competitively inhibited the VWF binding to coated bitiscetin with IC_{50} = 13.6 nM, while botrocetin also inhibited the binding at the relatively higher concentration with IC_{50} = 280 nM. Ristocetin, which was examined up to 2 mg/mL, showed no inhibitory effect on the binding between VWF and bitiscetin (data not shown). These results suggest that bitiscetin binds to the A1 domain of VWF in close proximity to the botrocetin- and NMC-4-binding sites rather than the A3 domain close to the main collagen-binding site.

Bitiscetin-Binding Site on the VWF Molecule. To elucidate the bitiscetin-binding site on the VWF molecule, alanine scanning mutagenesis was performed for the bitiscetin binding of the VWF A1 domain. Since bitiscetin has a very basic nature in contrast to botrocetin, we have focused on the charged amino acid residues (arginine, lysine, histidine, glutamic acid, and aspartic acid) in the A1 domain and substituted them for neutral alanine residues as previously examined for botrocetin (*11*, *12*). Targeted amino acid residues within the A1 domain, singly or clustery, were described by Matsushita et al. (*11*, *12*) and shown in Figure 3. Mutants with 11 cluster and 33 single substitutions were assayed. All Ala-rVWFs have been reported to be normally secreted into the medium with a normal multimeric pattern (*12*). Ala-rVWFs in a fixed concentration were captured by mAb VW40-1, which binds to the C-terminal region of VWF without showing an inhibitory effect on the binding between

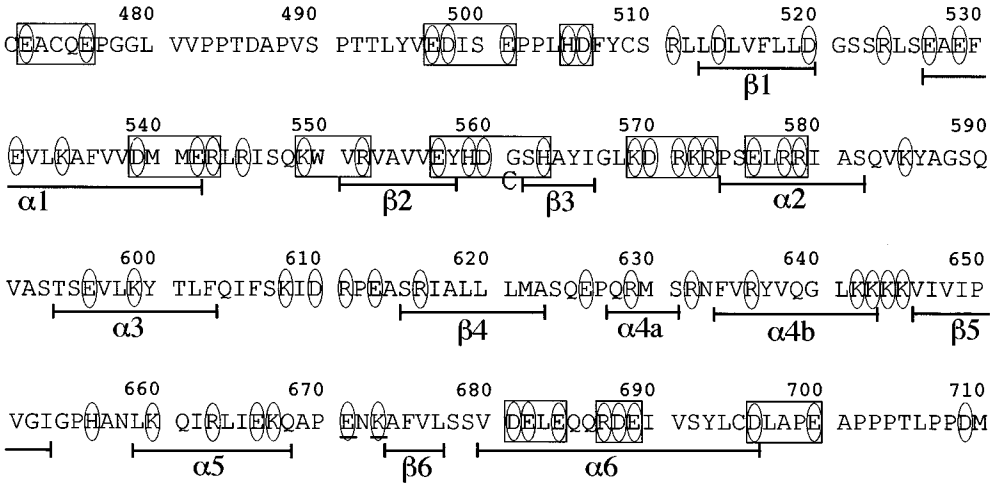

FIGURE 3: Amino acid residues substituted to alanine in the VWF A1 domain. The amino acid sequence containing the A1 domain (residues 711−716 were omitted) was shown with the secondary structure according to Celikel et al. (*30*) under the sequence (α, α-helix; *β*, *β*-strand). Charged residues (His, Arg, Lys, Glu, and Asp) targeted for the mutagenesis are circled, and the mutants containing more than two targeted residues (cluster) are boxed (*12*). Two additional constructs are underlined.

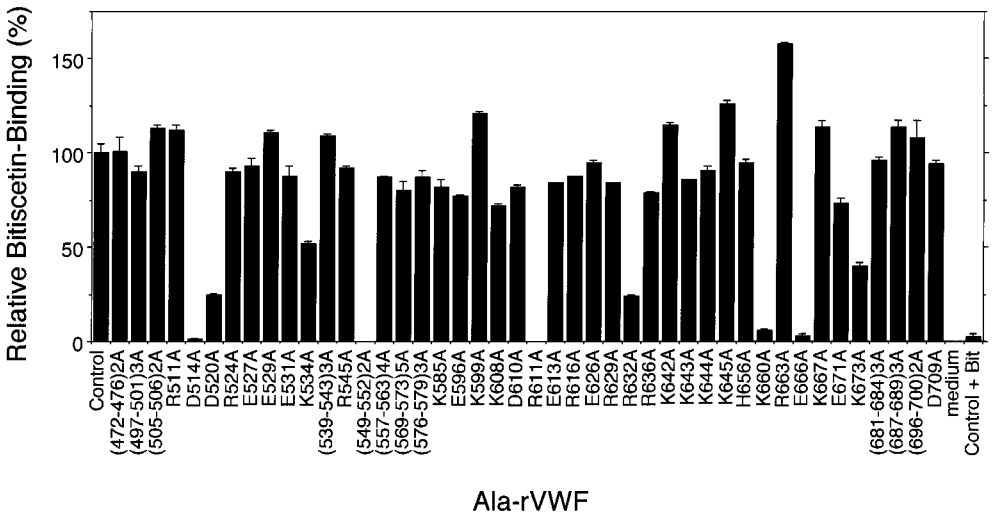

FIGURE 4: Binding of bitiscetin to Ala-rVWF. Ala-rVWFs at 1 μg/mL or culture medium was immobilized on an ELISA plate coated with mAb VW40-1. Biotin-conjugated bitiscetin at 1 μg/mL was added to the plate, and the binding was detected with HRP-conjugated streptavidin followed by HRP reaction. The binding was normalized to the values obtained for wild-type rVWF (Control) as 100%. Wild-type rVWF was also assayed in the presence of 50 μg/mL bitiscetin (Control + Bit). The data represent the mean ± SE of at least three independent experiments.

VWF and bitiscetin (Figure 1D), and the binding capacity of each Ala-rVWF for bitiscetin was compared with wild-type recombinant VWF. The binding of biotin-labeled bitiscetin to wild-type rVWF was almost canceled in the presence of a 50-fold higher amount of unlabeled bitiscetin, indicating the specific binding (Figure 4). Ala-rVWFs mutated at Asp514, Asp520, Lys549 + Arg552, or Arg611 showed decreased binding activities against bitiscetin, but these residues are embedded or located at the basal surface of the A1 domain, suggesting that these residues might not be related to the bitiscetin-binding site but might influence the molecular conformation of the A1 domain.

As summarized in Figure 4, the bitiscetin-binding activities of Ala-rVWFs were decreased or reduced when Lys534, Arg632, Lys660, Glu666, or Lys673 was substituted for alanine. In particular, substitutions at Arg632, Lys660, and Glu666 showed a very low binding activity to bitiscetin. In contrast, Ala-rVWFs substituted at Arg629, Arg636, and Lys667, which had been shown to be responsible for the reduced botrocetin binding of VWF (*12*), showed no significant effect on the binding for bitiscetin. Substitution at Arg663 showed an increased effect on the bitiscetin binding. These results indicate that bitiscetin binds to the VWF A1 domain at very close proximity to, but distinct from, the botrocetin-binding sites.

Effects of mAbs against Bitiscetin on Platelet Agglutination. We have obtained five mAbs against bitiscetin (ABIS). Three of them (ABIS-2, -5, and -8) recognized the reduced α-subunit (16 kDa), and two (ABIS-1 and -4) recognized the reduced *β*-subunit (13 kDa) of bitiscetin (Figure 5) as well as the nonreduced bitiscetin with an apparent molecular mass of about 28 kDa. No cross-reaction of these mAbs was observed against botrocetin by western blotting under either reducing or nonreducing conditions (Figure 5). When the bitiscetin binding to immobilized VWF was examined in the presence of various concentrations of ABISs, none of these mAbs showed an inhibitory effect on the binding between bitiscetin and VWF (data not shown), suggesting that these

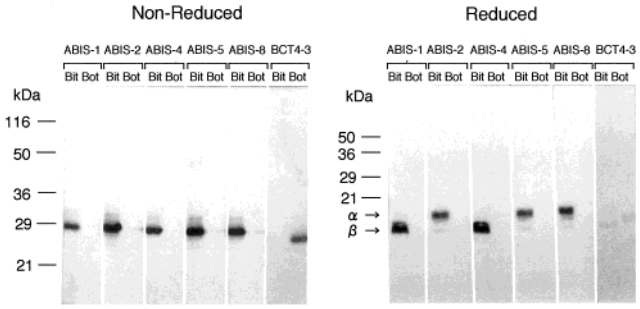

FIGURE 5: Binding specificity of anti-bitiscetin mAbs (ABIS). Bitiscetin (Bit) and botrocetin (Bot) (1 μg each) were electrotransferred to a PVDF membrane after SDS−PAGE under reducing or nonreducing conditions. Each blot was incubated with anti-bitiscetin mAbs (ABIS) or anti-botrocetin mAb (BCT4-3), and the binding was detected with HRP-conjugated second antibody. Bitiscetin and botrocetin showed a single band under nonreducing conditions at about 28 and 27 kDa, respectively. Arrows indicate the positions of the α- and β-subunits of bitiscetin at SDS−PAGE under reducing conditions. Numbers at the left indicate the molecular masses of standard marker proteins.

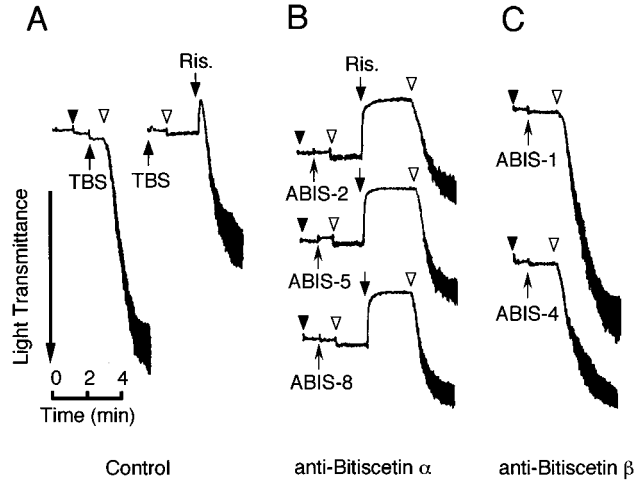

FIGURE 6: Effects of ABIS on platelet agglutination. Formalin-fixed platelets were mixed successively with bitiscetin (black arrowheads, 1 μg/mL), ABIS (thin arrows, 5 μg/mL), and VWF (white arrowheads, 5 μg/mL) at 1 min intervals. Platelet agglutination was monitored as a light transmittance using an aggregometer. (A) Control platelet agglutination induced by bitiscetin and ristocetin in the absence of ABIS. (B) Effects of anti-bitiscetin α-subunit mAbs (ABIS-2, -5, and -8) on the bitiscetin-induced platelet agglutination. Ristocetin (Ris, 1.3 mg/mL) was introduced 2 min after VWF addition, and subsequently extra VWF (10 μg/mL) was added to the mixture. (C) Effects of anti-bitiscetin β-subunit mAbs (ABIS-1 and -4) on the bitiscetin-induced platelet agglutination.

antibodies do not interact with the VWF-binding sites of bitiscetin.

The effects of ABIS on the bitiscetin-induced platelet agglutination were assayed using formalin-fixed platelets (Figure 6). In contrast to the direct bitiscetin binding to VWF, ABIS-2, -5, and -8, which specifically recognize the bitiscetin α-subunit, inhibited the bitiscetin-induced platelet agglutination (Figure 6A,B), but ABIS-1 and -4 recognizing the β-subunit showed no effect on the platelet agglutination (Figure 6C). The inhibitory effects of ABIS-2, -5, and -8 were not canceled by subsequent addition of ristocetin, but further addition of an abundant amount of VWF resumed the platelet agglutination (Figure 6B). These results suggest that the complex of mAb and bitiscetin still binds to VWF but makes VWF unable to access platelet GPIb possibly due to steric hindrance. In contrast, mAbs for the β-subunit did not have such an effect, suggesting that the mAbs for the α-subunit can perturb the access of VWF to GPIb.

DISCUSSION

We explored the bitiscetin-binding site on the VWF molecule using function-blocking mAbs against VWF, competition assays with collagen and botrocetin, and alanine scanning mutagenesis of VWF. Although bitiscetin has recently been reported to bind the A3 domain of VWF and interfere with the binding between collagen and VWF (*17*), our experiment showed that bitiscetin has no significant effect on the binding between coated type III collagen and soluble VWF. It is not clear why our results did not coincide with those obtained by Obert et al. (*17*), but it might be due to the preparation of bitiscetin, since there exist several structurally and functionally similar proteins (isotypes) in the same venom (Matsui, unpublished observations). Their preparation was not separated into two distinct subunits after SDS−PAGE under reduced conditions (*17*), in contrast to our results (*14*).

The binding of bitiscetin to VWF was inhibited by mAb NMC-4 and by botrocetin, both of which bind to the A1 domain (*10*, *12*, *24*). Furthermore, several recombinant VWFs mutated at specific residues of the A1 domain lost their bitiscetin-binding activity. Our results indicate that bitiscetin does not compete with the binding site of collagen but binds to the A1 domain by means of its very close position to botrocetin and NMC-4.

Matsushita et al. (*11*, *12*) clearly indicated the essential residues of the A1 domain of VWF for its binding to GPIb, botrocetin, and conformation-dependent anti-VWF mAbs by alanine scanning mutagenesis. They showed that Arg629, Arg632, Arg636, and Lys667 are important for the binding to botrocetin. They suggested that Asp514, Asp520, Arg552, and Arg611 are necessary for the proper folding of the A1 domain, even though each substitution decreased the binding to botrocetin, since these substitutions also decreased the binding of conformation-dependent anti-VWF mAbs. Lys599 was proposed to be the binding site for GPIb because the mutation at Lys599 abolished both the ristocetin- and botrocetin-induced GPIb binding but bound normally to botrocetin (*12*). Furthermore, the crystal structures of the A1 domain (*29*, *30*) have shown that Asp514, Asp520, Arg552, and Arg611 are located on the inner or bottom chain (the β1, β2 strands and α3−β4 loop) of the A1 domain (Figure 3) and form a salt bridge with surface residues necessary for the correct folding (*29*) and that the affecting residues for the botrocetin binding are located at the surface of α-helix 4 and 5 (Figure 7) (*12*).

The bitiscetin binding was significantly decreased when Lys534, Arg632, Lys660, Glu666, or Lys673 was substituted for alanine (Figure 4). Lys534 is located in the middle of helix α1 (rather than embedding itself in the molecule), and others are closely located within helix α4a, α5 and the α5−β6 loop facing the surface (Figure 7), suggesting that the substitution at Lys534 may influence the conformation rather than the bitiscetin binding of the A1 domain as reported for botrocetin (*12*). Substitution at Arg629, Arg636, or Lys667

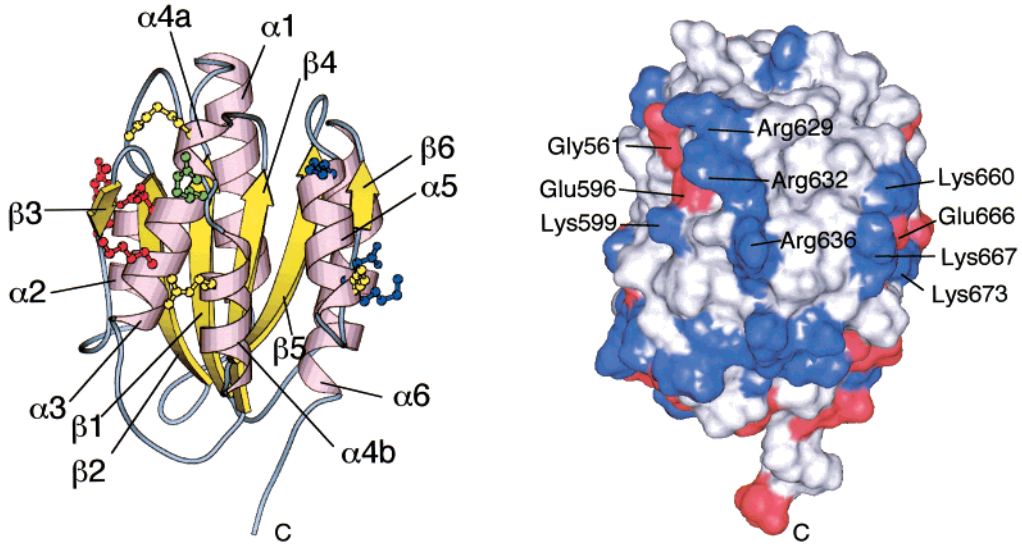

FIGURE 7: Location of amino acid residues important for bitiscetin binding on the VWF A1 domain. The VWF A1 domain structure was represented as a ribbon model (left) by MOLSCRIPT (*40*) and a surface representation model (right) using the program Insight II (Molecular Simulations, San Diego, CA) on the basis of the Protein Data Bank identification number 1auq. Both models are in the same orientation. Amino acid residues important for the binding of bitiscetin (Lys660, Glu666, and Lys673), botrocetin (Arg629, Arg636, and Lys667), and both (Arg632) are expressed in blue, yellow, and green as a ball-and-stick model on the left, respectively. Residues required for the GPIb binding (Lys599, Glu596, and Gly561) are expressed in red in a ribbon model. The α-helices (pink) and *β*-strands (yellow) are numbered according to Celikel et al. (*30*). Blue corresponds to positive and red to negative potentials on the right model. The letter C indicates the carboxyl terminus.

for Ala, which decreases the botrocetin binding (*12*), had no effect on the bitiscetin binding. Lys673 and Glu666 are important for bitiscetin binding but not for botrocetin binding (*11*, *12*), suggesting that bitiscetin interacts with the α5−*β*6 loop of the A1 domain. It is noteworthy that those basic amino acid residues, Arg632, Lys660, and Lys673, are important for the bitiscetin binding similar to botrocetin although bitiscetin has a basic nature (*14*). In addition to our results, Figure 7 suggests that the binding site of bitiscetin is not identical to, but overlaps with, that of botrocetin. As indicated in Figure 2, the competition of botrocetin on the binding between bitiscetin and VWF might be attributed to this overlapping in the binding site. Since Arg632 and Arg636 in helix α4 play an important role in the interaction with the NMC-4 Fab fragment (*12*, *29*), it is plausible that the binding of bitiscetin was inhibited by NMC-4 (Figure 1D) as in the case of botrocetin (*24*). These results suggest that the bitiscetin-binding area is around α4a, α5 helices and in the loop to the *β*6 strand including Arg632, Lys660, Glu666, and Lys673.

Hirotsu et al. (*31*) recently determined the crystal structure of bitiscetin. The overall structure of bitiscetin resembles those of C-type lectin-like proteins such as coagulation factor IX/X-binding protein (*32*), flavocetin (*33*), and botrocetin (*13*). Each subunit was composed of a globular domain and a central loop, and each loop was extended into the globular domain of the adjoining subunit. The central concave structure formed by the two subunits is a candidate binding site for each ligand, as was recently verified with the crystal structure of the complex between coagulation factor X-binding protein and the Gla (*γ*-carboxyglutamic acid) domain of factor X (*34*). The specificity of each ligand-binding property corresponds to the differences in the surface potential on the central concave structure (*31*). In botrocetin, Sen et al. (*13*) showed that a negatively charged patch on the concave surface of botrocetin is a candidate site for the binding to the positively charged surface of the VWF A1 domain. The crystal structure of bitiscetin indicated that it also has a negatively charged patch in the central concave surface similar to botrocetin (*31*). Furthermore, a positively charged patch was also present on the surface of the *β*-subunit of bitiscetin. These results suggest that bitiscetin has both binding sites, a central negative patch for the positive surface of the A1 domain and a positive patch for the negative surface of the A3 domain. Our results suggest that the basic residues, such as Arg632, Lys660, and Lys673 in the A1 domain, interact with a negative patch of bitiscetin similar to botrocetin. Although the present data indicate that the A3 domain is not sufficient to bind bitiscetin, it is possible that a positive patch of bitiscetin synergistically stabilizes the complex by interacting with the A3 domain. Recently, the collagen-binding site has been proposed not to be located on the negatively charged top face of the A3 domain but on the bottom face of the domain (*35*, *36*), suggesting that bitiscetin can interact with a different site on the A3 domain from the collagen-binding site.

Fujimura et al. (*6*) suggested that the α-subunit of botrocetin is responsible for VWF binding on the basis of the results obtained with an α-subunit-specific mAb. The crystal structures of botrocetin and bitiscetin suggest that both of the subunits are responsible for constructing the negative patch of the central concave surface (*13*, *31*). In the present study using mAbs against each subunit of bitiscetin, we could not obtain any mAb which blocks the binding between bitiscetin and VWF but found that only the α-subunit recognizing mAb inhibits the bitiscetin-induced platelet agglutination. Because this mAb−bitiscetin complex can bind to VWF but this VWF became insensitive to the subsequent addition of ristocetin, mAb in the complex might interfere with the binding of GPIb to VWF. The putative GPIb-binding site is located at helix α3 of the A1 domain including Lys599 (*11*, *12*) and Glu596 as well as at a region around strand *β*3

including Gly561 and Tyr 565 (*37*) (Figure 7). NMC-4 indirectly perturbs the access of GPIb by binding to helix α4 near the GPIb-binding site (*30*). The binding of an anti-α-subunit mAb to the bitiscetin might bring about a steric hindrance to the GPIb-binding site, suggesting that the α-subunit of bitiscetin is located on VWF closer to the GPIb-binding site than the *β*-subunit is.

The induction of VWF−GPIb binding in vivo has been attributed to the immobilization of VWF on the subendothelial surface and the dynamic flow of platelets. It is proposed that VWF changes from a globular shape under static or continuous flow conditions to a stretched shape when immobilized under shear stress (*3*) which might increase the accessibility of the A1 domain to GPIb. In vivo, GPIb−VWF binding seems rather weak, but its continuous attaching and detaching may stimulate platelets to undergo the activation of GPIIb/IIIa, which gives rise to a higher binding affinity (*4*, *38*). It remains to be determined whether bitiscetin, botrocetin, and ristocetin may also induce the stretched conformation of the VWF molecule by binding to the A1 domain in the same manner as that under high shear stress, because the binding of GPIb to the VWF−modulator complex seems more rigid than the binding under physiological conditions. It seems likely that the GPIb-binding site (around helix α3 and strand *β*3 of the A1 domain) may become an exposed state by a conformational change induced by the bitiscetin or botrocetin binding, leading to an increased accessibility to platelet GPIb. There is another possibility that in vitro VWF modulators have a sub- or synergistic binding site for GPIb after being complexed with VWF. Structural similarities of several GPIb-binding venom proteins also suggest that a similar structure can interact with GPIb. Kawasaki et al. (*39*) reported that the GPIb-binding site of GPIb-binding protein from the *Bothrops jararaca* venom (which shows high similarities to bitiscetin and botrocetin) resides on its *β*-subunit. Further analysis, such as an X-ray crystal structural analysis using the complex of the A1 domain, GPIb, and bitiscetin will be a prerequisite to understand the structure−function relationships and the regulation mechanisms of these in vitro modulators.

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