Functional Domains on Von Willebrand Factor

Recognition of Discrete Tryptic Fragments by Monoclonal Antibodies that Inhibit Interaction of Von Willebrand Factor with Platelets and with Collagen

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Abstract. We have identified two functional domains on the von Willebrand factor (VWF) moiety of the Factor VIII-von Willebrand factor complex (FVIII-VWF), one interacting with blood platelets, and one interacting with vessel wall collagens, by means of two monoclonal antibodies directed against the VWF molecule, CLB-RAg 35 and CLB-RAg 201.

The monoclonal antibody CLB-RAg 35 inhibited virtually all platelet adherence to artery subendothelium and to purified vessel wall collagen type III, at relatively high wall shear rates. CLB-RAg 35 also inhibited the ristocetin-induced platelet aggregation and the binding of FVIII-VWF to the platelet in the presence of ristocetin but did not affect the binding of FVIII-VWF to collagen. The monoclonal antibody CLB-RAg 201 inhibited the binding of FVIII-VWF to purified vessel wall collagen type I and III and all platelet adherence to collagen type III and the platelet adherence to subendothelium that was mediated by FVIII-VWF in plasma.

The two functional domains on FVIII-VWF that were recognized by CLB-RAg 35 and CLB-RAg 201 were identified by means of immunoprecipitation studies of trypsin-digested FVIII-VWF. The domains resided on different polypeptide fragments, with a M_r of 48,000 for

the collagen binding domain and a M_r of 116,000 for the platelet binding domain. The 116,000-mol wt fragment consisted of subunits of 52,000/56,000 mol wt and 14,000 mol wt after reduction. The 52,000/56,000-mol wt subunits possessed the epitope for CLB-RAg 35.

Introduction

Adherence of blood platelets to perivascular connective tissue is one of the initial and crucial steps in the formation of a hemostatic plug (1). Platelet adherence to subendothelium at high shear rates is almost completely dependent on Factor VIII-von Willebrand factor complex (FVIII-VWF)[1] (2, 3). von Willebrand factor (VWF) antigen is already present in the subendothelium, deposited by endothelial cells (4), and this supports platelet adhesion, but plasma FVIII-VWF is also needed for optimal adhesion (5).

FVIII-VWF consists of two molecules: Factor VIII, the procoagulant molecule that is absent in hemophilia A, and VWF, which is responsible for the adhesion of platelets to subendothelium and collagen at high wall shear rates (2, 3) and is absent or abnormal in von Willebrand's disease.

Recently, we have described a series of monoclonal antibodies against VWF, among which two cause complete inhibition of platelet adherence (6). These two antibodies were directed against the same or closely related epitopes. One of

1. *Abbreviations used in this paper:* CLB-RAg 35 and 201, code names for monoclonal antibodies directed against VWF; ELISA, enzyme-linked immunosorbent assay; FVIII-VWF, Factor VIII-von Willebrand factor complex; HAS, human albumin solution: 4 mM KCl, 107 mM NaCl, 20 mM NaHCO$_3$, 2 mM Na$_2$SO$_4$, 19 mM trisodium citrate, 2.5 mM CaCl$_2$, 5 mM glucose and 4% (w/v) human albumin, pH 7.4; IPB, immunoprecipitation buffer; NP40, Nonidet P40; PAA, polyacrylamide; VWF, von Willebrand factor; TPCK, L-(tosylamido 2-phenyl) ethyl chloromethyl ketone.

these antibodies, CLB-RAg 35, has been studied in more detail (7). It appears to inhibit the interaction of VWF with blood platelets, as indicated by the inhibition of ristocetin-induced platelet aggregation and the binding of VWF to platelets in the presence of ristocetin.

In this paper, we describe another monoclonal antibody to VWF, CLB-RAg 201, which caused partial inhibition of platelet adhesion, and which was found to inhibit the binding of FVIII-VWF to collagen.

We have employed both antibodies CLB-RAg 35 and CLB-RAg 201 in immunoprecipitation tests in order to identify tryptic fragments of VWF that contain the corresponding epitopes. We found that the epitope for CLB-RAg 35, the antibody that blocks the interaction of VWF with platelets, was located on a different fragment than the epitope for CLB-RAg 201, which was the antibody that blocked the interaction of VWF with collagen.

Methods

All chemicals obtained from commercial sources were of the highest purity grade obtainable. Phenylmethylsulfonylfluoride, soybean trypsin inhibitor (type I-S), benzamidine, and acetyl salicylic acid were from Sigma Chemical Co. (St. Louis, MO). ϵ-amino caproic acid was from Nogepha (Alkmaar, The Netherlands). L-(tosylamido 2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin was obtained from Worthington Biochemical Corp. (Freehold, NJ). Carrier-free ^{125}I ($\sim$17 Ci/mg) was from Radiochemical Centre (Amersham, United Kingdom). ^{111}Indium-oxinate was obtained from Byk-Mallinckrodt, C.I.L. BV (Petten, The Netherlands).

Isolation of FVIII-VWF. FVIII-VWF was isolated from fresh cryoprecipitates by agarose gel filtration on Sepharose CL-4B as described previously (8). The void volume fraction was precipitated by dialysis against 1.6 M ammonium sulfate, pH 7.0, at 4°C for 18 h and stored as ammonium sulfate suspension at 4°C until use (usually within 48 h).

Radiolabeling of FVIII-VWF. FVIII-VWF was collected from the ammonium sulfate suspension by centrifugation (10,000 g, 4°C, 2 min). The pellet was dissolved in 0.05 M sodium phosphate buffer, pH 7.0, and dialyzed against this buffer to remove remaining ammonium sulfate. Radiolabeling with ^{125}I was performed with the chloramine T method (9). Noncovalently linked ^{125}I was removed by dialysis overnight at 4°C against 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.0. ^{125}I-labeled preparations of FVIII-VWF with a specific activity of $\sim$10 μCi/mg were obtained.

Tryptic digestion of FVIII-VWF. A sample of 0.65 ml was taken from 3.5 ml 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.0, which contained 1.4 mg ^{125}I-FVIII-VWF and added to 40 μl of a solution of 0.25 M benzamidine, 0.25 M ϵ-aminocaproic acid, and 0.4 mg/ml soybean trypsin inhibitor. The remaining 2.85 ml was warmed to 37°C and 90 μl of a trypsin solution (166 U/ml TPCK-treated trypsin, active site titrated 0.439 μmol/μmol, dissolved in 10 mM HCl and 10 mM CaCl$_2$) was added. Samples of 0.65 ml were collected after 10 min, 90 min, 6 h, and 24 h of incubation at 37°C and added to 40 μl of a solution of 0.25 M benzamidine, 0.25 M ϵ-aminocaproic acid, and 0.4 mg/ml soybean trypsin inhibitor.

Characteristics of the monoclonal antibodies. The monoclonal antibodies CLB-RAg 35 and CLB-Rag 201 have been described

elsewhere (6). These antibodies are directed against VWF on the basis of the following criteria: they react in an enzyme-linked immunosorbent assay (ELISA) with purified FVIII-VWF, normal plasma, and a cross-reacting material (negative CRM$^-$) plasma of a hemophilia A patient, whereas they do not react with plasma of a patient with von Willebrand's disease subtype III or with a FVIII fraction that was obtained after dissociation of FVIII-VWF with 0.25 M CaCl$_2$. Immunofluorescence studies of cultured endothelial cells that were derived from human umbilical veins with these antibodies showed the granular-staining patterns that were characteristic for VWF in endothelial cells.

The antibody CLB-RAg 35 completely inhibited ristocetin-induced platelet aggregation and the binding of FVIII-VWF to platelets in the presence of ristocetin (7), but had no effect on platelet aggregation that was induced by ADP or collagen. This antibody inhibited almost completely the adhesion of blood platelets to subendothelium in an annular perfusion chamber at high shear rates (7).

The monoclonal antibody CLB-RAg 201 had no inhibitory effect on the ristocetin-induced platelet aggregation or on the binding of FVIII-VWF to platelets in the presence of ristocetin. The adhesion of blood platelets to subendothelium in an annular perfusion chamber at high shear rates was only partially inhibited by CLB-RAg 201 (6). CLB-RAg 201 did not compete with CLB-RAg 35 in a competitive radioimmunoassay, which indicated that the epitope for CLB-RAg 201 differed from that for CLB-RAg 35 (Table I). Labeling of a SDS-agarose multimeric pattern of VWF showed staining of all bands with both CLB-RAg 35 and CLB-RAg 201.

Inhibition of binding of FVIII-VWF to collagen. Collagen type I and III were isolated from human umbilical arteries by pepsin digestion according to Chung and Miller (10). Separation of the collagen types was achieved by the method of Chandrarajan (11). The homogeneity of the purified collagen was evaluated with SDS-polyacrylamide (PAA) electrophoresis. Only the characteristic collagen bands were observed

Table I. Competitive Inhibition Study of CLB-RAg 201 on CLB-RAg 35 Binding

Competing antibody	
	cpm
Buffer	1,250
Control IgG	1,180
CLB-RAg 201	1,210
CLB-RAg 35	90

Purification and radiolabeling of monoclonal antibodies was performed as described (33). Polystyrene tubes were coated with purified FVIII-VWF, 20 μg/ml in 0.02 M sodium phosphate buffer, pH 7.2, by incubation overnight at 4°C. The tubes were washed three times with phosphate-buffered saline (PBS; 9 vol 0.15 M NaCl, 1 vol 0.1 M phosphate buffer, pH 7.4 that contained 0.05% [v/v] Tween 20) and then incubated for 24 h at room temperature with 0.002 μg radiolabeled CLB-RAg 35-IgG ($\sim$10,000 cpm in PBS that contained 3% [w/v] bovine serum albumin and 0.05% [v/v] Tween 20) together with 10 μg of the unlabeled antibodies to be tested (CLB-RAg 201, CLB-RAg 35 and control IgG), which was a monoclonal antibody directed against cat allergen (courtesy of Dr. Aalberse, Central Laboratory). The tubes were then washed three times and bound radioactivity was estimated in a gamma counter.

after Coomassie Brilliant Blue staining when 10 μg of protein was applied to a gel. Nonfibrillar collagen was dissolved in 50 mM acetic acid and stored at 4°C. Collagen fibrils were prepared by dialysis of a collagen solution (1 mg/ml in 50 mM acetic acid) against 0.02 M sodium phosphate buffer, pH 7.4, for 48 h at 4°C. The fibril formation had come to completion at this time as checked turbidimetrically in a spectrophotometer at 313 nm (11). Collagen fibrils were separated from nonpolymerized collagen by centrifugation (5 min, 10,000 g, 4°C).

50 μl of 0.02 M sodium phosphate buffer, pH 7.4, that contained various concentrations of collagen fibrils and either 6-μl ascites that contained monoclonal antibodies or 6 μl phosphate-buffered saline were added to 250 μl platelet-poor plasma from a pool of 40 normal donors. This mixture was incubated for 15 min at 37°C under occasional stirring and then centrifuged (5 min, 10,000 g, 4°C). The supernatant was pipetted off and residual VWF was estimated by ELISA as previously described (6). Addition of ascites that contained monoclonal antibodies directed against VWF had no influence on the concentration of VWF in plasma as estimated by ELISA.

Inhibition of platelet adherence with CLB-RAg 201. In order to study the adherence of blood platelets in flowing blood to subendothelium, perfusion experiments were performed under steady flow (12) in an annular perfusion chamber according to Baumgartner (13). Segments of a human umbilical artery that was denuded of endothelium were everted and mounted on the central rod and exposed for 5 min to flowing blood at a flow rate of 107 ml/min, corresponding to a wall shear rate of 2,500 s^{-1}.

The artery segments were ~1.0 cm long and the endothelium was removed by brief air exposure before eversion of the segments. Before perfusion the arteries were treated for 1 h with 0.1 mM acetyl salicylic acid in 0.2 M Tris–HCl, pH 7.35, in order to prevent prostacyclin production by smooth muscle cells which may inhibit platelet adherence (14) and subsequently washed four times with the Tris–HCl buffer.

Interaction of blood platelets with nonfibrillar collagen in flowing blood was studied with a rectangular perfusion chamber which has been described elsewhere (15). A glass microscope coverslip was coated with 30 μg/cm^2 of collagen type III by spraying of a solution of 1 mg/ml in 50 mM acetic acid with a retouching air brush (Badger Model No. 100 IL, Badger Air Brush Co., Franklin Park, IL.[2] The collagen coated coverslip was inserted into the perfusion chamber, and perfusion during 5 min was performed at a flow rate of 107 ml/min, which corresponded to a wall shear rate of 960 s^{-1}. For inhibition studies, 50-μl ascites were added to 12 ml of perfusate and incubated for 5 min at 37°C before the perfusion started.

Immunoprecipitation with CLB-RAg 35 and 201. Immunoprecipitation of the trypsin digest of ^{125}I-labeled FVIII-VWF with CLB-RAg 35 and CLB-RAg 201 was performed according to Borst et al. (17). To 300 μl digested ^{125}I–FVIII-VWF (300 μg/ml) was added 20 μl of a preformed complex between normal mouse serum and rabbit anti-mouse serum (Nordic, Tilburg, The Netherlands) for preclearing. This mixture was incubated under continuous rotation for 1 h at 4°C and centrifuged (15 min, 10,000 g, 4°C). This procedure was repeated twice for the supernatant. 10 μl of a specific complex between CLB-RAg 35 or CLB-RAg 201 and rabbit anti-mouse serum was then added to the supernatant and incubated under continuous rotation for 3 h or overnight at 4°C. This mixture was centrifuged (15 min, 10,000

g, 4°C) and the pellet was resuspended in 100 μl 10 mM triethanolamine, pH 7.8, 150 mM NaCl, 1% (w/v) Nonidet P40 (NP40) with 1 mM phenylmethylsulfonylfluoride, and 20 μg/ml soybean trypsin inhibitor (immunoprecipitation buffer [IPB]) and applied to a discontinuous sucrose gradient of 800 μl of 20% (w/v) sucrose in IPB without NP40 and 400 μl of 10% (w/v) sucrose in IPB that contained 0.5% (w/v) NP40, and centrifuged (15 min, 10.000 g, 4°C). The pellet was resuspended in 50 μl IPB plus 17 μl sample buffer (0.25 M Tris–HCl, pH 6.8, 20% (w/v) glycerol, 8% (w/v) SDS, 0.1% (w/v) bromophenol blue), boiled for 5 min, and applied to a 3–30% gradient PAA gel.

Polyacrylamide gel electrophoresis. SDS slab gel electrophoresis was performed on a PAA gradient of 3–30% (280 × 140 × 0.75 mm) essentially according to Laemmli (18). The gels were run at 200 V (constant voltage) for 20 h using running tap water for cooling. After electrophoresis the gels were stained for protein with Coomassie Brilliant Blue R-250 and dried between Cellophane sheets (Bio-rad Laboratories, Richmond, CA).

Autoradiography of the dried gels was performed using Kodak-X-Omat AR5 (Eastman Kodak Co., Rochester, NY) film and DuPont Cronex Lightening Plus intensifying screens (DuPont Instruments, Wilmington, DE). A mixture of low molecular weight proteins (Pharmacia, Uppsala, Sweden) and high molecular weight proteins (Pharmacia or Bio-rad Laboratories) was analyzed on every gel as molecular weight markers.

Two-dimensional PAA-gel electrophoresis was essentially performed as described by Phillips and Agin (19). The first dimension was run on cylindrical gradient gels of 3–25% PAA (3 mm ∅ 10 cm long). The gels were run at 100 V constant voltage for 16 h. The gels were removed from the tubes and reduced for 15 min at 60°C in sample buffer that contained 10% (v/v) glycerol, 2.3% (w/v) SDS, 0.0625 M Tris–HCl, pH 6.8, and 5% (w/v) β-mercaptoethanol. The gel was then

Table II. Inhibition of Binding of FVIII-VWF to Fibrillar Collagen Type I and III by Monoclonal Antibodies

	% Residual VWF-antigen	
Code of monoclonal	Collagen type I	Collagen type III
No collagen	100	100
No antibody	64	51
Control antibody	65	54
CLB-RAg 35	56	52
CLB-RAg 201	86	92

50 μl of 0.02 M sodium phosphate buffer, pH 7.4, containing 100 μg of fibrillar collagen type I or 50 μg of fibrillar collagen type III and 6-μl ascites or PBS were added to 250 μl platelet-poor plasma from a pool of 40 normal donors. This mixture was incubated for 15 min at 37°C under occasional stirring and then centrifuged (5 min, 10,000 g, 4°C). VWF was estimated in the supernatant by ELISA. The control antibody was a monoclonal antibody that was raised against a cat allergen (see legend Table I). In the 'no collagen' incubation, both collagen and ascites were omitted. In the 'no antibody' incubation we used PBS instead of ascites. CLB-RAg 35 is shown as representative for the 20 other monoclonal antibodies against VWF that had no effect on the interaction of VWF and collagen. The data shown are the mean values of two determinations.

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placed on top of a gradient slab gel and subjected to electrophoresis as described above. Molecular weight markers were run in parallel in the first and second dimension.

Results

Inhibition of interaction of VWF with collagen and subendothelium by CLB-RAg 201. A series of 23 monoclonal antibodies against VWF was screened for inhibition of binding of FVIII-VWF to fibrillar collagen type I and III. Only one monoclonal antibody, CLB-RAg 201, inhibited the interaction of FVIII-VWF with collagen, whereas the other antibodies, including the two antibodies that inhibited the interaction between platelets and VWF (CLB-RAg 35 and 34), had no effect (Table II).

The inhibition of binding of FVIII-VWF by CLB-RAg 201 was studied in more detail. Various concentrations of fibrillar collagen types I and III were added to plasma. More binding of FVIII-VWF was observed to collagen type III than to collagen type I at low concentrations of collagen. Half-maximal
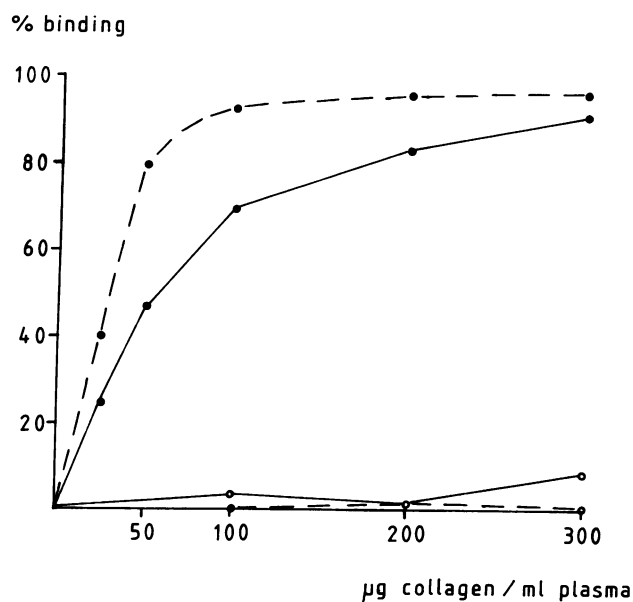
% binding

μg collagen / ml plasma

Figure 1. Inhibition of binding of FVIII-VWF to collagen type I and III by CLB-RAg 201. 50 μl of 0.02 M sodium phosphate buffer, pH 7.4, which contained 6.25, 12.5, 25, 50, or 75 μg of collagen fibrils type I and type III were added to 250 μl platelet-poor plasma from a pool of 40 normal donors. This mixture was incubated for 15 min at 37°C under occasional stirring and then centrifuged (5 min, 10,000 g, 4°C). VWF was estimated in the supernatant by ELISA. 3 μl of ascites that contained CLB-RAg 201 was added to study the inhibition of binding by this monoclonal antibody. The results are expressed as the percentage of VWF present in plasma, bound to collagen type I (● —— ●), or to type III (● - - - ●). The results in the presence of CLB-RAg 201 are indicated for collagen type I (o —— o) and type III (o - - - o).

binding was observed at ~30 μg/ml of collagen type III and at 60 μg/ml of collagen type I (Fig. 1). CLB-RAg 201, added as ascites in a final dilution of 1:100, inhibited binding of FVIII-VWF to both collagen types at all concentrations. Addition of ascites that contained CLB-RAg 201, diluted 1:1,000 and 1:10,000, also completely inhibited binding to collagen type III (165 μg/ml).

Inhibition of platelet adhesion to collagen and subendothelium by CLB-RAg 201. The effect of CLB-RAg 201 on platelet adhesion to nonfibrillar collagen and to arterial subendothelium in a perfusion system were compared in Table III. CLB-RAg 201 was able to inhibit the platelet adhesion to collagen type III completely, whereas the platelet adhesion to subendothelium was only partially inhibited. CLB-RAg 35 inhibited adhesion to collagen as well as adhesion to arterial subendothelium almost completely. The most likely explanation for the difference in inhibition of platelet adhesion to collagen and to subendothelium by CLB-RAg 201 is that subendothelium already contained sufficient VWF to support some adhesion and CLB-RAg 201 did not inhibit the action of this VWF, whereas in the case of collagen all FVIII-VWF had first to bind to the collagen, and this is the step that is inhibited by CLB-RAg 201. This hypothesis was further tested by first

Table III. Inhibition of Platelet Adhesion to Nonfibrillar Collagen Type III and Subendothelium by CLB-RAg 35 and CLB-RAg 201

	Platelets × 10⁻⁵/cm² (Mean±SEM)	
	Platelet adhesion to collagen	Platelet adhesion to subendothelium
Normal plasma	32.7±1.3 (n = 4)	65.9±1.8 (n = 17)
Normal plasma + CLB-RAg 35	2.8±0.5 (n = 3)	3.3±0.6 (n = 4)
Normal plasma + CLB-RAg 201	3.1±0.6 (n = 3)	39.5±3.4 (n = 5)

For the study of platelet adhesion to subendothelium, umbilical arteries 1-cm long were briefly exposed to air, everted, and mounted on the central rod of an annular perfusion chamber. For the adhesion to collagen, collagen type III that was purified from human umbilical arteries was sprayed with the retouching air brush on a glass microscope coverslip (30 μg/cm²). This collagen-coated coverslip was inserted into a rectangular perfusion chamber (15). Both surfaces were exposed to a perfusate that consisted of washed red blood cells (40% hematocrit), normal citrated plasma, and ¹¹¹In-labeled blood platelets (final concentration 114 × 10³/μl). The perfusion was performed for 5 min at a wall shear rate of 960 s⁻¹ for collagen and 2,500 s⁻¹ for subendothelium. Monoclonal antibodies were added as 50-μl ascites to 12 ml of perfusate and incubated for 5 min at 37°C before the perfusion started. No control ascites studies were performed in this experiment because previous parallel studies (6, 7) had shown no effect. The adhesion in the presence of CLB-RAg 35 and 201 was significantly decreased (P < 0.001).

blocking the VWF that was present in the subendothelium with CLB-RAg 35. Platelet adhesion to subendothelium became thereby also completely dependent on plasma FVIII-VWF. The action of this plasma FVIII-VWF could be inhibited by CLB-RAg 201, which supported the above mentioned hypothesis (Table IV).

The effect of CLB-RAg 201 on binding of VWF to collagen-coated surfaces and subendothelium were directly tested with radiolabeled FVIII-VWF. A considerable amount of ^{125}I–FVIII-VWF bound to the collagen coated coverslip, and this binding was blocked by CLB-RAg 201 but not by CLB-RAg 35. The binding to subendothelium was in the same range as previously reported (5) and much lower than to a collagen-coated surface. CLB-RAg 201 and 35 had no effect on this binding (Table V).

Tryptic digestion of FVIII-VWF and immunoprecipitation. Before digestion (0 h), FVIII-VWF did not migrate into the gel; neither did the VWF precipitated by CLB-RAg 35 and

Table IV. Inhibition of Platelet Adherence to Subendothelium Incubated with CLB-RAg 201 and 35

Preincubation	Perfusion fluid	Platelet adherence $\times 10^{-5}/cm^2$
		Mean±SEM
1 Buffer	Normal plasma	51.1±4.0 ($n = 12$)
2 Buffer	HAS	21.8±3.0 ($n = 12$)
3 CLB-RAg 201	HAS	21.3±4.8 ($n = 9$)
4 CLB-RAg 35	HAS	4.4±0.6 ($n = 9$)
5 CLB-RAg 35	HAS + FVIII-VWF*	22.2±5.5 ($n = 6$)
6 CLB-RAg 35	HAS + FVIII-VWF* + CLB-RAg 201	5.9±0.5 ($n = 8$)

Umbilical artery segments 1 cm long were briefly exposed to air everted and mounted on the central rod of an annular perfusion chamber. The mounted vessel segments were incubated for 1 h at 37°C in 2 ml of Krebs-Ringer buffer (4 mM KCl, 107 mM NaCl, 20 mM NaHCO$_3$, 2 mM Na$_2$SO$_4$, pH 7.4, that contained 19 mM trisodium citrate, 5 mM glucose, and 2.5 mM CaCl$_2$). The appropriate antibodies were added as ascites in a final dilution of 1:10. The mounted arteries were then rinsed with Krebs-Ringer buffer that contained glucose, sodium citrate, and calcium, as mentioned above, and placed in a standard annular perfusion chamber. Perfusion was performed with a perfusate that consisted of human albumin solution (HAS: 4 mM KCl, 107 mM NaCl, 20 mM NaHCO$_3$, 2 mM Na$_2$SO$_4$, 19 mM trisodium citrate, 2.5 mM CaCl$_2$, 5 mM glucose, and 4% (w/v) human albumin, pH 7.4), red blood cells (40% hematocrit), and ^{111}In-labeled platelet (114 × 10^3/μl) for 5 min at a wall shear rate of 1,800 s^{-1}. n equals number of perfusions.
* The FVIII-VWF added was in a concentration of 3 U/ml. CLB-RAg 201 was added to the perfusate as ascites in a final dilution of 1:100. The differences between 1 and 2 and 1 and 3 were significant. ($P < 0.001$). The differences between 2 and 4 and 2 and 6 were also significant ($P < 0.001$). Adhesion was significantly increased in five as compared with 4 and 6 ($P < 0.005$).

Table V. Inhibition of Binding of ^{125}I-labeled FVIII-VWF to Nonfibrillar Collagen Type III and Subendothelium in the Perfusion System

	^{125}I-FVIII-VWF bound (ng/cm^2)	
	Collagen type III	Subendothelium
Control	242±22 (6)	24±7 (5)
CLB-RAg 201	13±3 (6)	23±10 (6)
CLB-RAg 35	195±11 (4)	18±8 (5)

Nonfibrillar human collagen type III (20 μg/cm^2) or subendothelium were exposed to the recirculating perfusate in the perfusion systems (see Methods) for 5 min at a wall shear rate of 800 s^{-1}. The perfusate consisted of washed platelets in HAS (1.9 × 10^5/μl) that contained 10 μg/ml ^{125}I–FVIII-VWF. This FVIII-VWF was radiolabeled with the lactoperoxidase method (34). Monoclonal anti–FVIII-VWF antibodies CLB-RAg 201 and 35 ascites were added to this HAS in a dilution of 1:200. Finally, washed red cells were added to a hematocrit of 0.4. The perfusate was prewarmed for 5 min at 37°C. The amount of FVIII-VWF that was bound to the surfaces was calculated from the specific radioactivity and the radioactivity on the surfaces after perfusion. The values represent the mean±SD with the number of perfusions in parentheses.

201. A faint 60,000-mol wt band was caused by contamination with albumin; this band remained unchanged upon further digestion (Fig. 2 *A*). After 10 min of digestion (not shown) a 36,000-mol wt band was observed and several high molecular weight bands, the lowest of which had a M_r of 240,000. CLB-RAg 35 immunoprecipitated only the high molecular weight material and bands at 170,000 and 120,000 mol wt. CLB-RAg 201 immunoprecipitated only high molecular weight material. After 90 min, at least eight bands were observed in the total digest. CLB-RAg 35 immunoprecipitated a band at 120,000 mol wt. CLB-RAg 201 immunoprecipitated a band at 240,000 mol wt and faint bands at 58,000 and 48,000 mol wt. After 6 h of digestion (not shown), three prominent bands were observed at 170,000, 116,000, and 48,000 mol wt in the total sample. CLB-RAg 35 immunoprecipitated a 116-mol wt band. CLB-RAg 201 precipitated a band at 48,000 mol wt. After 24 h of digestion, the total sample contained two principal bands at 116,000 and at 48,000 mol wt. CLB-RAg 35 immunoprecipitated a band at 116,000 mol wt. CLB-RAg 201 immunoprecipitated a band at 48,000 mol wt.

The 116,000-mol wt fragment immunoprecipitated by CLB-RAg 35 and the 48,000-mol wt fragment immunoprecipitated by CLB-RAg 201 were analyzed further by nonreduced/reduced two-dimensional electrophoresis (Fig. 3, *A* and *B*). The 116,000-mol wt fragment consisted after reduction of fragments at 52,000–56,000 mol wt (double spot), 14,000 mol wt, and a minor fragment at 10,500 mol wt. The 48,000-mol wt fragment shifted after reduction to a slightly higher molecular weight.
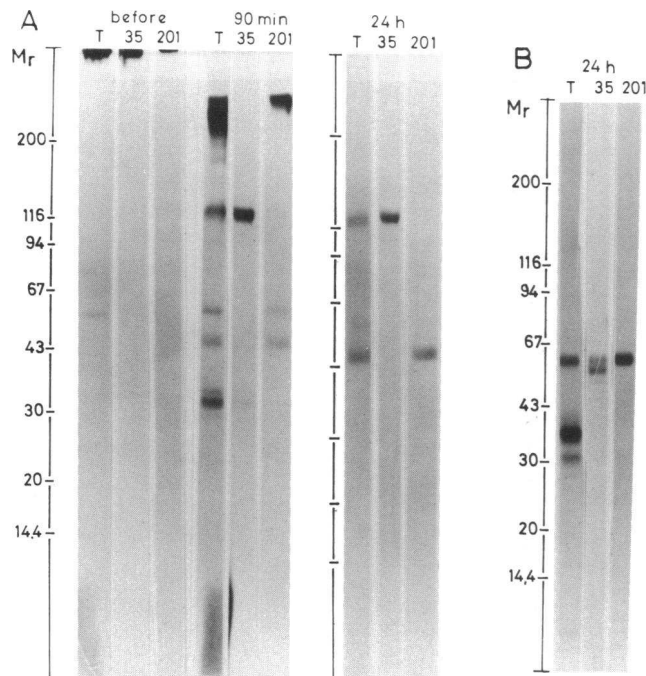
Figure 2. Autoradiogram of a SDS-PAA gel of tryptic fragments of VWF. (*A*) Nonreduced sample. 1.4 mg ^{125}I–FVIII-VWF in 3.5 ml Tris–HCl, 0.1 M NaCl, pH 7.0, was digested by addition of 90 μl TPCK-treated trypsin (166 U/ml in 10 mM HCl and 10 mM CaCl$_2$, active site titration 0.43 μmol/μmol). Samples of 0.65 ml were collected before and 10 min, 90 min, 6 h, and 24 h after trypsin addition and added to 40 μl of 0.25 M benzamidine and 0.25 M ε-aminocaproic acid with 0.4 mg/ml soybean trypsin inhibitor. For immunoprecipitation, 20 μl of preformed complex between CLB-RAg 35 or 201 and rabbit anti-mouse serum was added to 300 μl of digested ^{125}I–FVIII-VWF which had first been treated three times with complexes from normal mouse serum and rabbit anti-mouse serum (see Methods). The mixture was incubated under continuous rotation for 3 h or overnight at 4°C and centrifuged (15 min, 10,000 g, 4°C). The pellet was washed over a discontinuous sucrose gradient as described in Methods and subjected to SDS-electrophoresis on a PAA gradient of 3–30%. The figure shows the autoradiograms of the sample collected before and after 90 min and 24 h of digestion. The *left* lane marked T shows the total sample. The lane marked 35 shows the material immunoprecipitated with CLB-RAg 35; the lane marked 201 shows the material immunoprecipitated by CLB-RAg 201. (*B*) Reduced sample. ^{125}I–FVIII-VWF was digested for 24 h with trypsin as described in Methods and Fig. 2 *A*. The digest was reduced with 2 mm dithiothreitol at 37°C for 1 h, and alkylated with 4 mM iodoacetic acid at 37°C for 1 h. Immunoprecipitation was performed as described in Fig. 2 *A*. The samples were applied to a SDS-PAA 3–30% gradient gel without further reduction. The indication of the lanes are as in Fig. 2 *A*.

Partial reduction of tryptic fragments of FVIII-VWF followed by immunoprecipitation with CLB-RAg 35 and 201. In order to find out which of the subunits of the tryptic fragments

shown in Fig. 3 carried the epitope for CLB-RAg 35 or 201, reduction of tryptic fragments was performed before immunoprecipitation. The total sample before immunoprecipitation after 24 h showed main bands at 58,000, 36,000, and 32,000 mol wt (Fig. 2 *B*). The material immunoprecipitated by CLB-RAg 35 had bands at 56,000 and 52,000 mol wt, with a weak band at 49,000 mol wt after 6 and after 24 h of digestion. The material that was immunoprecipitated by CLB-RAg 201 showed a single band at 58,000 mol wt.

Discussion

Studies on structure-function relationship of FVIII-VWF have indicated that the ability to aggregate platelets in the presence of ristocetin is dependent on the degree of polymerization of FVIII-VWF (20–24). A relatively lower degree of polymerization may be required for support of platelet adhesion but a minimal size of above two million was still needed (25). It was therefore not surprising that only a low residual functional activity was observed after disruption of the structure of FVIII-VWF by limited proteolysis (26–28). This activity resided on a 400,000-mol wt fragment after plasmin digestion (26) and on a 116,000-mol wt fragment after trypsin digestion (28).

The recent availability of monoclonal antibodies directed against a limited number of precisely recognizable, antigenic determinants on a protein has provided an alternative approach for the study of structure-function relations. Such approach has been successful in the recognition of specific domains on plasminogen (29) and fibronectin (30).

We have used two monoclonal antibodies directed against discrete epitopes that were directly associated with the function of VWF. Previously we have shown that one antibody, CLB-RAg 35, completely blocks ristocetin-induced platelet aggregation, inhibits binding of FVIII-VWF to platelets in the presence of ristocetin, and inhibits platelet adherence to subendothelium at high shear rates (6, 7).

Another antibody described in this study, CLB-RAg 201, inhibits the interaction of FVIII-VWF with collagen fibrils and with collagen-coated surfaces in the perfusion system. Binding of FVIII-VWF to collagen fibrils has been described previously (31, 32). We have recently been able to demonstrate that preincubation of collagen types I and III with FVIII-VWF abolished the need for FVIII-VWF in the perfusion fluid for platelet adhesion.[2] Our present studies indicate that inhibition of interaction of FVIII-VWF with collagen by CLB-RAg 201 causes an almost complete impairment of adherence of blood platelets to collagen (Table III). We have previously shown that exposure of subendothelium to FVIII-VWF abolished the need for FVIII-VWF in plasma (5). In that study we found a correlation between the FVIII-VWF bound and the subsequent platelet adherence. The binding of FVIII-VWF that was observed in the present study (Table V) was in the same range. CLB-RAg 201 did not inhibit this binding but it caused partial inhibition of platelet adherence (Table III) and it completely
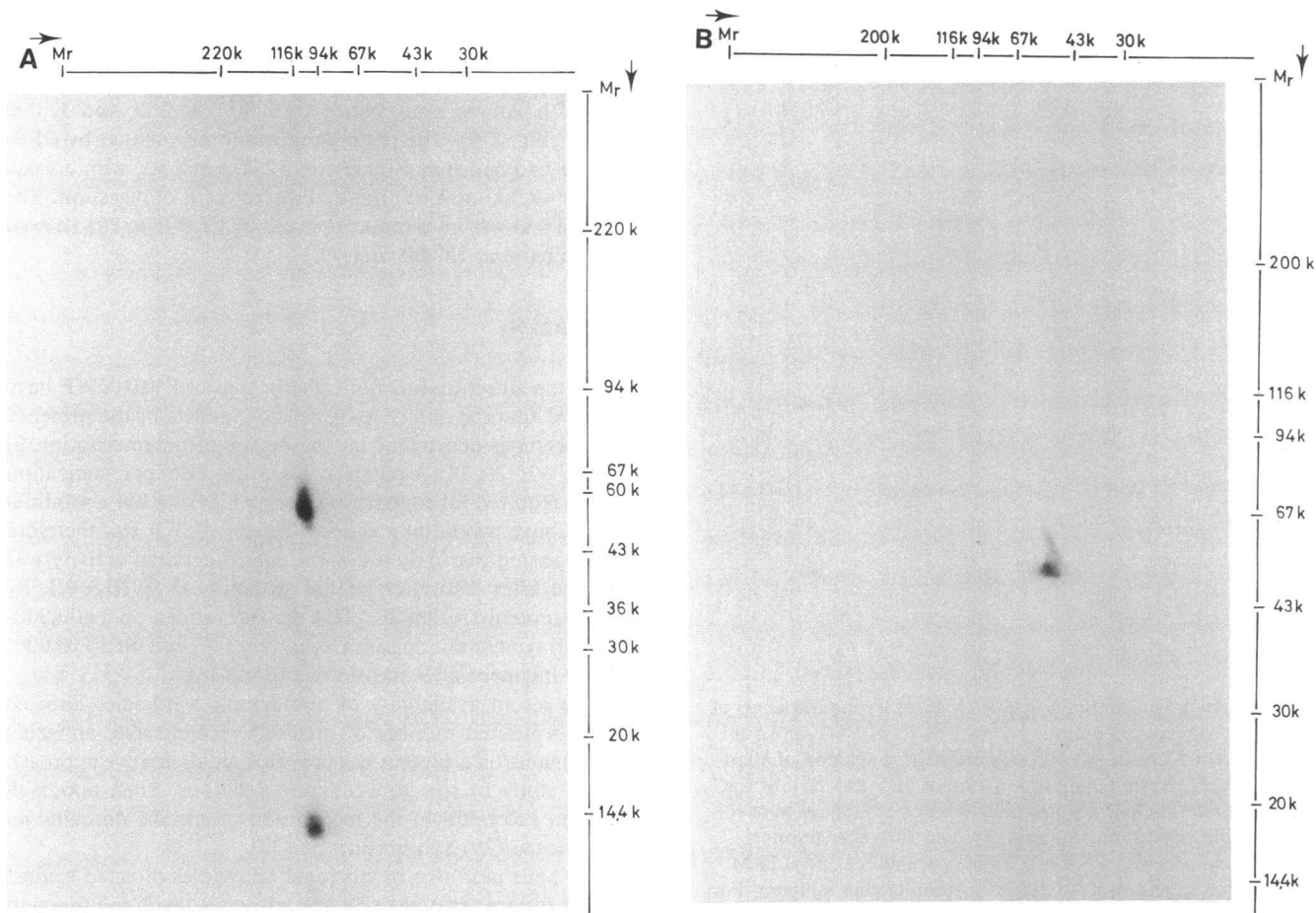
Figure 3. Autoradiogram of two-dimensional PAA electrophoresis of immunoprecipitate with CLB-RAg 35 (*A*) or 201 (*B*) of 24-h trypsin digest. Trypsin digestion and immunoprecipitation were performed as described in the legend of Fig. 2. The immunoprecipitates were electrophoresed on cylindrical gradient gels of 3–25% PAA. The gels were removed from the tube and reduced for 15 min at 60°C in 10% (v/v) glycerol, 2.3% (w/v) SDS, 0.0625 M Tris–HCl, and 5% w/v

β-mercaptoethanol. The gel was then placed on top of a 3–30% PAA-gradient slab gel and electrophoresed. Molecular weight markers were run in parallel in the first and second dimension. The arrows indicate the direction of electrophoresis. Electrophoresis in the first dimension was from left to right; in the second dimension from above downward.

inhibited the effect of plasma FVIII-VWF once the VWF already present in the subendothelium had been blocked by CLB-RAg 35 (Table IV). The most likely explanation of this apparent discrepancy is that only relatively few VWF molecules bind to sites in the subendothelium, which makes them reactive to platelets. Most other molecules bind to irrelevant sites and this binding is not influenced by CLB-RAg 201. The functional inhibition by CLB-RAg 201 suggests that similar domains on VWF are important for the relevant binding to subendothelium as to collagen, but these data do not necessarily indicate that collagen is the reactive substance in the subendothelium because the same domain on VWF may be involved in the binding to various components.

The monoclonal antibody CLB-RAg 35 was used for the identification of tryptic fragments that contained the domain on VWF that was involved in the interaction with platelets. Before digestion, the epitope was present on high molecular weight material. After 10 min, bands at 180,000, 120,000, and 36,000 mol wt were precipitated. The 36,000-mol wt band was the first fragment that was split off. It was immunoprecipitated, although weakly, by both CLB-RAg 35 and 201 after 10 min of digestion and by CLB-RAg 35 after 90 min, but not at later times. This fragment may have become immunoprecipitated due to an association by noncovalent bonds to epitope-containing peptides. The 120,000-mol wt fragment was the main fragment immunoprecipitated at 90 min. The fragment immunoprecipitated at 24 h had a slightly lower molecular weight, of 116,000. It consisted of peptides of

Table VI. Comparison of the Properties of CLB-RAg 35 and 201

	CLB-RAg 35	CLB-RAg 201
Inhibition of ristocetin-induced aggregation	+++	—
Inhibition of ristocetin-induced binding of VWF	+++	—
Inhibition of binding of VWF to collagen	—	+++
Inhibition of platelet adherence to subendothelium	Complete	Partial
Inhibition of platelet adherence to collagen-coated surfaces	Complete	Complete
Tryptic peptide immuno-precipitated	116,000 mol wt (52,000–56,000-mol wt subunit)	48,000 mol wt (58,000 mol wt after reduction)

56,000/52,000 and 14,000 mol wt. It is probably identical to the 116,000-mol wt fragment previously reported, which was found to contain some residual ristocetin cofactor activity (28). Immunoprecipitation of FVIII-VWF fragments that were first reduced showed that the 56,000/52,000-mol wt fragments contained the epitope for CLB-RAg 35.

Immunoprecipitation with CLB-RAg 201 precipitated high molecular weight material at 10 min, but not the 180,000 and 120,000-mol wt fragments. At 90 min, the epitope was localized on a 240,000-mol wt fragment and on 58,000 and 48,000-mol wt fragments. These latter two fragments may represent a single entity because the 48,000-mol wt fragment turns into a 58,000-mol wt fragment upon reduction and alkylation. The most likely explanation for this shift in apparent molecular weight is the presence of intrachain disulfide bonds.

The epitopes for CLB-RAg 35 and 201 are evidently located on totally different fragments. This was supported by radioimmunoassays which showed no competition, and by functional studies of the antibodies which showed no cross-reactivity. These data are summarized in Table VI.

More precise information on the localization of both domains on the intact VWF molecule can be obtained by limited proteolytic degradation and isolation of the respective fragments, followed by functional studies. Such studies are currently in progress.

Acknowledgments

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