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# Interaction of von Willebrand Factor with the Extracellular Matrix and Glycocalicin under Static Conditions[1]

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**The binding of human von Willebrand factor (vWF) to a variety of extracellular matrix components immobilized on plates and the binding of vWF to platelet glycoprotein Ib (GPIb) after interacting with these matrix components were examined by means of an enzyme-linked immunosorbent assay. vWF preferably bound to type III collagen, whereas it did not significantly bind to type I, IV, V, or VI collagen, fibronectin, laminin, elastin, or proteoglycans. Soluble type III collagen did not bind to vWF coated on plates and showed a little effect on the vWF binding to the immobilized collagen, suggesting that solid-phase collagen is important for the interaction with vWF. When glycocalicin, the N-terminal carbohydrate-rich extracellular domain of GPIb$\alpha$ exhibiting the vWF-binding activity, was added to vWF bound to collagen type III, no significant binding of glycocalicin was observed, but it bound to vWF in the presence of botrocetin, a vWF modulator protein isolated from *Bothrops jararaca* snake venom. These results indicate that vWF immobilized on collagen can interact with GPIb but that the binding of vWF to the collagen matrix alone is insufficient for modulating vWF so that it interacts with GPIb under static conditions. Another unknown physiological modulator functionally mimicking botrocetin or high-shear stress may be involved in the platelet adhesion to extracellular matrix in the early stage of hemostasis.**

**Key words: botrocetin, collagen, ELISA, glycocalicin, von Willebrand factor.**

The prompt formation of a platelet plug at the site of vascular injury is an essential process in the early stage of hemostasis. This process comprises three major molecular interactions; firstly, anchoring of plasma von Willebrand factor (vWF) to the exposed subendothelial extracellular matrix (ECM), secondly, interaction between vWF and platelet membrane glycoprotein Ib (GPIb) leading to platelet adhesion to the injury site followed by platelet activation, and thirdly, platelet aggregation through the binding of $\alpha$IIb/$\beta$III integrin to vWF and/or fibrinogen (*1, 2*). Since vWF never interacts with GPIb exposed on the surface of platelets in plasma with normal vascular flow, the interaction between vWF and GPIb must occur through a rapid conformational change of vWF upon vWF binding to ECM.

vWF is known to bind to heparin (*3*), collagens (*4, 5*), and sulfatides (*6*). Recently, type VI collagen was postulated to be a vWF-binding component in ECM *in vivo* (*7-9*). It has also been postulated that once vWF has interacted with ECM component(s) it may attain the GPIb-binding activity through a conformational change leading to uncovering of the GPIb-binding site of vWF (*10, 11*). Binding studies involving proteolytic fragments of vWF (*12, 13*), recombinant vWF (*14*), or site-directed monoclonal antibodies (MoAbs) (*15*) indicated that the GPIb-binding site is located in the A1 loop domain (residues 479-717) of vWF, especially at residues 514-542 (*16*). Antibiotic ristocetin and snake venom botrocetin, *in vitro* modulators of vWF inducing platelet agglutination, interact with the negatively-charged flanking region of the A1 loop and the inner loop domain, respectively (*16-18*). Interestingly, tentative binding sites for collagen (*5*), heparin (*19*), sulfatides (*20*), and botrocetin (*18*) overlap or are close to each other within the A1 domain. Moreover, all the amino acid substitutions found in type 2B von Willebrand disease causing increased affinity to GPIb are localized in the same region (*1, 2*). These results imply that the interaction of vWF with ECM components may affect the A1 domain, leading to a conformational change of the GPIb binding site.

We have developed a simple assay system for investigating the molecular interactions among vWF, ECM, and GPIb

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Abbreviations: ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; GPIb, glycoprotein Ib; HRP, horseradish peroxidase; MoAb, monoclonal antibody; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; vWF, von Willebrand factor.

under static conditions. In the present study, we screened the binding activity of vWF as to immobilized ECM components, and examined whether or not ECM-bound vWF acquires the GPIb-binding activity using glycocalicin, the N-terminal carbohydrate-rich extracellular domain of GPIb$\alpha$ exhibiting the vWF-binding activity.

## MATERIALS AND METHODS

*Materials*—Citrated human normal plasma was prepared as described (*21*). Botrocetin was purified from *Bothrops jararaca* venom as described (*22*). vWF was purified from outdated factor VIII concentrates (Haemate P, donated by Hoechst Japan, Tokyo) as described (*23*). Pepsin-digested soluble human placental collagens (type I, III, IV, and V) were purchased from Sigma (St. Louis, MO), Seikagaku (Tokyo), or Gibco BRL (Gaithersburg, MD). The various products of human placental type VI collagen were from Telios Pharmaceuticals (San Diego, CA), Southern Biotechnology Associates (Birmingham, AL), and HEYL (Berlin, Germany). Human placental laminin and cellular fibronectin were from Takara Shuzo (Otsu) and Fibrogenex (Morton Grove, IL), respectively. Human plasma fibronectin and vitronectin were from Iwaki Glass (Tokyo). Human soluble aortic elastin was from Elastin Products (Owensville, MO). Human umbilical hyaluronic acid and shark cartilage chondroitin sulfate C were from Seikagaku, and bovine cornea keratan sulfate and kidney heparan sulfate from Sigma. All ECM proteins used were of above 90% pure grade and used without further purification. Anti-human vWF polyclonal antibodies conjugated with horseradish peroxidase (HRP), and site-specific MoAbs (*24*) were obtained from Dakopatts (Glostrup, Denmark), and a generous gift from Takara Shuzo, respectively. MoAb against human GPIb$\alpha$ (HPL-16), which does not inhibit ristocetin-induced platelet agglutination, was prepared as previously described (*25*). MoAb against botrocetin (BCT107-3), which does not inhibit the botrocetin binding to vWF, was a gift from Takara Shuzo. Other antibodies against human ECM, plasma proteins, and HRP-conjugated second antibodies were from Medical and Biological Laboratory (Nagoya), Fuji-Yakuhin (Toyama), or TAGO Immunologicals (Camarillo, CA).

*Preparation of Glycocalicin*—Glycocalicin was prepared as described (*26*). Briefly, washed platelets were sonicated in 10 volumes of 3 M KCl and then incubated at 37°C for 30 min. After centrifugation, the supernatant was dialyzed against Tris-buffered saline (TBS: 150 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 5 mM EDTA, and then applied to a column of wheat germ agglutinin conjugated agarose (Seikagaku) equilibrated with the same buffer. The fraction eluted with 100 mM acetate buffer, pH 5.0, containing 200 mM GlcNAc was further applied to a Mono Q-FPLC column (Pharmacia Biotech, Uppsala, Sweden) and eluted with a salt gradient. In general, 200 $\mu$g of glycocalicin was purified from 4 ml of platelet pellet.

*Enzyme-Linked Immunosorbent Assay (ELISA)*—The wells of an immuno-module (8 wells; Nunc Intermed, Kamstrup, Denmark) were coated with 50 $\mu$l aliquots of TBS containing 10 $\mu$g/ml of each protein at 4°C overnight (pH adjusted to 7.5) and then blocked with 200 $\mu$l of TBS containing 1% BSA (B-TBS) at 4°C overnight. The binding of each protein to the surfaces of wells was immunologically confirmed with the antibodies specific to each protein or monitored as the reduction in the protein concentration on coating. The wells were incubated with 50 $\mu$l of B-TBS containing 1-5 $\mu$g/ml of vWF or plasma diluted to 1:10 with B-TBS in the presence or absence of anti-vWF MoAbs (25 $\mu$g/ml) at room temperature for 1.5 h, followed by washing 5 times with 200 $\mu$l of TBS containing 0.1% BSA. To detect the binding of vWF, HRP-conjugated anti human vWF polyclonal antibodies (50 $\mu$l, diluted 1:1,000) were added to each well, followed by incubation for 1 h. Non-specific binding of the anti-vWF antibodies to each protein-coated plate was also examined. After washing several times as described above, 100 $\mu$l of a solution comprising 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5 mg/ml of *o*-phenylenediamine, and 0.03% of $H_2O_2$ was added. The HRP reaction was performed in the dark for 10-30 min and terminated by the addition of 100 $\mu$l of 8 M $H_2SO_4$. After 10 min, the absorbance at 490 nm was measured with a plate reader (Intermed Immuno Mini NJ-2201). In a separate experiment, the wells were further incubated with glycocalicin (1 $\mu$g/ml in B-TBS) in the presence or absence of botrocetin (5 $\mu$g/ml in B-TBS) after incubation with vWF (or plasma). After washing, the wells were incubated with anti-glycocalicin MoAb (HPL-16), followed by HRP-conjugated 2nd antibodies to detect the binding of glycocalicin.

## RESULTS

*Binding of vWF to Immobilized ECM Components*—Among various ECM components examined by ELISA on ECM component-coated plates, vWF preferentially bound to collagen-coated plates, especially type III (Fig. 1). The same results were obtained when several different products

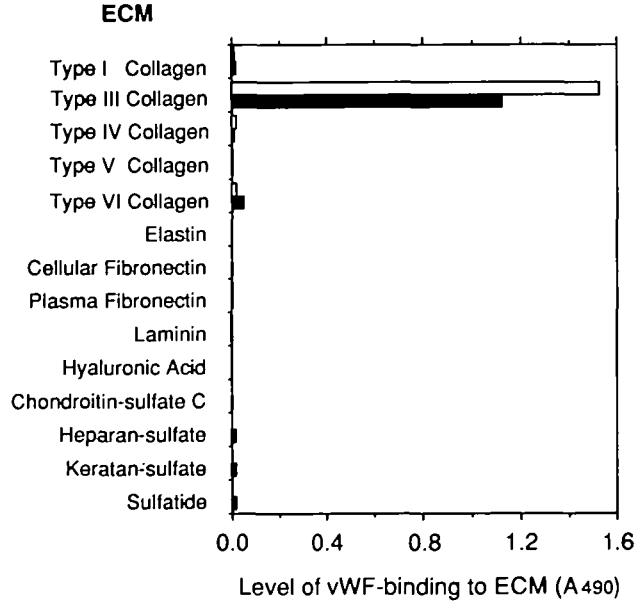


Fig. 1. **Binding of vWF with immobilized ECM components.** An ELISA plate coated with each ECM component (10 $\mu$g/ml) was incubated with 1:10 diluted plasma (white columns) or vWF (1 $\mu$g/ml) in B-TBS (black columns). The binding of vWF was detected with HRP-conjugated anti-vWF polyclonal antibodies, followed by the HRP-reaction, and then the absorbance at 490 nm ($A_{490}$) was read.

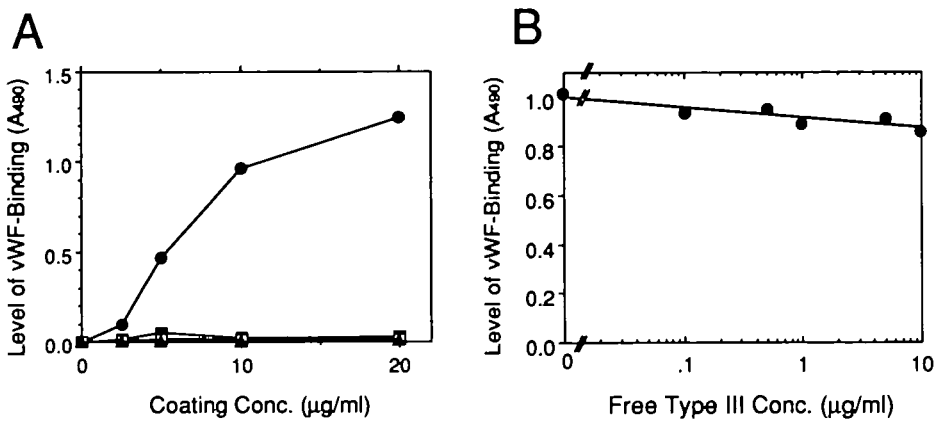


Fig. 2. **Binding of vWF to immobilized collagens.** A: ELISA plates coated with collagen type I (○), type III (●), type IV (■), type V (□), or type VI (▲) at the concentrations indicated were incubated with vWF (1 μg/ml) in B-TBS, and then the binding of vWF was detected with anti-vWF polyclonal antibodies as described in Fig. 1. B: ELISA plates coated with 10 μg/ml of type III collagen in TBS were incubated with vWF (1 μg/ml) in B-TBS in the presence of soluble type III collagen (0-10 μg/ml). After washing, the binding of vWF was detected with HRP-conjugated anti-vWF polyclonal antibodies, followed by the HRP-reaction.

TABLE I. **Effect of plasma fibronectin on vWF-binding to type III collagen.** Type III collagen (10 μg/ml) coated plates were preincubated with B-TBS (buffer) or buffer containing human plasma fibronectin for 1 h. After washing, each plate was incubated with vWF in the presence or absence of fibronectin, and then the binding of vWF was detected with HRP-conjugated anti-vWF polyclonal antibodies.

| 1st incubation | 2nd incubation | vWF-binding (%)[a] |
|---|---|---|
| Buffer | vWF (1 μg/ml) | 100 |
| Fibronectin (30 μg/ml) | vWF (1 μg/ml) | 107 |
| Buffer | vWF (1 μg/ml) + Fibronectin (30 μg/ml) | 96 |

[a]Binding of vWF ($A_{490}$) to collagen-coated plates in the absence of fibronectin was expressed as 100% binding.

of type III collagen were examined, whereas the specific binding of vWF to immobilized type I and VI collagen was a little, even when the products from several different companies were examined. The binding of vWF was dependent on the concentration of type III collagen used for coating (Fig. 2A). Reversely, when soluble type III collagen was added to a plate coated with vWF through anti-vWF antibodies, no significant binding of collagen was detected with the anti-collagen antibodies (data not shown). Soluble type III collagen showed a little inhibitory effect on the vWF binding to a immobilized collagen (Fig. 2B).

Among several plasma proteins examined, human plasma fibronectin and vitronectin also showed binding affinity to type III collagen (data not shown). The concentrations of fibronectin and vitronectin are usually 15-20 times higher than that of vWF in the plasma. In a separate experiment, however, the binding of purified vWF was not affected by the presence or absence of a 30 times higher concentration of fibronectin (Table I).

The effects of several MoAbs specific to different domains of vWF on the vWF-binding to type III collagen were examined. Two MoAbs among 12 clones, VW53-2 and 9-10, which specifically bind to *Staphylococcus aureus* V-8 protease fragment III (residues 1-1365) containing both A1 (residues 479-717) and A3 (residues 910-1111) domains, but not to fragment I (residues 911-1365) containing an A3 but not an A1 domain (*24*), and fragment I (Katayama, M., unpublished observation), respectively, clearly inhibited the binding of vWF to type III collagen-coated plates (Fig. 3).

*Binding of Glycocalicin to vWF Immobilized on Collagen*—To determine whether or not vWF acquires the

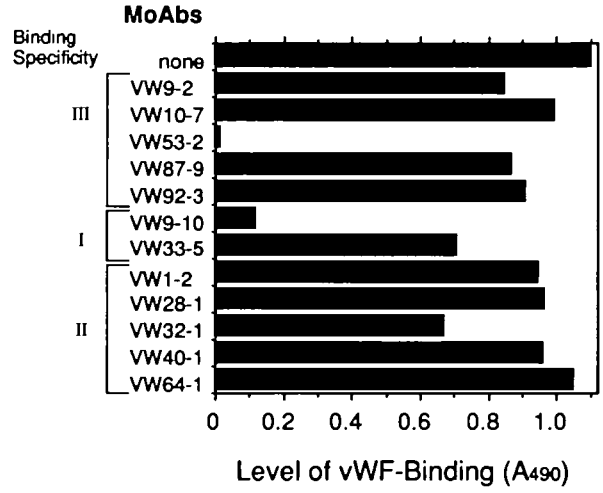


Fig. 3. **Effects of MoAbs against several vWF domains on vWF binding to immobilized type III collagen.** vWF (1 μg/ml) in B-TBS was added to type III collagen (10 μg/ml) coated plates in the presence or absence of MoAbs (25 μg/ml) which specifically bind to V-8 fragments III (residues 1-1365), I (residues 911-1365), and II (residues 1366-2050) of vWF. After washing, the binding of vWF was detected with anti-vWF polyclonal antibodies, followed by the HRP-reaction.

GPIb-binding activity after binding to type III collagen, we used glycocalicin purified from GPIb as a probe. As shown in Fig. 4A, the binding of vWF to plates coated with type III collagen increased in a dose-dependent manner, however, there was no significant binding of glycocalicin to either type III collagen alone or vWF immobilized on type III collagen. The same results were obtained with other types of collagen. It is possible that the binding of type III collagen to vWF interferes with the glycocalicin binding site of vWF. However, when glycocalicin was added to vWF immobilized on collagen in the presence of botrocetin, the binding of glycocalicin was observed (Fig. 4B). Glycocalicin bound to the immobilized vWF in a botrocetin-dependent manner, and the binding in the two assay systems reached a plateau in a similar manner after the addition of 10 μg/ml of botrocetin. Botrocetin bound to vWF on a type III collagen plate but not to a type III collagen plate alone. Glycocalicin did not bind to immobilized botrocetin or coated type III collagen. There was no binding of glyco-

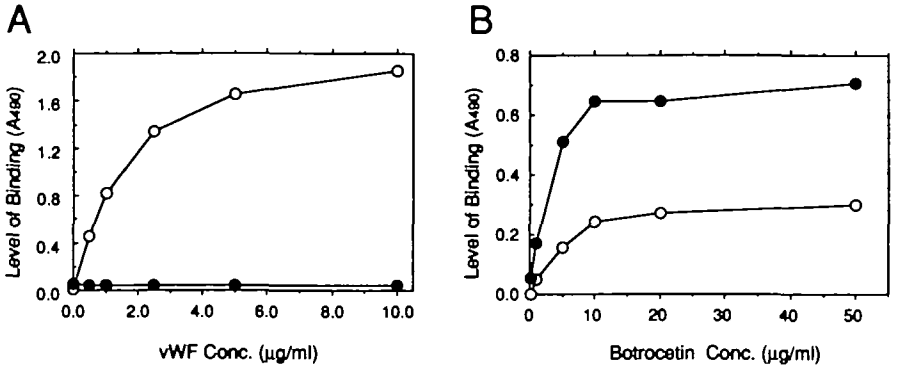


Fig. 4. **Binding of glycocalicin to vWF immobilized on type III collagen.** The binding of glycocalicin to vWF immobilized on type III collagen was examined in the absence (A) or presence (B) of botrocetin. A: ELISA plates coated with type III collagen (10 μg/ml) were incubated with vWF (0-10 μg/ml) in B-TBS containing glycocalicin (1 μg/ml) for 1 h. After washing, the binding of glycocalicin (●) and vWF (○) was detected with anti-glycocalicin (HPL-16) MoAb and HRP-conjugated anti-vWF polyclonal antibodies, respectively. B: Type III collagen (10 μg/ml) coated plates were incubated with vWF (2.5 μg/ml) in B-TBS for 1 h. After washing, glycocalicin (1 μg/ml) in B-TBS was added together with botrocetin (0-50 μg/ml) for 1 h. The binding of glycocalicin (●) and botrocetin (○) was detected with anti-glycocalicin (HPL-16) and anti-botrocetin (BCT107-3) MoAbs, respectively.

calicin when antibiotic ristocetin, another vWF modulator, was used instead of botrocetin (data not shown).

## DISCUSSION

Although vWF has been reported to bind to several types of collagen and heparin (*3-5, 8, 27*), none of the reports mentioned whether or not the binding of vWF to these ECM components induces any conformational change of vWF to trigger platelet adhesion. Since native platelets are unstable and easily aggregate in response to many stimuli including collagen, it is not easy to see the effect of vWF-binding to ECM on the accessibility of GPIb. In the present study, we examined *in vitro* molecular interactions among vWF, ECM, GPIb (glycocalicin), and botrocetin under static conditions. We found that type III collagen is the most effective ECM component as to immobilization of vWF. Type I (*4, 27*) and VI collagens (*8*) were also reported to bind vWF, but they showed much less binding activity toward vWF than type III collagen under our assay conditions. Other types of collagen showed vWF binding activity at much higher concentrations than that used in the present study, but they also showed increases in nonspecific binding (data not shown).

Soluble free type III collagen neither bound to vWF nor interfered with the vWF binding to immobilized collagen under the conditions used, suggesting that solid-phase type III collagen is important for the interaction with vWF. Since vWF has a multimeric structure, the multivalency of vWF as well as the immobilization of collagen may facilitate the interaction. The vWF binding was not affected significantly by other plasma proteins. Since plasma fibronectin and vitronectin also bind to type III collagen, but do not compete with vWF, it is suggested that collagen has different binding sites for vWF and fibronectin (or vitronectin). vWF has no collagen-binding motif found in plasma fibronectin (*28*). These results imply that type III collagen can specifically anchor vWF, even if abundant plasma fibronectin and vitronectin bind to it.

We postulated that glycocalicin may interact with vWF if the GPIb-binding site of vWF is accessible. In our assay system, no significant binding of glycocalicin to vWF bound to type III collagen was observed. Since two distinct binding sites for collagen have been postulated to be located in the A1 (residues 542-622) and A3 (residues 948-998) domains (*5*), and the former is close to the tentative GPIb binding site (residues 514-542) (*16*), it is possible that the binding of collagen may cause steric hindrance at the GPIb binding site rather than modulation. However, this is not likely, because vWF interacts with botrocetin, which also binds to three distinct sites (residues 539-553, 569-583, and 629-643) in the same A1 domain (*18*), even after the binding to collagen. Moreover, glycocalicin binding occurs in the presence of botrocetin. These results indicate that at least the GPIb binding site is not blocked or inactivated by binding to collagen. Recently, Cruz *et al.* (*29*) reported that the A3 domain is the major binding site for collagen. We found that two MoAb clones, reacting with the A1 and A3 domains, respectively (*24*), inhibit vWF binding to type III collagen. Our data suggest that both the A1 and A3 domains are important for the binding to collagen and that this binding does not interfere with either GPIb or botrocetin binding to vWF.

It is not clear why ristocetin has no modulatory effect on vWF bound to type III collagen. Recently, it was reported that ristocetin binds to GPIb as well as the negatively-charged Pro-rich hinge region of the A1 loop of vWF (*17, 30*). Although glycocalicin is rich in negatively-charged groups, because it contains highly sialylated sugar chains, a certain density or immobilization of the binding site on glycocalicin may be important for the interaction with ristocetin.

Our present data indicate that vWF preferentially binds to a type III collagen matrix. However, this binding is not sufficient to induce the binding of vWF to GPIb. After vWF is anchored to ECM through binding to type III collagen, another factor inducing a conformational change of the vWF molecule may be still required. It has been reported that loosening of the tertiary structure of the A1 domain actually enhances the affinity to GPIb (*31*). Recently, the tertiary structure of the A1 domain was predicted from the sequence structural homology to the GTP-binding fold of ras-p21 (*32*) and leukocyte integrin component receptor type 3 (CR3) (*33*) of known crystal structure. From this prediction, the botrocetin binding sites (*18*) and mutation sites of von Willebrand disease type 2B (*1, 2*) seem to be localized on one side of the A1 domain near the tentative ligand binding crevice (GPIb-binding site) (*32*). Under flow conditions, high-shear stress induces the adhesion of platelets to a collagen-coated plate (*34-36*). However, it

remains unknown whether or not the shear stress changes the conformation of vWF and is solely enough to activate vWF adhered to ECM *in vivo*. Plasma components may not participate in the modulation of vWF directly, as shown by our experiments. There is no evidence that one of the selectin family proteins, such as P-selectin or E-selectin (*37*), having a C-type lectin motif like botrocetin, modulates the immobilized vWF. Recently, Katayama *et al.* (*38*) reported that a 130 kDa component and fibronectin complex was obtained with an anti-botrocetin MoAb column from a human umbilical vein extract. It is possible that this unknown complex may mimic the structure of botrocetin and thus modulate vWF *in vivo*. The present assay system involving immobilized vWF and glycocalicin is simple and useful for screening candidate(s) for the *in vivo* vWF modulator. We are now attempting to isolate this component on a large scale to determine whether or not it shows any vWF modulating activity in our assay system.

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## REFERENCES

1. Sadler, J.E. (1991) von Willebrand factor. *J. Biol. Chem.* **266**, 22777-22780
2. Fujimura, Y. and Titani, K. (1993) Structure and function of von Willebrand factor in *Haemostasis and Thrombosis* (Bloom, A.L., Forbes, C.D., and Thomas, D., eds.) pp. 379-395, Churchill Livingstone, Edinburgh
3. Fujimura, Y., Titani, K., Holland, L.Z., Roberts, J.R., Kostel, P., Ruggeri, Z.M., and Zimmerman, T.S. (1987) A heparin-binding domain of human von Willebrand factor: Characterization and localization to a tryptic fragment extending from amino acid residue Val-449 to Lys-728. *J. Biol. Chem.* **262**, 1734-1739
4. Scott, D.M., Griffin, B., Pepper, D.S., and Barnes, M.J. (1981) The binding of purified factor VIII/von Willebrand factor to collagens of differing type and form. *Thromb. Res.* **24**, 467-472
5. Roth, G.J., Titani, K., Hoyer, L.K., and Hickey, M.J. (1986) Localization of binding sites within human von Willebrand factor for monomeric type III collagen. *Biochemistry* **25**, 8357-8361
6. Roberts, D.D., Williams, S.B., Gralnick, H.R., and Ginsburg, V. (1986) von Willebrand factor binds specifically to sulfated glycolipids. *J. Biol. Chem.* **261**, 3306-3309
7. Rand, J.H., Patel, N.D., Schwatz, E., Zhou, S.-L., and Potter, B. J. (1991) 150-kDa von Willebrand factor binding protein extracted from human vascular subendothelium is type VI collagen. *J. Clin. Invest.* **88**, 253-259
8. Denis, C., Baruch, D., Kielty, C.M., Ajzenberg, N., Christophe, O., and Meyer, D. (1993) Localization of von Willebrand factor binding domains to endothelial extracellular matrix and to type VI collagen. *Arterioscler. Thromb.* **13**, 398-406
9. Ross, J.M., McIntire, L.V., Moake, J.L., and Rand, J.H. (1995) Platelet adhesion and aggregation on human type VI collagen surfaces under physiological flow conditions. *Blood* **85**, 1826-1835
10. Baruch, D., Denis, C., Marteaux, C., Schoevaert, D., Coulombel, L., and Meyer, D. (1991) Role of von Willebrand factor associated to extracellular matrices in platelet adhesion. *Blood* **77**, 519-527
11. Stel, H.S., Sakariassen, K.S., de Groot, P.G., van Mourik, J.A., and Sixma, J.J. (1985) von Willebrand factor in the vessel wall mediates platelet adherence. *Blood* **65**, 85-90
12. Fujimura, Y., Titani, K., Holland, L.Z., Russell, S.R., Roberts, J.R., Elder, J.H., Ruggeri, Z.M., and Zimmerman, T.S. (1986) von Willebrand factor: A reduced and alkylated 52/48 kDa fragment beginning at amino acid residue 449 contains the domain interacting with platelet glycoprotein Ib. *J. Biol. Chem.* **261**, 381-385
13. Andrews, R.K., Gorman, J.J., Booth, W.J., Corino, G.L., Castaldi, P.A., and Berndt, M.C. (1989) Cross-linking of a monomeric 39/34-kDa dispase fragment of von Willebrand factor Leu-480/Val-481 to the N-terminal region of the $\alpha$-chain of membrane glycoprotein Ib on intact platelets with bis (sulfosuccinimidyl) suberate. *Biochemistry* **28**, 8326-8336
14. Matsushita, T. and Sadler, J.E. (1995) Identification of amino acid residues essential for von Willebrand factor binding to platelet glycoprotein Ib: Charged-to-alanine scanning mutagenesis of the A1 domain of human von Willebrand factor. *J. Biol. Chem.* **270**, 13406-13414
15. Fujimura, Y., Usami, Y., Titani, K., Niinomi, K., Nishio, K., Takase, T., Yoshioka, A., and Fukui, H. (1991) Studies on anti-von Willebrand factor (vWF) monoclonal antibody NMC-4, which inhibits both ristocetin- and botrocetin-induced vWF binding to platelet glycoprotein Ib. *Blood* **77**, 113-120
16. Berndt, M.C., Ward, C.M., Booth, W.J., Castaldi, P.A., Mazurov, A.V., and Andrews, R.K. (1992) Identification of aspartic acid 514 through glutamic acid 542 as a glycoprotein Ib-IX complex receptor recognition sequence in von Willebrand factor: mechanism of modulation of von Willebrand factor by ristocetin and botrocetin. *Biochemistry* **31**, 11144-11151
17. Hoylaerts, M.F., Nuyts, K., Peerlinck, K., Deckmyn, H., and Vermylen, J. (1995) Promotion of binding of von Willebrand factor to platelet glycoprotein Ib by dimers of ristocetin. *Biochem. J.* **306**, 453-463
18. Sugimoto, M., Mohri, H., McClintock, R.A., and Ruggeri, Z.M. (1991) Identification of discontinuous von Willebrand factor sequences involved in complex formation with botrocetin: A model for the regulation of von Willebrand factor binding to platelet glycoprotein Ib. *J. Biol. Chem.* **266**, 18172-18178
19. Sobel, M., Soler, D.F., Kermode, J.C., and Harris, R.B. (1992) Localization and characterization of a heparin binding domain peptide of human von Willebrand factor. *J. Biol. Chem.* **267**, 8857-8862
20. Christophe, O., Obert, B., Meyer, D., and Girma, J.-P. (1991) The binding domain of von Willebrand factor to sulfatides is distinct from those interacting with glycoprotein Ib, heparin, and collagen and resides between amino acid residues Leu512 and Lys673. *Blood* **78**, 2310-2317
21. Matsui, T., Fujimura, Y., Nishida, S., and Titani, K. (1993) Human plasma $\alpha_2$-macroglobulin and von Willebrand factor possess covalently linked ABO(H) blood group antigens in subjects with corresponding ABO phenotype. *Blood* **82**, 663-668
22. Usami, Y., Fujimura, Y., Suzuki, M., Ozeki, Y., Nishio, K., Fukui, H., and Titani, K. (1993) Primary structure of two-chain botrocetin, a von Willebrand factor modulator purified from venom of *Bothrops jararaca*. *Proc. Natl. Acad. Sci. USA* **90**, 928-932
23. Chopek, M.W., Girma, J.-P., Fujikawa, K., Davie, E.W., and Titani, K. (1986) Human von Willebrand factor: A multivalent protein composed of identical subunits. *Biochemistry* **25**, 3146-3155
24. Katayama, M., Hirai, S., Kato, I., and Titani, K. (1994) Immunoenzymometric analysis for plasma von Willebrand factor degradation in diabetes mellitus using monoclonal antibodies recognizing protease-sensitive sites. *Clin. Biochem.* **27**, 123-131
25. Kunishima, S., Hayashi, K., Kobayashi, S., Naoe, T., and Ohno, R. (1991) New enzyme-linked immunosorbent assay for glycocalicin in plasma. *Clin. Chem.* **37**, 169-172
26. Michelson, A.D., Loscalzo, J., Melnick, B., Coller, B.S., and Handin, R.I. (1986) Partial characterization of a binding site for von Willebrand factor on glycocalicin. *Blood* **67**, 19-26
27. Pareti, F.I., Niiya, K., McPherson, J.M., and Ruggeri, Z.M. (1987) Isolation and characterization of two domains of human von Willebrand factor that interact with fibrillar collagen type I and III. *J. Biol. Chem.* **262**, 13835-13841
28. Owens, R.J. and Baralle, F.E. (1986) Mapping the collagen-binding site of human fibronectin by expression in *Escherichia coli*. *EMBO J.* **5**, 2825-2830
29. Cruz, M.A., Yuan, H., Lee, J.R., Wise, R.J., and Handin, R.I.

(1995) 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.* **270**, 10822-10827
30. Scott, J.P., Montgomery, R.R., and Retzinger, G.S. (1991) Dimeric ristocetin flocculates proteins, binds to platelets, and mediates von Willebrand factor-dependent agglutination of platelets. *J. Biol. Chem.* **266**, 8149-8155
31. Miyata, S., Goto, S., Federici, A.B., Ware, J., and Ruggeri, Z.M. (1996) Conformational change in the A1 domain of von Willebrand factor modulating the interaction with platelet glycoprotein Ib$\alpha$. *J. Biol. Chem.* **271**, 9046-9053
32. Edwards, Y.J.K. and Perkins, S.J. (1995) The protein fold of the von Willebrand factor type A domain is predicted to be similar to the open twisted $\beta$-sheet flanked by $\alpha$-helices found in human ras-p21. *FEBS Lett.* **358**, 283-286
33. Lee, J.-O., Rieu, P., Arnaout, M.A., and Liddington, R. (1995) Crystal structure of the A domain from the $\alpha$ subunit of integrin CR3 (CD11b/CD18). *Cell* **80**, 631-638
34. Alevriadou, B.R., Moake, J.L., Turner, N.A., Ruggeri, Z.M., Folie, B.J., Phillips, M.D., Schreiber, A.B., Hrinda, M.E., and McIntire, L.V. (1993) Real-time analysis of shear-dependent thrombus formation and its blockade by inhibitors of von Willebrand factor binding to platelets. *Blood* **81**, 1263-1276
35. Sakariassen, K.S., Fressinaud, E., Girma, J.-P., Baumgartner, H.R., and Meyer, D. (1986) Mediation of platelet adhesion to fibrillar collagen in flowing blood by a proteolytic fragment of human von Willebrand factor. *Blood* **67**, 1515-1518
36. Savage, B., Saldivar, E., and Ruggeri, Z.M. (1996) Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell* **84**, 289-297
37. Bevilacqua, M.P. and Nelson, R.M. (1993) Selectins. *J. Clin. Invest.* **91**, 379-387
38. Katayama, M., Nagata, S., Hirai, S., Miura, S., Fujimura, Y., Matsui, T., Kato, I., and Titani, K. (1995) Fibronectin and 130-kDa molecule complex mimics snake venom botrocetin-like structure potentially modulating association between von Willebrand factor and vascular vessel wall. *J. Biochem.* **117**, 331-338