Conformational Changes in the A3 Domain of von Willebrand Factor Modulate the Interaction of the A1 Domain With Platelet Glycoprotein Ib

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Bitiscetin has recently been shown to induce von Willebrand factor (vWF)-dependent aggregation of fixed platelets (Hamako J, et al, Biochem Biophys Res Commun 226:273, 1996). We have purified bitiscetin from Bitis arietans venom and investigated the mechanism whereby it promotes a form of vWF that is reactive with platelets. In the presence of bitiscetin, vWF binds to platelets in a dose-dependent and saturable manner. The binding of vWF to platelets involves glycoprotein (GP) Ib because it was totally blocked by monoclonal antibody (MoAb) 6D1 directed towards the vWF-binding site of GPIb. The binding also involves the GPIb-binding site of vWF located on the A1 domain because it was inhibited by MoAb to vWF whose epitopes are within this domain and that block binding of vWF to platelets induced by ristocetin or botrocetin. However, in contrast to ristocetin or botrocetin, the binding site of bitiscetin does not reside within the A1 domain but within the A3 domain of vWF. Thus, among a series of vWF fragments, bitiscetin only binds to those that overlap the A3 domain, ie, SpII (amino acid [aa] 1-1365), SpI (aa 911-1365), and vWF-A3 domain (aa 920-1111). It does not bind to SpII corresponding to the C-terminal part of vWF subunit (aa 1366-2050) nor to the 39/49/kD disperse species (aa 480-718) or T116 (aa 449-728) overlapping the A1 domain. In addition, bitiscetin that does not bind to DeltaA3-rvWF (deleted between aa 910-1113) has no binding site outside the A3 domain. The localization of the binding site of bitiscetin within the A3 domain was further supported by showing that MoAb to vWF, which are specific for this domain and block the interaction between vWF and collagen, are potent inhibitors of the binding of bitiscetin to vWF and consequently of the bitiscetin-induced binding of vWF to platelets. Thus, our data support the hypothesis that an interaction between the A1 and A3 domains exists that may play a role in the function of vWF by regulating the ability of the A1 domain to bind to platelet GPIb.

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Materials and Methods

Fixed human platelets. Fixed platelets were prepared from fresh human blood as previously described. Blood was collected from healthy volunteers into one-tenth volume of 3.8% sodium citrate. Platelet-rich plasma was prepared by centrifugation of blood for 10 minutes at 100g at 22°C. Platelets were fixed with paraformaldehyde as described.

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**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot.** SDS-PAGE was performed according to the method of Laemmli using vertical slab gels. Molecular weight (MW) markers were low and high MW standards from Pharmacia (Uppsala, Sweden). After migration the proteins were stained with Coomassie blue or electrotransferred onto nitrocellulose paper.

Electrotransfer of proteins from SDS-PAGE was performed using nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) according to the method of Towbin et al. After transfer, the membranes were saturated with 2% skim milk (Merck, Damstadt, Germany) in 25 mmol/L Tris-HCL buffer, 0.15mol/L NaCl, pH 7.4 (TBS), before incubation with the selected ligands in TBS containing 1% skim milk, for 18 hours at 22°C. After extensive washing, the bound radioactivity was displayed by autoradiography.

**Purification of bitiscetin.** Bitiscetin was purified from Bothris arianesi venom (Sigma, St Louis, MO; batch 13H0760) essentially as described by Hamako et al using a series of chromatographies onto Phenyl-Superose columns, Superose columns, and monoFPLC columns (Pharmacia). Bitiscetin activity was followed by testing the capacity of the eluted fractions to induce binding of purified 125I-vWF to fixed platelets. Bitiscetin was identified on SDS (3.5% to 20% polyacrylamide gradient) gels by comparing Coomassie blue staining of the gels and autoradiography performed after electropherogram on nitrocellulose sheets, incubation with purified vWF (10 µg/mL), and staining with immunopurified 125I-polyclonal antibody raised in a goat and directed against vWF.

**Purification of botrocetin.** Two-chain botrocetin was purified from Bothrops jararaca venom (Sigma; batch 40H0911) as previously reported.

**Radiolabeling of proteins.** Purified vWF, bitiscetin, antibodies were labeled using Na125I (Amersham, Les Ulis, France) and Iodo-Gen (Pierce Chemical Co, Rockford, IL) according to the method of Fraker and Speck. Specific radioactivities varied from 8 to 15 μCi/μg for bitiscetin, 1 to 2 μCi/μg for vWF, and were 4.5 μCi/μg for IgG.

**Recombinant vWF (rvWF).** The bits was tested in this study. Expression vectors for wild-type rvWF (WT–rvWF) and mutant rvWF[Lys 875 were constructed and characterized as previously described. Both rvWF were expressed in COS-7 cell cultures after electroporation of the appropriate plasmid DNA (10 μg) mixed with training DNA (50 μg), using an electric shock (280 V, 1,500 μF, 7 Ohm). Cells were cultured for 24 hours in MCDB-105 medium (Sigma) containing 10% fetal calf serum and 1% antibiotics, and 48 hours in serum-free medium. rvWF was produced in conditioned medium at the level of 0.1 U/mL vWFAg. It was concentrated to 1 U/mL vWFAg using Aquacit II (MW 500 000, Calbiochem) and dialyzed against 5 mmol/L Tris-HCl buffer, pH 8. WT-rvWF and rvWF[Lys875 exhibited a normal multimeric structure and normal binding to platelets in the presence of ristocetin or botrocetin and to purified botrocetin.

Delta A3–vWF and the A3 domain of A3 were gifted from Professor J.J. Sixma and Dr E.G. Huizinga (Department of Haematology, University Hospital Utrecht, Utrecht, The Netherlands). Delta A3–vWF was expressed in a BHK cell line overexpressing furin. Its characterization was performed by Lankhof et al. Delta A3–vWF was deleted of the A3 domain (aa 910-1113). This recombinant vWF also exhibited a normal distribution of multimers, a normal binding to platelets when induced by ristocetin or botrocetin but a strikingly decreased capacity to bind to collagen as compared with WT–vWF. The A3 domain of vWF (aa 920-1111) was expressed in Escherichia coli. Its purification and characterization were performed by Huizinga et al.

**Purification of vWF and its proteolytic fragments.** Human vWF was purified from outdated high-purity vWF concentrates kindly donated by LFB (Les Ulis, France) essentially as previously described.

The three fragments SpI (monomer, aa 911-1365), SpII (dimer, aa 1366-2050), and SpIII (dimer, aa 1-1365) were produced by digestion of purified vWF with Staphylococcus aureus V8 protease (Miles, Paris, France) and purified as previously described. The monomeric 39/34 kD species (aa 480-718) overlapping the A1 domain of vWF was prepared by digestion of purified vWF with dispase (Boehringer, Meylan, France) and purified according to the method of Andrews et al. The T116 fragment (dimer, aa 449-728) was produced by digestion of purified vWF by trypsin (Sigma) and isolated using affinity chromatography on heparin coupled to Sepharose.

MoAb. A series of 10 murine MoAb to vWF were used as IgG fractions in this study. MoAb 701, 710, and 724 were raised by immunization with the 39/34 kD fragment dispase. MoAb 701 blocks the interaction between GPIb and vWF induced by either ristocetin or botrocetin. MoAb 710, whose epitope is localized between Ser 593 and Ser 678, blocks the vWF-GPIb interaction when induced by ristocetin but not by botrocetin. MoAb 724 is directed against a conformational epitope of the A1 loop. It inhibits the binding of sulfatides, botrocetin, and heparin to vWF as well as botrocetin- but not ristocetin-induced binding of vWF to platelets. MoAb 724 does not inhibit vWF binding to collagen but exhibits a strikingly decreased affinity for the vWF-collagen complex in comparison with free vWF. In addition, MoAb 724 promotes shear-dependent platelet aggregation.

MoAb 322 blocks the binding of vWF to platelets in the presence of ristocetin but not of botrocetin. It recognizes an epitope of one of the flanking regions of the A1 loop of vWF.

MoAbs 201, 400, 505, and 535 are directed against fragment SpI (aa 911-1365) overlapping the A3 domain of vWF. Each of these antibodies blocks the binding of vWF to collagen. MoAbs 454 and 487 are directed against fragment SpII (aa 1366-2050). They have no known effect on the functions of vWF.

MoAb 6D1, a gift from Dr B.S. Collier (State University of New York, Stony Brook, NY) directed against GPIb and that blocks ristocetin- and botrocetin-induced binding of vWF to platelets, was used as a control of specificity of the interactions.

**Synthetic peptides.** Five peptides derived from the sequence of human vWF were synthetized by the method of Merrifield et al and purified by high-performance liquid chromatography using a reverse-phase system. Peptides Cys 474 to Pro 488 (CQEPGGLVYPPTDAP) and Lys 569 to Gin 583 (KDRKRPSELRRIASQ) were from Neosystem Laboratoire, Strasbourg, France. Peptides Lys 494 to Arg 511 (LYVE-DISEPPLHDFYCSR), Gin 628 to Pro 655 (QRMSNFVRYVQGLKKKVKIVPGVIP) and Ser 692 to Pro 708 (SYLCILAPEAPPTLP) were a gift from Dr D. Diaz (Sanofi Recherche, Montpellier, France).

Peptides had free N- and C-terminus. The thiol group of Cys 474, 509, and 695 was S-carboxamidomethylated.

**Binding of 125I-vWF to fixed platelets in the presence of bitiscetin and its inhibition by MoAb.** Binding isotherm of 125I-vWF to fixed platelets was performed essentially as previously described using various concentrations of 125I-vWF (0 to 25 µg/mL), 10^6 cells/mL, and 2 µg/mL of purified bitiscetin as inducer. After 30 minutes at 22°C, aliquots of the mixture reaction (100 µL) were layered at the top of 400 µL conical tubes containing 250 µL of 25% sucrose. Bound and free radioactivity was separated by centrifugation at 12,000g for 3 minutes and cutting the tips of the tubes containing the platelet pellets and was counted. Nonspecific binding was estimated under identical conditions but in the absence of bitiscetin. Binding isotherm and binding parameters (Kd and number of binding sites) were derived from the best fitted Langmuir hyperboler calculated from the experimental data by nonlinear least-squares, regression analysis using the computer-assisted program Kaleidagraph (Synergy Software, Reading, PA).

**Inhibition of bitiscetin-induced binding of 125I-vWF to fixed platelets** by MoAb was performed as described using 2 µg/mL of purified bitiscetin as inducer, 10^6 cells/mL, and 125I-vWF (1 µg/mL final concentration) prexiced with various concentrations of competitor. Nonspecific binding was estimated under the same conditions but in the
absence of inducer. After 30 minutes at 22°C the bound and free radioactive material were separated as described above and counted. Results were expressed as the percent of the specific binding estimated in the absence of competitor.

Localization of the bitiscetin-binding domain on vWF subunit. In a first set of experiments the localization of the vWF binding domain of bitiscetin was performed by SDS-PAGE (3.5% to 20% polyacrylamide gradient gel) and electroblotting of proteolytic or recombinant vWF fragments, followed by binding of 125I-bitiscetin (× 10^6 cpm/mL) to vWF containing 1% skin milk and NaCl to the nitrocellulose membrane. Purified fragments were loaded on the gel at 5 µg/well. In parallel analysis, purified fragments were loaded at 10 µg/well and shown by Coomassie blue staining.

In another set of experiments, purified vWF or its proteolytic or recombinant fragments (10 µg/mL) in TBS were immobilized by coating onto wells of polyvinyl chloride microtiter plates (Dynatech, Marne-la-Coquette, France) by incubating 200 µL/well overnight at 4°C. Negative control was performed by incubating buffer alone. After postcoating for 30 minutes at 22°C with TBS containing 2% bovine serum albumin (BSA fraction V, Calbiochem, La Jolla, CA), 100 µL/well of various concentrations of purified 125I-bitiscetin (specific radioactivity × 20 × 10^6 cpm/µg) in TBS containing 1% BSA were incubated for 5 hours at 37°C. After washing with buffer, bound radioactive material was counted.

In some experiments, to compare the material bound to immobilized vWF and the starting material, 125I-bitiscetin bound to coated vWF was extracted after washing by incubating wells with 100 µL of 1% SDS in 0.125 mol/L Tris-HCl buffer, pH 6.8. The radioactive material was compared with the starting labeled material by SDS-15% PAGE followed by autoradiography.

The localization of the binding site of bitiscetin on the vWF subunit was also analyzed by comparing the binding of 125I-bitiscetin to recombinant wild type and to recombinant Delta A3-vWF immobilized onto a MoAb to vWF. For this purpose, coating of MoAb 454 (10 µg/mL in 0.05 mol/L sodium carbonate/bicarbonate buffer, pH 9.6) was performed by incubating buffer (100 µL/well) in microtiter plates by coating as described above. After postcoating for 5 hours at 37°C after washing, the bound radioactivity was counted in the absence of competitor.

Characterization of purified bitiscetin and vWF-bound 125I-bitiscetin by SDS-PAGE. Figure 1 compares labeled purified bitiscetin and bound material extracted from immobilized vWF analyzed by SDS-15% PAGE. When unreduced, purified bitiscetin migrates as a major single band at 29 kD. A faint band was also observed at 18 kD. Analysis of the material bound to immobilized vWF showed that only the major 29 kD band reacted with vWF. Under reducing conditions, labeled purified bitiscetin appears as a major single band at 15 kD that had the same mobility as the single band observed in the bound material after reduction. Thus, in our hands bitiscetin appeared as a dimeric protein composed of chains of identical molecular weight.

Binding isotherm of 125I-vWF to platelets in the presence of bitiscetin. The concentrations of purified bitiscetin that induced maximum binding of vWF to fixed platelets were determined at room temperature using 10⁶ cells/mL, 1 µg/mL of 125I-vWF, and increasing concentrations of bitiscetin (0 to 30 µg/mL). Maximal binding (B/T ≠ 30%) was reached at 2 µg/mL (not shown). This concentration was chosen for the binding assays of 125I-vWF to fixed platelets.

Figure 2A shows binding data for one representative experiment with various concentrations of 125I-vWF (1.4 × 10⁶ cpm/µg). Results are interpretable as saturable binding to platelets in the presence of bitiscetin. Total binding increased from 0.055 to 0.33 µg/10⁶ platelets when vWF concentration increased from 0.01 to 25 µg/mL. Nonspecific binding linearly increased to 0.03 µg/10⁶ platelets at the highest concentration of vWF. Parameters of binding were derived from the best-fitted Langmuir hyperbolic for the specific binding (correlation coefficient, r = 0.98). The maximal binding was 0.30 µg/10⁶ platelets and the apparent Kd was 0.45 µg/mL (ie, Kd = 1.6 × 10⁻⁹ mol/L assuming a molecular mass of 275 kD for the vWF subunit).

Bitiscetin-induced binding of vWF to platelets involves the interaction of GP Ib with the A1 domain. Figure 2B shows the effect of MoAb 701, 710, 724, and 322 directed against the A1 domain of vWF and of MoAb 6D1 against the vWF-binding domain on GP Ib, upon the bitiscetin-induced binding of 125I-vWF to platelets. Binding of 125I-vWF to platelets was inhibited in a similar way by MoAbs 701, 710, 724, and 6D1. It was totally inhibited at a concentration of 1 µg/mL and 50% displacement required ≥ 0.05 µg/mL. MoAb 322 appeared as a less-potent inhibitor with a 50% displacement reached at ≥ 1 µg/mL and a total inhibition at ≥ 10 µg/mL. As a control, MoAb 454 directed against the C-terminal part of vWF had no effect on the binding.
Localization of the bitiscetin-binding site on the A3 domain of vWF.

The staining of purified fragments of vWF by 125I-bitiscetin after SDS-PAGE and electroblotting is shown on Fig 3. 125I-bitiscetin recognized SpIII and SpI. In contrast, no binding site of bitiscetin was detected on fragments overlapping only the A1 domain, like the 39/34 kD dispase and the T116 species or the C-terminal portion of the vWF subunit (SpII).

These results were confirmed using purified vWF, proteolytic fragments (SpIII, SpII, SpI, 39/34 kD, and T116 fragments) or the recombinant A3 domain immobilized on microtiter plates and incubated with increasing concentrations of 125I-bitiscetin. A specific binding that linearly increased as a function of bitiscetin concentration was observed using immobilized vWF and SpIII fragments (not shown), SpI fragment, or the A3 domain (Fig 4). Binding observed in the presence of the other immobilized fragments was not distinguishable from the nonspecific binding estimated using buffer. However, comparison of the ratio of the bound to total 125I-bitiscetin shows that the relative affinity of the ligand for the vWF (B/T ≠ 10%) is significantly higher than for SpIII (B/T ≠ 4%) whereas it is similarly low for SpI or the recombinant A3 domain (≠ 0.5%). In contrast, as shown on Fig 3, SpI was still detectable after SDS denaturation and western blotting whereas the A3 domain was no longer detectable under similar conditions (not shown). Thus, our data suggest an effect of the conformation of the binding site for its recognition by bitiscetin.

To confirm that bitiscetin recognizes a binding site within the A3 domain and to establish that it had no other binding site outside this domain, we tested the reactivity of 125I-bitiscetin with various concentrations of WT-vWF or Delta A3-vWF immobilized onto MoAb 454. vWF Lys875 was used as control. In a parallel experiment the amount of immobilized vWF was estimated using 125I-MoAb 487. As shown in Fig 5, even though the bound 125I-MoAb 487 (≠ 1,800 to 19,000 cpm/well) indicated that a similar amount of the three vWFs was immobilized, the binding of 125I-bitiscetin to Delta A3-vWF was similar to the nonspecific binding (conditioned medium). In contrast, the dose-response curves for binding of bitiscetin linearly increased with the amount of immobilized WT-vWF and vWF Lys875, reaching ≥ 10^4 cpm/well and 8,000 cpm/well at the highest concentration, respectively.

Inhibition of 125I-bitiscetin binding to immobilized vWF by MoAb, botrocetin, bitiscetin, and peptides.

The localization of the bitiscetin binding site on the A3 domain of vWF was further confirmed by experiments of inhibition of 125I-bitiscetin binding to immobilized vWF by MoAb.

Four MoAbs (201, 400, 505, and 535) directed against the A3 domain (SpI fragment) and that blocked the vWF binding to collagen were tested. Figure 6A shows that three of them (MoAb 201, 400, and 535) totally blocked binding of 125I-bitiscetin to vWF at the concentration of ≥ 10 μg/mL. 50% inhibition was reached at the concentrations of 1.8 μg/mL for MoAb 535 and 4.4 μg/mL for MoAb 201 and 400, respectively. In contrast, the occupancy of epitope 505 appeared to significantly increase (40%) the binding of 125I-bitiscetin to vWF. MoAb 454 used as control had no effect on the binding.

As a control, the effect of MoAb 201, 400, 505, and 535 (0.01 to 10 μg/mL) was also tested on the bitiscetin-induced binding of 125I-vWF to platelets (not shown). As expected this binding was totally abolished by MoAb 201, 400, and 535 but at concentrations much lower than those required to abolish 125I-bitiscetin binding to immobilized vWF (ie, total inhibition
At $\neq 1$ µg/mL and 50% inhibition at $\neq 0.1$ µg/mL. It thus appears that the affinity of these MoAb is conformation dependent and higher for vWF in solution than for vWF immobilized on plastic. MoAb 505 did not significantly decrease the binding of $^{125}$I-vWF to platelets.

In contrast with the effect observed using MoAb directed to the A3 domain of vWF, MoAb 701 and 710, directed to the A1 domain and blocking bitiscetin-induced binding of $^{125}$I-vWF to platelets, had no effect on the interaction of $^{125}$I-bitiscetin with vWF (Fig 6B). MoAb 322 increased by 50% the binding of $^{125}$I-bitiscetin. In contrast, MoAb 724 partially blocked this binding. Thus, it appears that an interaction occurred between the A1 and A3 domains of vWF, which can be influenced by modifying the conformation of either domain.

To further investigate the role of selected sequences of the A1 domain on the binding of bitiscetin to the A3 domain we tested the effect of peptides in experiments of inhibition. Among the six peptides tested, Fig 7A shows that only two of them significantly inhibited $^{125}$I-bitiscetin binding to vWF: peptide Gln 628 to Pro 655, which belongs to the predominantly basic region of the A1 loop, and peptide Cys 474 to Pro 488, previously involved in the binding of vWF to GPIb. Other peptides (Fig 6A), Lys 569 to Gln 583, Lys 494 to Arg 511, and Ser 692 to Pro 708, had almost no effect on the binding.

Similarly (Fig 7B), the binding of botrocetin to the A1 domain had only a low effect on the binding of bitiscetin in the range of concentrations tested (0.1 to 20 µg/mL). The residual binding remained higher than 70% of the binding estimated in the absence of competitor. In contrast, bitiscetin totally abolished the binding of $^{125}$I-bitiscetin at the concentration of 10 µg/mL.

**Inhibition of $^{125}$I-vWF binding to human type-III fibrillar collagen by bitiscetin or botrocetin.** Figure 8 shows that bitiscetin blocked the interaction of vWF with collagen in a dose-dependent fashion. Binding of $^{125}$I-vWF to collagen was totally inhibited by increasing the concentration of bitiscetin to 100 µg/mL and 50% inhibition was reached at the concentration of 42 µg/mL of bitiscetin. In contrast, increasing the concentration of botrocetin had no significant effect on the binding of $^{125}$I-vWF to collagen.

**DISCUSSION**

Bitiscetin purified from *Bitis arietans* venom was recently identified as a new inducer of vWF-dependent platelet agglutination.7 Like botrocetin it was shown to interact with vWF allowing binding of the latter to platelet GPIb. However, bitiscetin was found to be distinct from the negatively charged botrocetin in particular in regard to its highly basic nature suggesting that it acted on vWF through a different mechanism.

Previous studies have established that botrocetin binds to sequences of the A1 domain of vWF.17,18 This binding modifies the structural environment of the A1 domain that promotes its GPIb-binding capacity. In the present paper we have established that bitiscetin induces GPIb-binding of the A1 domain by binding to sequences of the A3 domain. Therefore, we show that a conformational interaction exists between the A1 domain and the bitiscetin-A3 complex and a series of arguments indicate that such an interaction may be modulated by acting on either of the counterparts, ie, on sequences of the A1 or of the A3 domain.

We first showed that bitiscetin-induced binding of vWF to platelets is specific, dose-dependent, and saturable. Inhibition experiments were performed using MoAb directed against the GPIb-binding site of the A1 domain (MoAb 701) or against the
vWF-binding site of GPIb (MoAb 6D1). Both antibodies were shown to totally inhibit ristocetin-, botrocetin-, and bitiscetin-induced binding of vWF. Our results thus indicate that the bitiscetin-induced binding involves the same contact sites between the A1 domain and GPIb as those already implicated in the presence of the two other inducers. In addition, our data showing that MoAb 701 had no effect on the binding of bitiscetin to vWF ruled out the possibility that the inhibition of

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**Fig 3.** SDS-(3.5% to 20% gradient) PAGE of purified fragments produced by proteolysis of vWF. (Left panel) Fragments were loaded at 10 μg/well. After migration the gel was stained using Coomassie blue. (Right panel) The fragments were loaded at 5 μg/well. After migration the proteins were electrophoresed on nitrocellulose sheet, stained by incubating with 125I-purified bitiscetin, and showed by autoradiography. Fragments were SpI (lanes 1 and 6); SpII (lanes 2 and 7); T116 (lanes 3 and 8); 39/34 kD (lanes 4 and 9); and SpIII (lanes 5 and 10).

**Fig 4.** Binding of 125I-bitiscetin to SpI (○), SpII (●) and to the recombinant A3 domain (■) coated on plastic.

**Fig 5.** Binding of 125I-bitiscetin to various concentrations of recombinant vWF immobilized onto MoAb 454. The amount of immobilized rWF was estimated in parallel experiments using 125I-MoAb 487. Recombinant vWF was WT-rvWF (●); rvWFlys 875 (○) and Delta A3-vWF (□).
this interaction was responsible for blocking the binding of vWF to GPIb.

On the other hand, several pieces of evidence showed that bitiscetin-induced binding of the A1 domain to GPIb is promoted by the binding of this inducer to the A3 domain. In one set of experiments we tested the reactivity of 125I-bitiscetin with a series of purified proteolytic or recombinant fragments of vWF that were immobilized by either blotting or coating onto wells of microtiter plates. We observed that the two A3 domain-containing fragments SpIII and SpI as well as the recombinant A3 domain, positively reacted with bitiscetin. In contrast, other fragments lacking the A3 domain, ie, SpII, T116, and 39/34 kD dispase fragment, were not recognized by bitiscetin. The absence of potential binding site for bitiscetin outside the A3 loop was further shown by showing that recombinant delta A3-vWF, deleted from the A3 domain and known to normally interfere with ristocetin and botrocetin,29 totally fails to bind to bitiscetin.

The presence of the binding site of bitiscetin within the A3 loop was also shown by inhibition of binding using MoAb directed against the A3 domain. Four MoAb (201, 400, 505, and 535) that all blocked the collagen binding to that region, were analyzed. Three of these MoAb (201, 400, and 535) totally inhibited binding of bitiscetin to vWF and consequently binding of vWF to platelets. Thus, together with results showing the inhibition of vWF binding to collagen by bitiscetin, our data suggest that the two ligands of vWF, collagen and bitiscetin, share at least in part a common binding site on the A3 loop. However, bitiscetin and collagen are not equivalent ligands of vWF because the mechanism by which bitiscetin induces binding of vWF to platelet GPIb is not observed using collagen under static conditions.32 Our results showing that MoAb 505 increases bitiscetin binding but blocks collagen binding to vWF also discriminate between the two ligands and suggest that epitope 505 is...
a conformational site of the A3 loop that is not directly involved in the ligand-vWF interactions.

More information on the conformational interaction between the A1 domain and the bitiscetin-A3 loop complex comes from experiments performed using selected MoAbs against the A1 region and synthetic peptides. MoAb 322 is directed against one of the flanking regions of the A1 loop and MoAb 710 reacts with an epitope located within the A1 loop involving Ser 593 and Ser 678. Both MoAbs were previously shown to block ristocetin- but not botrocetin-induced binding of vWF to GPIb. In the present study, both MoAbs also inhibit bitiscetin-induced binding of vWF to platelets suggesting that the mechanisms of the binding of vWF to GPIb in the presence of bitiscetin or ristocetin involved at least in part similar sites within the A1 domain. In addition, the significant increase of the binding of bitiscetin to vWF observed in the presence of MoAb 322 discriminates its epitope from that of MoAb 710 and strongly supports its involvement in the potential interaction between A1 and A3 domains. Experiments using MoAb 724 also suggested the involvement of the corresponding epitope in the A1-A3 loop interaction. Epitope of MoAb 724 was previously identified as a conformational site of the A1 loop playing a key role in the interactions of vWF with GPIb in the presence or absence of ristocetin. In addition, this antibody was shown to inhibit the binding of heparin, sulfatides, and botrocetin to vWF, suggesting that its epitope overlaps the common binding site (aa 569-584) for the three ligands. In the present study we observed that MoAb 724 totally inhibits bitiscetin-induced binding of vWF to platelets and partially blocks binding of bitiscetin to vWF in a way similar to that observed using botrocetin. However, we observed that addition of botrocetin has no significant effect on the binding of bitiscetin to vWF. Thus, because it is unlikely that the occupancy of the same site on the A1 domain by either botrocetin or MoAb 724 is not responsible for the same effect on the bitiscetin binding to the A3 loop, we assume that epitope 724 is distinct from the common binding site for heparin, botrocetin, and sulfatides. It is likely that MoAb 724 acts in all cases by disrupting the folding of the A1 and A3 regions through a conformational change of the A1 loop that blocks bitiscetin- and botrocetin-induced binding of vWF to GPIb as well as heparin, sulfatides, and botrocetin binding to the A1 domain; and a conformational change of the A3 domain that at least in part prevents the binding of bitiscetin to vWF.

Interaction of the A1 domain with the A3 loop was further shown by testing the effect of synthetic peptides corresponding to sequences of the A1 domain on the binding of bitiscetin to vWF. Sequences corresponding to vWF residues Cys 474-Pro 488, Leu 494-Arg 511, and Ser 692-Pro 708 were shown to play a role in modulating vWF binding to GPIb. Peptides 474-488 and 692-708 blocked ristocetin-induced binding of vWF to GPIb and the spontaneous binding of asialo-vWF but they had no effect on botrocetin-induced binding of vWF to platelets. Both peptides have been proposed to be ristocetin binding sites on vWF. In addition, experiments using deleted recombinant fragments of vWF and mutated recombinant vWF as well as studies of the natural mutations of vWF leading to the 2B subtype of vWD suggest that sequences 494-511 and 692-708 play a role on the conformation of the A1 loop by limiting exposure of the GPIb-binding site. In the present study only peptide 474-488 blocked the binding of bitiscetin to the A3 domain suggesting that the interaction of the sequence Cys 474-Pro 488 with the A3 loop also regulates the binding of vWF to GPIb. Binding of vWF to GPIb was proposed to be regulated by both the amino acid sequences of contact sites and by an electrostatic mechanism involving positively charged regions of the A1 domain and negatively charged domains of GPIb. Two positively charged sequences of the A1 domain, i.e., aa 569-583 and 628-655, appeared to play an important role in the regulation of the interaction. Both are involved in the binding sites of vWF to its negatively charged ligands like botrocetin, sulfatides, or heparin. In addition, the sequence 628-655 of vWF was shown to contain two segments (aa 629-632 and 642-645) that belong to the contact site with GPIb. Because it is likely that the modulation of the interaction of GPIb with the A1 domain involving the negatively charged A3 domain also requires an electrostatic mechanism, we tested the effect of the corresponding synthetic peptides on the binding of bitiscetin to vWF. Our results showed that peptide 628-655 totally blocks bitiscetin binding whereas peptide 569-583 has no effect. Thus, our data are in agreement with the hypothesis that the electrostatic charges are an important feature of the interaction between the A1 and A3 loops. However, the discrepancy that we observed in the effect produced by each peptide also suggests an important role of the amino acid sequence.

In conclusion, we report that the conformation of the A1 domain is modulated by binding of bitiscetin to the A3 loop. Several hypothesis can be raised to explain the mechanism: (1) the binding of bitiscetin to the A3 loop may change or disrupt an
interaction preexisting in native vWF between the positively charged A1 loop and the negatively charged A3 domain. This hypothesis is in agreement with the fact that the 39/34 kD monomeric dispase fragment overlapping the A1 domain and free of A3 domain spontaneously interacts with GPIb^{42}; (2) the complex formation between bitiscetin and the A3 domain could facilitate a new interaction with the A1 loop leading to the exposure of the GPIb-binding site of vWF. On the one hand such an interaction may result from the binding of sequences of the A3 domain to the A1 region. On the other hand, bitiscetin could change its own conformation after its binding to the A3 loop, exposing a binding site for the A1 domain that in turn could activate that domain in a way similar to other inducers. Our data showing that the binding of bitiscetin occurs on purified A3 loop-containing fragments but can be inhibited by specifically acting on the A1 domain, together with the distribution of the electrostatic charges among the various reagents, are in favor of the hypothesis involving the modulation of a direct interaction between sequences of the A1 and A3 domains.

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Conformational Changes in the A3 Domain of von Willebrand Factor Modulate the Interaction of the A1 Domain With Platelet Glycoprotein Ib

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