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ORIGINAL ARTICLE

Characterization of bitiscetin-2, a second form of bitiscetin from the venom of *Bitis arietans*: comparison of its binding site with the collagen-binding site on the von Willebrand factor A3-domain

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Summary. *Background:* Bitiscetin, a heterodimeric snake venom protein purified from *Bitis arietans*, binds to the A1 domain of von Willebrand factor (VWF) and induces binding of this domain to platelet glycoprotein (GP) Ib. We previously purified a distinct form of dimeric bitiscetin (herein called bitiscetin-2) that also induces the VWF A1 domain-GPIb interaction, but does not bind to the A1 domain. Instead, it interacts with the collagen-binding A3 domain of VWF. *Methods:* In the current study we identify the amino terminal sequence of the bitiscetin-2 as DEGCLPDDSSRT, showing conclusively that the protein is distinct form the originally described bitiscetin. We further studied the interaction of bitiscetin-2 and VWF using DeltaA3 VWF and a series of 33 VWF point mutants previously prepared to map the collagen-binding site. *Results:* Our results confirm that DeltaA3 VWF, even though containing the A1-domain, is unable to interact with bitiscetin-2. Mutation of VWF-A3 residues Ile975, Asp979, Pro981, Ser1020 and His1023 reduces binding by 80% while mutation of residues Val980, Glu1001 and Arg1021 reduces binding by 30–60%. A 2- to 6-fold increase of binding is caused by mutation of residues Val985, Glu987, and Arg1016. *Conclusion:* Nearly all of these mutations also affect collagen binding showing that the binding sites for bitiscetin-2 and collagen type III in the VWF-A3 domain closely overlap.

Keywords: A3 domain, bitiscetin-2, collagen-binding, mutants, von Willebrand factor.

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Introduction

von Willebrand factor (VWF) is a multimeric glycoprotein (GP) that plays a key role, at high shear rates, in the initial attachment of platelets to the extracellular matrix of the endothelial cells after injury of the vessel wall. In this mechanism, VWF acts as a molecular bridge between platelet receptors, primarily GPIb and components of the subendothelium, such as collagen. The affinity of circulating VWF for platelet GPIb is low but can be increased under high shear conditions, which contribute to an adequate change of conformation of VWF [1]. It was also established that the shape change of VWF is facilitated when the protein is submitted to high-shear conditions after immobilization onto hydrophobic surfaces [2]. Therefore, it was suggested that a similar mechanism occurs when VWF is immobilized onto subendothelial collagen and submitted to high shear stress. However, so far under static conditions, no direct effect of the VWF-collagen interaction has been established on the ability of VWF to bind platelet GPIb. *In vitro*, interaction of VWF with GPIb can be promoted by various non-physiological inducers such as ristocetin, or snake venom proteins botrocetin [3] and bitiscetin [4,5].

VWF is composed of a linear arrangement of identical 275 kDa subunits linked through disulfide bridges in a head-to-head and tail-to-tail manner [6]. Each subunit contains 2050 amino acid (aa) residues [7] and five types of structural domains (A to D and CK). There are three adjacent copies of the A domains. The A1 and A3 domains contain a large loop of 185 aa closed by a disulfide bond between Cys 509 and 695 and Cys 923 and 1109, respectively (numbering of aa starting at the first Ser of the mature subunit). The A1 loop contains the VWF binding sites for platelet GPIb and for botrocetin while ristocetin interacts with its flanking regions. The A3 loop contains the major VWF binding site for type I and type III collagens [8–11]. The collagen-binding site of the

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A3 domain has been further determined by epitope mapping of functional monoclonal antibodies (MoAbs), by binding studies on recombinant VWF molecules with mutations in the A3 domain and by analyzing the crystal structure of VWF-A3 [12–17].

Hamako *et al.* [4] first purified bitiscetin from snake venom of *Bitis arietans*. The protein appears as a heterodimeric protein of 25 kDa with an alpha chain of 16 kDa and a beta chain of 13 kDa. Bitiscetin was identified as a potent inducer of VWF-dependent platelet agglutination. A study by Matsui *et al.* [18], showing that mutations in the A1-domain altered the binding of bitiscetin to VWF, suggested that the inducer directly acted on the A1 domain. More recently the crystal structure of the A1 domain-bitiscetin complex was established [19]. We purified a bitiscetin-2 from *B. arietans* venom and also established that it specifically induces the binding of VWF A1-domain to GPIb [5]. However, some differences were noted between properties of the purified bitiscetin used by Titani's group and our bitiscetin-2, which might be in part responsible for discrepant results. In our hands, the bitiscetin-2 (29 kDa) appeared composed of two chains with similar molecular mass (MM) ($\sim$15 kDa) suggesting that distinct but similar proteins were purified and used [18]. Using MoAbs against the VWF A1-domain, we also established the interrelationship between the structural change of the A1-domain and the binding of bitiscetin-2 to VWF. However, we demonstrated that bitiscetin-2 did not bind to the A1-domain but that its binding site on VWF was located within the A3-domain. In addition, competitive binding assays established that bitiscetin-2 interfered with the binding of VWF to collagen. We thus hypothesized that the bitiscetin variant that we purified might induce a long-range conformational change of the A1-domain by acting at the A3-domain level in a way similar to that of collagen under high shear stress conditions.

In the present study, we sequenced the N-terminal part of purified bitiscetin-2. Using a series of VWF mutants already tested for their capacity to interact with collagen, we mapped the bitiscetin-binding site within the VWF A3-domain. Our data established that bitiscetin-2 is a dimer distinct from bitiscetin originally identified by Hamoko *et al.* [4] and that its binding site is closely overlapping the collagen-binding site on the VWF-A3 domain.

Materials and methods

Purification of bitiscetin-2, sequencing and induced binding of ^{125}I-VWF to fixed platelets

Bitiscetin-2 (originally termed bitiscetin [5]) was purified from *B. arietans* venom (Sigma, St Louis, MO, USA) as previously described [5]. Purified bitiscetin-2 appeared as a dimer by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis performed according to Laemmli [20] with an apparent MM of 29 kDa when unreduced and a single band of $\sim$15 kDa after reduction. After SDS-15% polyacrylamide gel electrophoresis, the reduced protein was transferred onto polyvinyldene fluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, USA) and identified by staining with Ponceau's solution (Sigma). After distaining with water, the band was excised and subjected to amino acid sequence analysis of the N-terminal end. Automated Edman degradation was carried out in a Procise® Applied Biosystems protein sequencer Model 492A (Applied Biosystems, Courtaboeuf, France) by the Centre Commun de Sequençage, IBPC-CNRS, Lyon, France.

The capacity of purified bitiscetin-2 to induce the binding of ^{125}I-VWF to GPIb onto fixed platelets was performed as previously described [5] using 1 μg mL^{-1} of VWF, 10^8 cells mL^{-1} and 2 μg mL^{-1} of bitiscetin-2 as inducer.

Radiolabeling of proteins

Purified VWF, bitiscetin-2 and antibodies (IgG) were labeled using Na^{125}I (Amersham, Les Ulis, France) and Iodo-Gen (Pierce Chemical Co, Rockford, IL, USA) [21]. Specific radioactivities were about 2, 10, and 4 μCi μg^{-1}, respectively.

Monoclonal antibodies

Two murine MoAbs to VWF, MoAb 433 and 487 were used in this study. Each was directed against a distinct epitope localized within the sequence 1366–2050 of the VWF mature subunit. None of them is known to interfere with a function of VWF.

Recombinant VWFs (rVWFs)

Besides the wild-type (WT) rVWF, 34 rVWFs mutated in the A3-domain were constructed, stably expressed in BHK cells over-expressing furin, purified and used in this study. Lankhof *et al.* [10] produced and characterized Delta A3-VWF deleted from the A3-domain (aa 910–1113). Its multimeric distribution was similar to that of WT-rVWF, but it exhibited a strikingly decreased capacity to bind to collagen. The positions of the 33 other mutations used in this study are presented in Fig. 1. van der Plas *et al.* [12] characterized seven rVWFs with the following substitutions (residue numbering used here starts at the first residue of the mature VWF subunit): Asp934Ala/Ser936Ala, Val1040Ala/Val1042Ala, Asp1046Ala, Asp1066Ala, Asp1069Ala, Asp1069Arg, and Arg1074Ala. These rVWFs exhibited a normal distribution of multimers and a normal binding to human fibrillar collagen type III [12]. Five alanine mutants at position Arg963*, Glu987, His990, Arg1016* and His1023* (*indicates the mutations significantly modifying the collagen binding) were produced and characterized as described [14]. Other 21 rVWFs with the residues Gln966, Ser974, Ile975*, Thr977*, Asp979*, Val980, Pro981, Asn983, Val984, Val985, Ser993, Val997*, Gln999, Glu1001*, Gln1006, Asp1009, Ser1020* and Glu1021 mutated to Ala and Pro962, Pro981* and Pro1027 mutated to His were produced and characterized as described [15]. Multimeric distribution of all these rVWF mutants and WT-rVWF were indistinguishable [14,15].

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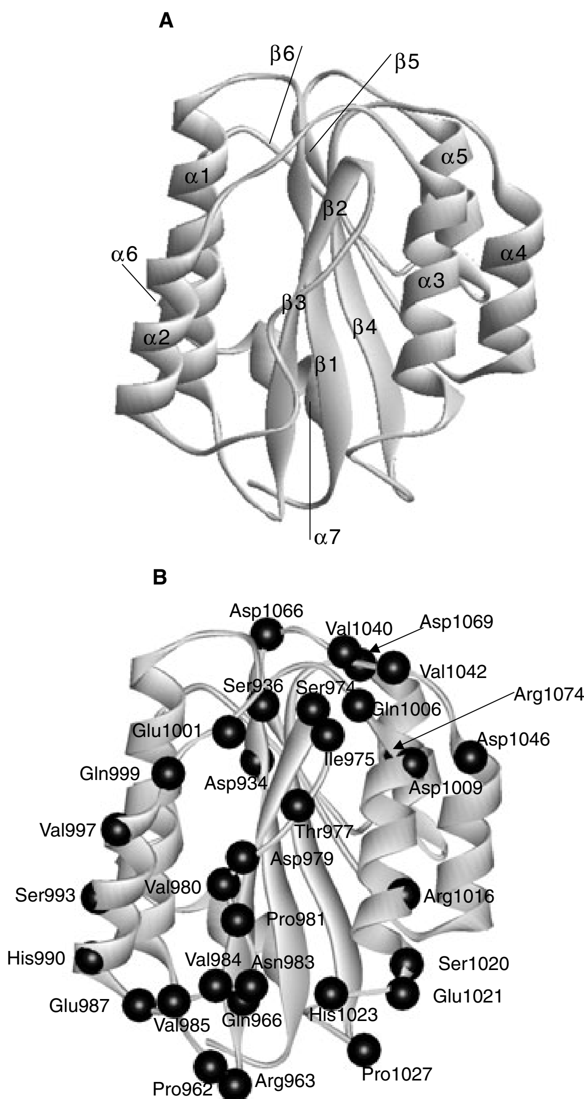

Fig. 1. Structure (ribbon diagram) of the von Willebrand factor (VWF) A3-domain (A) and localization of mutated amino acid residues (B) used in this study. Coordinates of the crystal structure of the VWF A-3 domain were taken from Brookhaven Protein Data Bank entry 1ATZ [17]. The figure was prepared using WebLab ViewerLite. The balls represent the position of the N-peptidic bond of the residues.

Binding of ^{125}I-bitiscetin-2 to rVWF

In our hands, the amount of purified bitiscetin-2 was not large enough to determine binding isotherms for each mutant. As an alternative method we used a two-site binding assay [22]. The method is based on the fact that binding isotherms are quite linear at low concentration of labeled ligand (below or in the range of K_d). Thus, under these conditions, using a single concentration of labeled ligand, a variation of the VWF concentration or of its binding constant (K_a) results in a proportional variation of ligand binding. While such a method does not allow determining absolute K_d values, it does provide a direct comparison of the capacity of binding of bitiscetin-2 to the series of mutants with respect to the WT-VWF. Binding of ^{125}I-bitiscetin-2 to purified rVWF was performed using such a two-site assay as described [5]. Briefly, coating of MoAb 433 (10 μg mL^{-1} in 0.05 M carbonate-bicarbonate buffer, pH 9.6) was performed by incubating 100 μL of the solution into wells of polyvinyl chloride microtiter plates for 18 h at 22 °C. Wells were blocked with 200 μL of 2% bovine serum albumin (BSA) (Sigma) in Tris-buffered saline (TBS) (25 mM Tris–HCl buffer, 0.15 M NaCl, pH 7.4) for 30 min at 37 °C. After washing, buffer or serial dilutions of rVWF in TBS containing 1% BSA (100 μL per well in the range of 0.004 to 0.5 U VWFAg mL^{-1}) were incubated for 18 h at 37 °C. After washing taking about 1 min, a constant amount of ^{125}I-protein (100 μL, 100 000 cpm, i.e. ~0.1 μg mL^{-1}, ~3×10^{-9} M) in TBS containing 0.1% BSA was incubated for 5 h at 37 °C. After washing (about 1 min) the bound radioactivity was counted. In parallel experiments the amount of rVWF immobilized into the well was estimated by using ^{125}I-MoAb 487 (30 000 cpm, 100 μL) instead of labeled bitiscetin-2.

Results were expressed as bound ^{125}I-bitiscetin-2 as a function of bound ^{125}I-MoAb 487. The ratio of slopes of the dose–response curves for mutated rVWF and for WT-rVWF was calculated to estimate the binding capacity of bitiscetin-2 to the various mutants. Statistical significance of the difference was estimated using *P*-values calculated with Student's *t*-test.

Results

By SDS-polyacrylamide gel electrophoresis, reduced bitiscetin-2 appears as a single band of about 15 kDa. After transfer onto PVDF membrane the protein was subjected to sequence analysis and the single N-terminal sequence identified was DEGCLPDDSSRT (single-letter amino-acid codes). Sequencing had an initial yield of 54 pmoles and a repetitive yield for residues D, E, G, and L of 91.2%. To our surprise the N-terminal sequence was very similar to that of the beta-subunit of bitiscetin differing only by a W → D and a Y → R substitution at positions 8 and 11, respectively. Comparison of the experimental retention times for D and W and for R and Y associated with a typical variation of retention time < 1% excluded all misinterpretation. This sequence was not found in other protein by computer search.

Figure 2 compares the capacity of binding of ^{125}I-bitiscetin-2 to a series of 34 mutated rVWFs with that of WT-rVWF. As previously observed the DeltaA3 rVWF lacking the VWF-binding site for bitiscetin-2 had no affinity for it. Among the 33 mutated rVWFs tested three groups can be distinguished. Alanine mutations of the residues I975, D979, P981, S1020, H1023, and mutation P981H resulted in significant loss (within 80–97%) of binding capacity of the A3 domain for bitiscetin-2 while alanine mutation of the residues V980, E1001 and E1021 resulted in a loss of 30%, 50% and 60%, respectively. Alanine mutations of residues V985, E987 and R1016 resulted in a significantly increased capacity of binding for bitiscetin-2 that was about 6- (mean ± SD = 580% ± 84%, $P < 0.001$) 2- (265% ± 80%, $P < 0.05$) and 3-fold (372% ± 62%, $P < 0.005$) greater than that of WT-rVWF, respectively.

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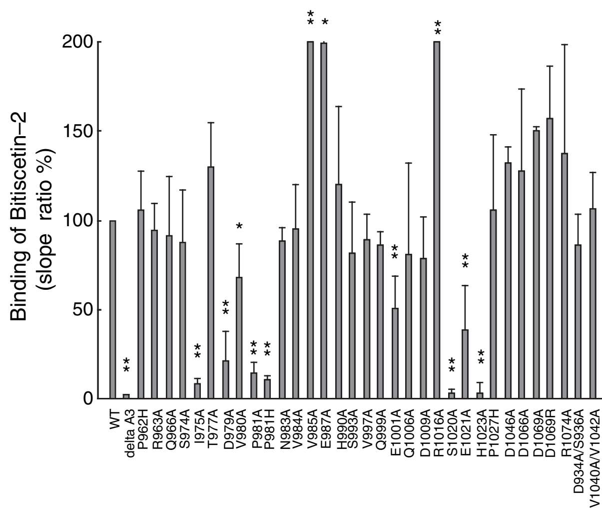

Fig. 2. Binding of ^{125}I-bitiscetin-2 to wild-type (WT)-rVWF, Delta A3-rVWF and rVWF with the indicated mutations in the A3-domain. Serially diluted rVWF were captured with MoAb 433, a constant amount of ^{125}I-bitiscetin-2 ($\sim 3 \times 10^{-9}$ M) was added and bound radioactivity was estimated. In parallel experiments, immobilized rVWF was estimated by IRMA using ^{125}I-MoAb 487. Results were expressed as bound ^{125}I-bitiscetin-2 as a function of bound ^{125}I-MoAb 487. The ratio of the slope of the dose-response curve for each mutant and WT-rVWF was calculated. Values are expressed as percentage. Each data point represents the mean $\pm$ SD of four independent experiments. $^{**}P < 0.005$, $^{*}0.005 \leqslant P < 0.05$ (unpaired Student's *t*-test).

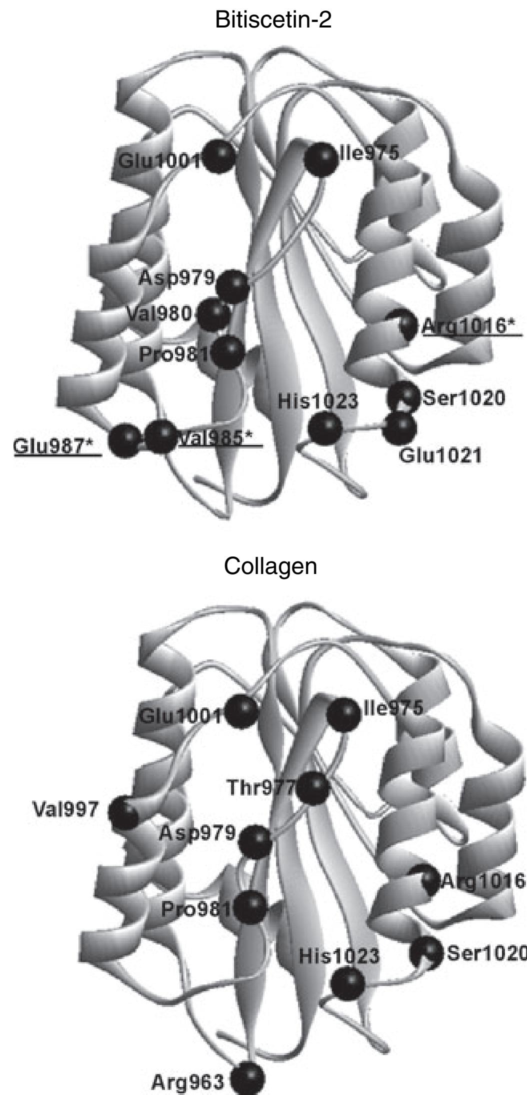

Fig. 3. Comparison of the amino acid residues of VWF A3-domain involved in the binding to bitiscetin-2 and to type III collagen as previously reported [14,15]. Mutation of underlined residues resulted in an increased binding of mutated VWF to bitiscetin-2. Other mutations led to a decreased binding of VWF to one of its ligands.

The other 21 mutations introduced within the A3-domain had no significant effect on the binding of VWF for the ligand.

As shown in Fig. 3, mutations that modified the binding of bitiscetin-2 are located at the front face of the A3-domain. This defines a flat central binding site. Mutation of residues in the antiparallel β_3 strand, loop $\alpha_2\alpha_3$ and loop $\alpha_3\beta_4$ decreased the binding. Mutations in loop $\beta_3\alpha_2$ and helix α_3 resulted in a significantly increased binding. Other tested mutations are located at the top and bottom faces of the A3 domain or within the surrounding α helices (Fig. 1). They did not have an effect.

Figure 3 also compares the binding site for bitiscetin-2 and for collagen as previously determined [14,15]. Seven residues (Ile975, Asp979, Pro981, Glu1001, Arg1016, Ser1020, and His1023) are involved in both interactions. Alanine mutations of Ile975, Asp979, Pro981, Ser1020, and His1023 resulted in significant loss of binding of the A3 domain to both bitiscetin-2 and collagen. Alanine mutations of Glu1001 resulted in a moderate decrease of binding in both cases, whereas mutation of Arg1016, which resulted in a decreased binding to collagen, induced a significant increase of the binding of the A3-domain to bitiscetin-2.

Discussion

We previously isolated from *B. arietans* venom a protein called bitiscetin-2 (formerly called bitiscetin) with characteristics and biological function close to but distinct from those reported for bitiscetin [4,5,18,19,23]. Both proteins induce VWF-dependent platelet agglutination by promoting the interaction of the A1-domain with platelet GPIb. However, bitiscetin binds to the VWF A1-domain [4,18,19] and has no effect on the binding of type III collagen to the A3-domain. In contrast, bitiscetin-2 binds to VWF A3-domain and interferes with the binding of VWF to collagen.

In the present paper, using Delta A3-rVWF as ligand, we confirmed our previous conclusion that bitiscetin-2 does not bind to the A1-domain but interacts primarily with the A3-domain. Partial aa sequencing of the bitiscetin-2 conclusively established that the two proteins are distinct species from the same venom. We obtained a single N-terminal sequence. There was notable similarity between the partial N-terminal sequence of the beta-subunit of bitiscetin [23] and that of bitiscetin-2, even though aromatic residues W and Y at position 8 and 11 of

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the beta-subunit of bitiscetin [23] were replaced by D and R, respectively. Thus, the similarity of their sequence and function suggest that the two proteins are homologous. Exactly how different forms of bitiscetin can be isolated from venom using similar methods [4] remains an unanswered question. Interestingly, a similar observation was previously reported for two-chain and one-chain botrocetin, two distinct platelet coagglutinins isolated from venom of *Bothrops jararaca* [24]. In this case, it was suggested that the composition of the venom collected for the starting preparation could vary from one lot to the other according to the life cycle of the snake or, as mentioned by the supplier (Sigma-Aldrich, St Quentin-Fallavier, France), the snakes might even have been misidentified. Similar factors may account for the isolation of two different forms of bitiscetin.

The A3-domain of VWF contains the binding site for bitiscetin-2 and the major binding site to type III collagen [8–11]. Binding and mutagenesis studies [12,14–16] combined with information from crystal structures have established that the collagen-binding site is located at the relatively flat and partly hydrophobic front face of the domain (Fig. 3). To explore the binding site of bitiscetin-2 we employed a set of 33 full-length rVWFs originally expressed to map the collagen-binding site [12,14,15]. We found that seven mutants (D934A/S936A, V1040A/V1042A, D1046A, D1066A, D1069A, D1069R, and R1074A) with substitutions in the top face, did not modify the affinity of rVWF for bitiscetin-2. Mutations that did affect binding are located at the front face of the domain and many of these mutations also affect collagen binding (Fig. 3). Residues Ile975, Asp979, and Pro981 located in strand β_3 play a key role in the interaction with bitiscetin-2 and are essential for the interaction with collagen [15,16]. However, not all residues of strand β_3 are involved in both interactions. Mutant V980A shows reduced binding to bitiscetin-2 but normal binding to collagen while the reverse is true for mutant T977A. Residues located in loop $\alpha_3\beta_4$ and the lower half of helix α_3 are also crucial for both interactions [13–16]. Mutations S1020A and H1023A (loop $\alpha_3\beta_4$) almost totally abolished the binding to both bitiscetin-2 and collagen. Again specific differences exist. Mutation E1021A (loop $\alpha_3\beta_4$), which had no effect on the interaction with collagen, had a minor effect on binding to bitiscetin-2 while mutation R1016A (helix α_3) decreased binding to collagen [14,16], but strongly increased binding to bitiscetin-2. Differential effects are also observed for mutations of residues in loop $\beta_2\alpha_2$ located in the lower half of the front face. Mutations in this loop (N983A, V984A, V985A, and E987A) had no effect on collagen binding [15,16] whereas two mutations (V985A and E987A) strongly increased binding affinity of bitiscetin-2 reaching 6- and 2-fold that of WT-rVWF. This finding may be interpreted as an alleviation of a steric hindrance, between Val985 and Glu987 and bitiscetin-2. Finally, mutation of residues located at the periphery of the collagen-binding site (R963, V997, and E1001) [13–16], which had a limited effect on collagen binding, also had only a minor or no effect on the binding of bitiscetin-2. In summary, despite the existence of some specific differences, a large set of residues of the VWF-A3 domain interact with collagen as well as bitiscetin-2, precluding simultaneous binding of both ligands to VWF.

Binding of VWF to platelet GPIb *in vivo* requires both VWF immobilization onto subendothelial collagen and high shear stress conditions. No effect of the VWF-collagen interaction on the binding to GPIb has been observed under static conditions. Results from our previous study [5] using a series of selected MoAbs directed against VWF and synthetic peptides established that binding of bitiscetin-2 to the A3-domain induced a conformational change of the A1-domain, exposing its binding site for platelet GPIb [5]. This observation combined with the extensive overlap between the binding sites for bitiscetin-2 and collagen type III observed in the present study strongly suggest that binding of bitiscetin-2 mimics the effects of both collagen binding and high shear stress conditions. Therefore, the mechanism whereby bitiscetin-2 interferes with VWF may serve as a model for understanding the *in vivo* behavior. Now, to gain insight into this mechanism, it appears that crystal structure analysis of a VWF-bitiscetin-2 complex should be undertaken.

References

1 Sadler JE. Biochemistry and genetics of von Willebrand factor. *Ann Rev Biochem* 1998; **67**: 395–424.
2 Siedlecki CA, Eppell SJ, Marchant RE. Interactions of human von Willebrand factor with a hydrophobic self-assembled monolayer studied by atomic force microscopy. *J Bio Mater Res* 1994; **28**: 971–80.
3 Brinkhous KM, Read MS, Fricke WA, Wagner RH. Botrocetin (venom coagglutinin): reaction with a broad spectrum of multimeric forms of factor VIII macromolecular complex. *Proc Natl Acad Sci USA* 1983; **80**: 1463–6.
4 Hamako J, Matsui T, Suzuki M, Ito M, Makita K, Fujimura Y, Ozeki Y, Titani K. Purification and characterization of bitiscetin, a novel von Willebrand factor modulator protein from *Bitis arietans* snake venom. *Biochem Biophys Res Commun* 1996; **226**: 273–9.
5 Obert B, Houllier A, Meyer D, Girma JP. Conformational changes in the A3 domain of von Willebrand factor modulate the interaction of the A1 domain with platelet glycoprotein Ib. *Blood* 1999; **93**: 1959–68.
6 Chopek MW, Girma JP, Fujikawa K, Davie EW, Titani K. Willebrand Factor: a multivalent protein composed of identical subunits. *Biochemistry* 1986; **25**: 3146–55.
7 Titani K, Kumar S, Ericsson LH, Wade RD, Ashida K, Walsh KA, Chopek MW, Sadler JE, Fujikawa K. Amino acid sequence of human von Willebrand Factor. *Biochemistry* 1986; **25**: 3171–84.
8 Kalafatis M, Takahashi Y, Girma JP, Meyer D. Localization of a collagen-interactive domain of human von Willebrand Factor between amino acid residues Gly 911 and Glu 1365. *Blood* 1987; **70**: 1577–83.
9 Pareti FI, Niiya K, McPherson JM, Ruggeri ZM. Isolation and characterization of two domains of human von Willebrand Factor that interact with fibrillar collagen types I and III. *J Biol Chem* 1987; **262**: 13835–41.
10 Lankhof H, van Hoeij M, Schiphorst ME, Bracke M, Wu YP, Ijsseldijk MJ, Vink T, de Groot PG, Sixma JJ. A3 domain is essential for interaction of von Willebrand factor with collagen type III. *Thromb Haemost* 1996; **7**: 950–8.
11 Cruz MA, Yuan HB, Lee JR, Wise RJ, Handin RI. Interaction of the von Willebrand factor (VWF) with collagen – localization of the primary collagen-binding site by analysis of recombinant vWF A domain polypeptides. *J Biol Chem* 1995; **270**: 10822–7.

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12 van der Plas RM, Gomes L, Marquart JA, Vink T, Meijers JCM, de Groot PG, Sixma JJ, Huizinga EG. Binding of von Willebrand factor to collagen type III: role of specific amino acids in the collagen binding domain of VWF and effects of neighboring domains. *Thromb Haemost* 2000; **84**: 1005–11.
13 Vanhoorelbeke K, Depraetere H, Romijn RA, Huizinga EG, De Maeyer M, Deckmyn H. A consensus tetra-peptide selected by phage display adopts the conformation of a dominant discontinuous epitope of a monoclonal anti-VWF antibody that inhibits the VWF-collagen interaction. *J Biol Chem* 2003; **278**: 37815–21.
14 Romijn RAP, Bouma B, Wuyster W, Gros P, Kroon J, Sixma JJ, Huizinga EG. Identification of the collagen-binding site of the von Willebrand factor A3-domain. *J Biol Chem* 2001; **276**: 9985–91.
15 Romijn RA, Westein E, Bouma B, Schiphorst ME, Sixma JJ, Lenting PJ, Huizinga EG. Mapping the collagen-binding site in the von Willebrand factor-A3 domain. *J Biol Chem* 2003; **278**: 15035–9.
16 Nishida N, Sumikawa H, Sakakura M, Shimba N, Takahashi H, Terasawa H, Suzuki EI, Shimada I. Collagen-binding mode of vWF-A3 domain determined by a transferred cross-saturation experiment. *Nat Struct Biol* 2003; **10**: 53–8.
17 Huizinga EG, van der Plas RM, Kroon J, Sixma JJ, Gros P. Crystal structure of the A3 domain of human von Willebrand factor: implications for collagen binding. *Structure* 1997; **5**: 1147–56.
18 Matsui T, Hamako J, Matsushita T, Nakayama T, Fujimura Y, Titani K. Binding site on human von Willebrand factor of bitiscetin, a snake venom-derived platelet aggregation inducer. *Biochemistry* 2002; **41**: 7939–46.
19 Maita N, Nishio K, Nishimoto E, Matsui T, Shikamoto Y, Morita T, Sadler JE, Mizuno H. Crystal structure of von Willebrand factor A1 domain complexed with snake venom, bitiscetin. Insight into glycoprotein Ibalpha binding mechanism induced by snake venom proteins. *J Biol Chem* 2003; **278**: 37777–81.
20 Laemmli UK. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 1970; **227**: 680–5.
21 Fraker PJ, Speck JC. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1, 3, 4, 6-tetrachloro-3a, 6a-diphenylglycoluril. *Biochem Biophys Res Commun* 1978; **80**: 849–57.
22 Kalmakoff J, Parkinson AJ, Crawford AM, Williams BR. Solid phase radioimmunoassays using labelled antibodies: a conceptual framework for designing assays. *J Immunol Methods* 1977; **14**: 73–84.
23 Matsui T, Hamako J, Suzuki M, Hayashi N, Ito M, Makita K, Fujimura Y, Ozeki Y, Titani K. Complete amino acid sequence of bitiscetin, a novel von Willebrand modulator protein purified from snake venom of *Bitis arietans*. *Res Com Biochem Cell Mol Biol* 1997; **1**: 271–84.
24 Fujimura Y, Titani K, Usami Y, Suzuki M, Oyama R, Matsui T, Fukui H, Sigimoto M, Ruggeri ZM. Isolation and characterization of two structurally and functionally distinct forms of botrocetin, the platelet coagglutinin isolated from the venom of *Bothrops jararaca*. *Biochemistry* 1991; **30**: 1957–64.

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