Crystal Structure of von Willebrand Factor A1 Domain Complexed with Snake Venom, Bitiscetic

INSIGHT INTO GLYCOPROTEIN Ibα BINDING MECHANISM INDUCED BY SNAKE VENOM PROTEINS*

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Bitiscetic, a platelet adhesion inducer isolated from venom of the snake Bitis arietans, activates the binding of the von Willebrand factor (VWF) A1 domain to glycoprotein Ib (GPIb) in vitro. This activation requires the formation of a bitiscetic-VWF A1 complex, suggesting an allosteric mechanism of action. Here, we report the crystal structure of bitiscetic-VWF A1 domain complex solved at 2.85 Å. In the complex structure, helix α5 of VWF A1 domain lies on a concave depression on bitiscetic, and binding sites are located at both ends of the depression. The binding sites correspond well with those proposed previously based on alanine-scanning mutagenesis (Matsui, T., Hamako, J., Matsuhashita, T., Nakayama, T., Fujimura, Y., and Titani, K. (2002) Biochemistry 41, 7939–7946). Against our expectations, the structure of the VWF A1 domain bound to bitiscetic does not differ significantly from the structure of the free A1 domain. These results are similar to the case of botrocetin, another snake-derived inducer of platelet aggregation, although the binding modes of botrocetin and bitiscetic are different. The modeled structure of the ternary bitiscetic-VWF A1-GPIb complex suggests that an electrostatic surface of bitiscetic may interact with a favorably positioned anionic region of GPIb. These results suggest that snake venom proteins induce VWF A1-GPIb binding by interacting with both proteins, and not by causing conformational changes in VWF A1.

Von Willebrand factor (VWF)1 plays a key role in hemostatic plug formation at sites of vascular injury by interacting with subendothelial matrix proteins and platelet glycoprotein Ib (GPIb) (1). VWF exists as a disulfide-linked multimer composed of identical 250-kDa subunits, each of which contains three adjacent A domains (A1, A2, and A3). The A1 and A3 domains have major binding sites for GPIb and collagens (types I and III), respectively, and the A2 domain contains the cleavage site for the plasma VWF-cleaving protease (ADAMTS13), which regulates the functional multimer size of VWF (2).

Binding between VWF A1 domain and GPIbα is evident only when blood is exposed to high shear stress in vivo (3, 4). This binding activation mechanism is proposed to reflect structural changes in the A1 domain (4, 5). A structural change in the Asp560–Gly561 region was observed in the structure of the VWD type 2B “gain-of-function” A1 domain mutant, which binds spontaneously to GPIb under physiological conditions (6). Binding of VWF to platelets in vitro occurs in the presence of various modulators, such as the antibiotic ristocetin (7) or snake venom proteins bitiscetic (8) or botrocetin (9). Snakes employ bitiscetic and botrocetin to disrupt hemostasis and thereby to kill or weaken their prey; these modulators have been used extensively as tools to study the complicated mechanisms involved in hemostasis.

Bitiscetic and botrocetin are members of C-type lectin-like proteins (CLPs). The first determination of the complete amino acid sequence of CLPs was carried out using coagulation factors IX- and X-binding protein (IX/X-bp) from Trimeresurus flavoviridis (habu snake) venom (10). Up to the present time, various CLPs have been sequenced and characterized with a variety of activities that affect plasma proteins, platelets, endothelial cells, and subendothelial structures. Thus, CLPs with diverse activities appear to be derived from a common ancestor. In this connection, IX-xbp and IX-bp from habu snake venom have diversified their amino acid sequences in an accelerating manner (11) as also observed for crotalinae snake venom gland phospholipases (12) and serine proteases (13). As shown for the digestive enzymes in venom, which vary predictably in response to differences in diet (14), CLPs have evolved possibly to gain functional diversity in response to important components in the hemostatic system of their prey.

CLPs are heterodimeric proteins consisting of homologous subunits A (α) and B (β) linked by a disulfide bond. Both subunits show a similarity to carbohydrate recognition domains of the classic C-type lectins. The crystal structure of IX-xbp (15) shows that the two subunits are tightly associated by domain swapping, and this dimerization results in the creation of the concave surface predicted to function as a coagulation factor binding site. Examination of the crystal structure of the complex between X-bp and the γ-carboxyglutamic acid (Gla) domain of factor X has revealed that the binding site is, in fact, the concave surface (16). The structures of CLPs with

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** The abbreviations used are: VWF, von Willebrand factor; GPIb, glycoprotein Ib; CLP, C-type lectin-like protein; IX-bp and X-bp, coagulation factors IX-binding protein and X-binding protein.

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EXPERIMENTAL PROCEDURES

Materials—Bitiscetin was purified from crude venom of Bitis arietans (purchased from ICM Biomedicals Inc., as described previously (5)). Recombinant VWF A1 domain (residues 497–708), expressed in Escherichia coli and purified as described (24), was further purified with MonoQ (Amersham Biosciences) and TSK-GEL G2000SW gel filtration column (Toosho). The affinity of VWF A1 binding to immobilized bitiscetin was measured by a surface plasmon resonance assay using Biacore 3000 (Biacore AB), and a high affinity with a Kd 2 nM was obtained. Bitiscetin and VWF A1 domain were mixed in a 1:1.3 molar ratio, placed on ice for 2–3 h, and then loaded on Superdex 200 column (Amersham Biosciences) equilibrated with sample buffer (10 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 5% 2-Mercaptoethanol, 0.1% CHAPS). The initial model was built by the O program (7) in a 1:1.3 molar ratio of reservoir solution. The crystal structure was determined by projection matching to the atomic coordinates of X-ray crystallography (8). The final model was refined by applying the corresponding symmetry-related reflections.

Crystalization and Data Collection—Needle-shaped crystals were obtained by mixing X-galactosidase solution with various concentrations of bitiscetin, botrocetin, and vWF A1 domain. The crystals were grown by the sitting-drop vapor diffusion technique by mixing 3.0 cm3 of reservoir solution. The data were collected at 100 K on an R-AXIS IV++ imaging plate detector (Rigaku). The data were integrated by using MOSFLM (25) followed by scaling with SCALA (26) in the resolution range of 30 to 2.85 Å.

Crystal Structure of Bitiscetin-VWF A1 Complex

Data Collection

Table 1

| Data collectiona | Photon Factory BL-6B |
|------------------|----------------------|
| X-ray source     |                      |
| Wavelength (Å)   | 1.000                |
| Detector         | R-AXIS IV++          |
| Space group      | P4_3                 |
| Unit cell (Å)    | a = b = 89.28 Å, c = 53.39 |
| Resolution (Å)   | 30–2.85 (2.99–2.85)  |
| Completeness (%) | 91.6 (91.6)          |
| Observed reflections | 51631 (8285) |
| Unique reflections| 9055 (1456)          |
| R.d. (sigma)     | 5.6 (5.7)            |
| Rsym (%)         | 10.7 (31.1)          |
| Refinement       |                      |
| Resolution (Å)   | 20–2.85              |
| RcrystalRfree (%)| 19.427.6             |
| r.m.s.d. bonds (Å)| 0.0552              |
| r.m.s.d. angles (%) | 1.21                |
| No. of non-H atoms | 3633/40             |
| (protein/water)  |                      |
| (B) (Å²) (protein/water) | 36.1/19.9 |
| Ramachandran plot (%) | Most favored 79.4 |
|                   | Allowed 19.9         |
|                   | Disallowed 0.7       |

RESULTS AND DISCUSSION

Overall Structure of Bitiscetin-VWF A1 Complex—Bitiscetin, as well as other CLPs, has a heterodimeric structure, and VWF A1 domain binds to the concave surface of bitiscetin (Fig. 1a). The concave surface often functions as a ligand-binding site as shown previously for the structure of X-bp complexed with the
factor X γ-carboxyglutamic acid domain (16). Helix α5 of the A1 domain is positioned near the center of the concavity, near the junction between the bitiscetin α and β subunit, but interacts mainly with the β-subunit, leaving a solvent-filled space between discontinuous binding sites at both ends of the concave surface of bitiscetin. Helix α4 of the A1 domain contacts the α-subunit of bitiscetin, burying a solvent accessible surface of \( -177 \text{ Å}^2 \), and sheet β6 and helices α5 and α6 of A1 contact the β-subunit of bitiscetin (contact 2), b and c, close-up views of the interaction site between the bitiscetin α-subunit and A1 domain (contact 1) (b) and the β-subunit and A1 domain (contact 2) (c). Hydrogen bond interactions are shown as broken lines. Residues and Ca traces are colored as described in a, c, inset, the red arrow shows different positions of Arg663 side chains when the A1 domain in the bitiscetin-A1 complex is superimposed on the uncomplexed A1 domain. This figure was generated with MOLSCRIPT (37) and RASTER3D (38).

A comparison with the structure of the botrocetin-VWF A1 complex shows that the botrocetin- and bitiscetin-binding sites overlap at A1 helices α4 and α5. The direction of the long-axis of bitiscetin is almost perpendicular to that of botrocetin when the bound A1 domains are superimposed. Although bitiscetin (516 Å\(^2\)) and botrocetin (520 Å\(^2\)) make contact with A1 domains, bitiscetin binds more tightly than botrocetin, with a solvent accessibility of ~431 Å\(^2\) (Fig. 1a).

Interaction of Bitiscetin and VWF A1 Domain—At the contact region on the α-subunit (contact 1), direct hydrogen bond interactions are observed between Tyr\(^{664}\) of bitiscetin and Arg\(^{629}\) of the VWF A1 domain (hereafter, the amino acid residues of bitiscetin and VWF A1 will be listed in the same order) and between the main chain carboxyl of His\(^{596}\) and Glu\(^{629}\), and indirect water-mediated interactions are observed between Asp\(^{65}\) and Glu\(^{628}\) and between the O atom of Val\(^{663}\) and Asp\(^{119}\) (Fig. 1b). In the contact region on the β-subunit (contact 2), hydrophobic and hydrogen bond interactions are found. A hydrophobic patch on the β-subunit composed of Leu\(^{59}\), Val\(^{64}\), Leu\(^{65}\), Phe\(^{104}\), and Ile\(^{109}\) contacts a hydrophobic surface of the A1 domain that is composed of Pro\(^{655}\), Leu\(^{659}\), Ile\(^{662}\), and Val\(^{676}\). Adjacent to the hydrophobic region, hydrogen bonded interactions are found between Trp\(^{111}\) and Lys\(^{117}\) in the β-subunit and Glu\(^{666}\) in A1, between Gln\(^{110}\) and Arg\(^{663}\), between Glu\(^{22}\) and Lys\(^{673}\), between Lys\(^{90}\) and both Glu\(^{686}\) and Glu\(^{689}\), and between Arg\(^{115}\) and the backbone oxygens of Glu\(^{666}\), Lys\(^{673}\), and Ala\(^{669}\) (Fig. 1c). In addition, a hydrogen bond between Glu\(^{96}\) in the bitiscetin α-subunit and Lys\(^{660}\) in the A1 domain is observed.

Comparison of the Bitiscetin-VWF A1 Structure with Mutagenesis Results—Recently, Matsui et al. (23) reported the localization of bitiscetin-binding sites on the VWF A1 domain by alanine-scanning mutagenesis. According to this report, three A1 domain mutations on helix α5 (K660A, E666A, and K673A) and one mutation on helix α4 (R632A) significantly reduced binding to bitiscetin. These results are in good agreement with our structural data, because all of these residues are involved in hydrogen bond interactions with bitiscetin (Fig. 1, b and c). Interestingly, the A1 mutant R663A showed 1.5-fold higher binding activity relative to the wild type A1 domain (23). The side chain of Arg\(^{663}\) interacts with Gln\(^{110}\) in the β-subunit of bitiscetin, one of the major interactions between the A1 domain and bitiscetin (Fig. 1c), and mutation of Arg\(^{663}\) might be expected to reduce the affinity of binding. This contrary result may be explained as follows. The orientation of Arg\(^{663}\) in
The stereo model was generated from the structures of complexes for bitiscetin-VWF A1 and GPIba-VWF A1. Bitiscetin ω-subunit (magenta), β-subunit (green), VWF A1 domain (cyan), and GPIba (gold) are drawn as ribbon representations. This figure was generated with MOLSCRIPT (37) and RASTER3D (38).

The stereo model was generated from the structures of complexes for bitiscetin-VWF A1 and GPIba-VWF A1. Bitiscetin ω-subunit (magenta), β-subunit (green), VWF A1 domain (cyan), and GPIba (gold) are drawn as ribbon representations. This figure was generated with MOLSCRIPT (37) and RASTER3D (38).

**Fig. 3.** Predicted model of bitiscetin-VWF A1-GPIba ternary complex. The stereo model was generated from the structures of complexes for bitiscetin-VWF A1 and GPIba-VWF A1. Bitiscetin ω-subunit (magenta), β-subunit (green), VWF A1 domain (cyan), and GPIba (gold) are drawn as ribbon representations. This figure was generated with MOLSCRIPT (37) and RASTER3D (38).

**Fig. 4.** Electrostatic surface potentials of the bitiscetin-VWF A1 complex (left) and the botrocetin-VWF A1 complex (right). The molecular surface is colored blue for a positive charge (+10 kT/e) and red for a negative charge (−10 kT/e). GPIba (Protein Data Bank entry 1M10) models are also shown in a possible bound position in both figures as Cα tube representations (green). The C terminus of the GPIba fragment (residues 284–288), including the anionic region (Protein Data Bank entry 1GWB), is colored in magenta for clarity. The positively charged patches on both venom proteins located near the C-terminal anionic region of GPIba are highlighted with yellow ovals. This figure was generated with GRASP (39).

**Fig. 5.** Hypothetical model of GPIba binding activation mechanism induced by bitiscetin. Step 1 (top), The VWF A1 domain (cyan) binds to the concave surface of bitiscetin, bringing the GPIba binding surface of the A1 domain and the positively charged patch on bitiscetin into proximity. Step 2 (middle), the anionic region of GPIba interacts with the positively charged patch of bitiscetin. Step 3 (bottom), the anionic region acts as an anchor, which enhances the GPIba to bind to A1 domain.

thermore, when the wild type or I546V A1 structures were superimposed onto the bitiscetin-complexed A1 structure, no significant structural changes were found at either the bitiscetin- or GPIba-binding sites (Fig. 2a). Large structural changes were observed only in the N-terminal and C-terminal regions of A1 domain that flank the Cys560–Cys695 disulfide bond. These regions are expected to be changeable because of their location outside of the disulfide loop, and indeed their flexibility is evident from the crystallographic B-factors in these regions.

**Predicted Model of the Bitiscetin-VWF A1-GPIba Ternary Complex**—The crystal structure of the VWF A1 (R543Q)-GPIba (M239V) complex reveals two binding sites on the A1 domain for GPIba: a major site at the “top” of A1 involves the β3 strand, α3 helix, and part of the α3-β4 loop; a minor site at the “bottom” of the domain involves loops α3-β4, β3-α2, and α1-β2. In the structures of A1 complexed with bitiscetin or botrocetin, however, no significant changes are observed in these regions. Moreover, in the structure of the botrocetin-A1 (I546V) complex, the main chain carbonyl of Asp560 has the same conformation as the uncomplexed wild type A1 domain, despite the use of a gain-of-function A1 mutant (21). These observations suggest that bitiscetin and botrocetin stimulate the binding of A1 to GPIba by a mechanism that does not require allosteric conformational changes in VWF A1.

The modeled structure of the bitiscetin-VWF A1-GPIba A1 complex (Fig. 3) indicates that the binding sites of bitiscetin and GPIba on the A1 domain do not overlap, so that GPIba could bind easily to the bitiscetin-VWF A1 complex. In this model, the β-subunit of bitiscetin is well separated from GPIba, whereas the α-subunit of bitiscetin and the C terminus of the GPIba fragment are approximated. This model is consistent with the observation that monoclonal antibodies to the α-subunit inhibit GPIba binding to the bitiscetin-A1 complex, but antibodies to the β-subunit do not (23).

**Implications for the Mechanism of GPIba-A1 Domain Binding**
Induced by Venom Proteins—The electrostatic potential of bitiscetin-A1 complex shows that there is a positively charged patch close to the C terminus of GPIb fragment in the modeled ternary complex (Fig. 4, left). The positively charged patch of bitiscetin is constructed from α-subunit residues Lys17, Lys20, Lys21, Lys8, and Lys30. A similar positively charged patch is also found in the botrocetin-A1 complex (Fig. 4, right), which consists of α-subunit residues Arg34, Lys103, and β-subunit residues Lys102, Trp109, Arg115, and Lys117 (botrocetin residues are numbered according to Ref. 19). We propose that these positively charged patches observed in both venom proteins are the key to the activation of GPIbα binding to the A1 domain.

At the C terminus of the GPIbα fragment, there is an anionic region (residues 269–279) characterized by three sulfated tyrosine residues, Tyr276, Tyr278, and Tyr279 (32). The removal of these sulfate moieties, whether by mutation or by inhibition of sulfation, severely impairs the ability of botrocetin to induce VWF-dependent platelet aggregation (33, 34) indicating that the anionic region of GPIb plays an important role in the high affinity binding of botrocetin-A1 complexes. The anionic region of GPIbα was disordered in the structure of the binary VWF A1-GPIbα complex (31). In the crystal structure of uncomplexed GPIbα, there were two molecules in the asymmetric unit; in one molecule the anionic region was observed but in the other it was disordered (35). These observations suggest that the anionic region of GPIbα is flexible but could adopt a specific conformation upon binding to a nearby positively charged site on bitiscetin or botrocetin complexed with VWF A1.

Based on their GPIbα-VWF A1 structure, Huizinga et al. (31) proposed that the N and C termini of the A1 domain, which are located at the bottom face, shield the binding site from the proposed that the N and C termini of the A1 domain, which are

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