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Alboaggregin-B: A New Platelet Agonist That Binds to Platelet Membrane Glycoprotein Ib†

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ABSTRACT: A new protein, called alboaggregin-B (AL-B), has been isolated from *Trimeresurus albolabris* venom by ion-exchange chromatography. It agglutinated platelets without the need for Ca^{2+} or any other cofactor. The purified protein showed an apparent molecular mass on SDS–PAGE and gel filtration of about 23 kDa under nonreducing conditions. Ristocetin did not alter the binding of AL-B to platelets or affect AL-B-induced platelet agglutination. Agglutinating activity was not dependent on either proteolytic or lectin-like activity in AL-B. Binding analysis showed that AL-B bound to platelets with high affinity (K_d = 13.6 ± 9.3 nM) at approximately 30 800 ± 14 300 binding sites per platelet. AL-B inhibited the binding of labeled bovine von Willebrand factor (vWF) to platelets. Monoclonal antibodies against the 45-kDa N-terminal domain of platelet glycoprotein Ib inhibited the binding both of AL-B and of bovine vWF to platelets, and also inhibited platelet agglutination induced by AL-B and bovine vWF. Specific removal of the N-terminal domain of GPIb by treatment of the plaelets with elastase or *Serratia marcescens* protease reduced the binding of labeled AL-B and bovine vWF to platelets and blocked platelet agglutination caused by both agonists. Monoclonal antibodies to glycoprotein IIb/IIIa, to bovine vWF, and to bovine serum albumin did not show any effect on the binding of AL-B to platelets. Our results indicate that the binding domain for AL-B on platelet GPIb is close to or identical with the one for vWF. This new protein may be a very useful tool for studying the interaction between platelets and vWF.

When blood vessels are damaged, von Willebrand factor (vWF)[1] is needed for platelets to recognize the damaged vascular endothelium and to form aggregates upon it. In vitro, the function of human vWF is assayed by its capacity to agglutinate platelets in the presence of the nonphysiological agonist ristocetin (Howard & Firkin, 1971). Bovine vWF agglutinates human platelets directly without the need for ristocetin (Forbes & Prentice, 1973). Agglutination of human platelets by bovine vWF, and by human vWF in the presence of ristocetin, is inhibited by antibodies to platelet membrane glycoprotein Ib (GPIb) (Tobelem et al., 1976). Bovine vWF and human vWF bind to platelets in a reversible manner and compete for binding to platelets (Suzuki et al., 1980).

Glycoprotein Ib is one of the major platelet membrane glycoproteins. There are approximately 25 000 copies of GPIb per platelet (Coller et al., 1983). This glycoprotein contains two disulfide-linked subunits, GPIbα (M_r 145K) and GPIbβ (M_r 24K), that are complexed with glycoprotein IX (M_r 22K) (Clemetson, 1985; Berndt et al., 1983, 1985). Two regions on GPIbα have been identified that are sensitive to proteolytic cleavage (Cooper et al., 1981; Berndt et al., 1986). One region is susceptible to hydrolysis by the platelet Ca^{2+}-dependent protease (calpain), and by *Serratia marcescens* protease. Cleavage at this site generates an N-terminal 135-kDa fragment termed glycocalicin, and a 25-kDa fragment that is disulfide-linked to GPIbβ. Another protease-sensitive region on GPIbα is located nearer to the N-terminal. Hydrolysis by elastase or by trypsin generates a soluble 45-kDa amino-terminal fragment, and a 100-kDa fragment that remains disulfide-linked to GPIbβ in the platelet membrane (Wicki & Clemetson, 1985). The external 45-kDa fragment may contain the binding sites for thrombin and vWF (Wicki & Clemetson, 1985; Kao et al., 1979; Handa et al., 1986). Proteolytic degradation of GPIb on platelet membranes is paralleled by a loss of vWF binding ability (Wicki & Clemetson, 1985; Coller, 1983) and a loss of responsiveness to bovine vWF and ristocetin (Cooper et al., 1977). Treatment of platelets with trypsin causes the GPIb–IX complex to be cleaved into four parts—an N-terminal domain (M_r 45K), a macroglycopeptide (M_r 85K), a remnant of the α-chain disulfide-linked to the β-subunit (M_r 41K), and undigested GPIX (M_r 22K) (Berndt et al., 1988; Du et al., 1987). Monoclonal antibodies AP1 and AK2, which are directed against the 45-kDa N-terminal

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[1] Abbreviations: AL-B, alboaggregin-B; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FWP, formalin-fixed washed platelet(s); GPIb, glycoprotein Ib; PRP, platelet-rich plasma; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; vWF, von Willebrand factor; WP, washed platelet(s).

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peptide of GPIbα, strongly inhibit the ristocetin-dependent binding of vWF to platelets. Antibodies AK1 (directed against an epitope on the membrane-associated region of the GPIb–IX complex), AK3 (directed against epitopes on the macroglycopeptide region of the GPIb–IX complex), and FMC 25 (directed against GPIX) do not inhibit vWF binding to GPIb (Berndt et al., 1988).

Botrocetin, a protein from the venom of the snake *Bothrops jararaca*, induces platelet agglutination by facilitating binding of vWF to GPIb, although botrocetin alone does not stimulate platelet agglutination directly (Sanders et al., 1988). Botrocetin appears to act in a two-step manner, first binding with vWF to form a complex, which then binds to GPIb, causing platelet agglutination (Read et al., 1989).

In this paper, we describe the purification and characterization of a new protein, AL-B (alboaggregin-B), which directly agglutinates platelets by binding to a site on platelet membrane GPIb close to or identical with the site for vWF binding.

Materials and Methods

Reagents. Trimeresurus albolabris venom was purchased from IBF Biotechnics, Inc. (Savage, MD). Elastase was from Elastin Products Co., Inc. (Pacific, MO). *Serratia marcescens* protease was purified according to the method described by Cooper et al. (1981). Ristocetin, α_1-antitrypsin, bovine serum albumin (BSA), *N*-ethylmaleimide, and soybean trypsin inhibitor were from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol (DTT) was from Eastman Kodak, Co. (Rochester, NY). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma Chemical Co.

Buffers. Phosphate-buffered saline contained 0.01 M phosphate, 0.15 M NaCl, and 0.02% NaN_3, pH 6.5. Tris-saline buffer contained 0.01 M Tris-HCl and 0.15 M NaCl, pH 7.4.

Determination of Protein Concentration. All protein concentrations were measured using the BCA protein assay (Pierce Chemical Co., Rockford, IL) on microtiter plates with bovine serum albumin as a standard. All concentrations described in this paper are final concentrations unless otherwise indicated.

Preparation of Platelet-Rich Plasma (PRP) and Washed Platelets (WP). Blood was obtained from healthy normal donors who denied having taken any medication for 2 weeks. Blood was collected into acid citrate/dextrose (1:8, v/v) and centrifuged at 150*g* for 15 min at room temperature to obtain PRP. Washed platelet suspensions were prepared according to the method of Mustard et al. (1972) and suspended in Tyrode's solution (pH 7.35) containing 3.5 mg/mL BSA.

Preparation of Formalin-Fixed Platelets. Fixed platelets were prepared as previously described (Kirby & Mills, 1975). Outdated platelet concentrates were a gift from the Penn-Jersey Regional Red Cross blood program. They were incubated with an equal volume of 2% formalin in Tris–saline buffer overnight at 4 °C. Platelets were then washed twice with Tris–saline buffer. The final platelet pellets were suspended in an equal volume of Tris–saline buffer containing BSA (20 mg/mL). Aliquots of these platelet suspensions were frozen at −80 °C. For each experiment, thawed platelets were diluted 1:10 with Tris–saline buffer without BSA. Fixed washed platelets were used for most of experiments unless otherwise indicated.

Purification of AL-B. Lyophilized *Trimeresurus albolabris* venom was dissolved in 0.05 M Tris buffer, pH 8.0, to a concentration of 20 mg/mL. Any undissolved material was removed by centrifugation at 12000*g* for 2 min followed by filtration through a 0.45-μm filter. This crude venom was fractionated by high-performance liquid chromatography on a Pharmacia Mono-Q column (5 × 50 mm) at a flow rate of 0.5 mL/min at room temperature. Proteins were eluted using a linear gradient of sodium chloride (0–0.7 M) in 0.05 M Tris, pH 8.0. Fractions were assayed for their absorbance at 226 nm and their ability to agglutinate formalin-fixed platelets. Pooled crude AL-B was dialyzed against 0.05 M Tris, pH 8.0, and subjected to rechromatography under the same conditions to remove trace contaminants. Pure AL-B was dialyzed against Tris–saline buffer and stored at 4 °C.

Platelet Agglutination. Agglutination of PRP, WP, and fixed platelets was performed according to the method described by Kirby (1982). Platelet agglutination was assayed at 37 °C with constant stirring at 1200 rpm in an aggregometer, and the extent of light transmission change was measured after 2 min.

Determination of Clotting Activity. Small amounts of clotting activity were routinely determined by mixing 10 μL of sample with 200 μL of human plasma in a microtiter plate. The rate of clot formation was determined by measuring the rate of the absorbance change at 660 nm on a microtiter plate reader at room temperature.

Determination of AL-B Molecular Weight. Gel filtration chromatography was performed on a column (0.8 × 15 cm) of Sephacryl S-200 Superfine (Pharmacia Fine Chemicals, Piscataway, NJ) preequilibrated with Tris–saline buffer. Concentrated AL-B, mixed with a trace of ^{125}I-AL-B, was loaded on the column at a flow rate of 0.1 mL/min. Fractions were assayed for radioactivity and tested for platelet agglutinating activity. SDS–PAGE was performed on 12% polyacrylamide gels according to the method of Fling and Gregerson (1986), in the presence or the absence of DTT. Low molecular weight standards (Sigma) were used as markers for molecular weight estimations.

Determination of the Isoelectric Point of AL-B. The isoelectric point was measured on polyacrylamide gels (4.8% acrylamide) with a preblended Ampholine solution, pH 3.5–9.5 (LKB Instruments, Inc., Rockville, MD). Purified AL-B (2.5 μg) was applied to the gel on rectangular pieces of filter paper (Whatman 3MM). The gel was prerun at a constant voltage of 350 V for 1 h and then was run at 900 V and 25 mA for 3 h. AL-B was detected by fixing the gel in sulfosalicylic acid and trichloroacetic acid, then staining with Coomassie Brilliant Blue R250 (0.5%), and destaining with 25% 2-propanol/10% acetic acid.

Purification of vWF. Bovine vWF was purified as described previously (Mascelli et al., 1986). The bovine vWF obtained was more than 90% homogeneous as revealed by SDS–PAGE using the method of Laemmli (1970).

Labeling of Proteins with Na^{125}I. Bovine vWF was labeled with Na^{125}I by the Iodogen procedure as described previously (Kirby, 1982). AL-B was labeled by the Chloramine T procedure. Purified AL-B (approximately 4.4 nmol in 0.5 mL of 0.05 M Tris buffer, pH 8.6) was mixed with 200 μCi of Na^{125}I. Iodination was initiated by the addition of chloramine T (0.9 μmol) and terminated after 10 s by the addition of sodium metabisulfite (1.2 μmol). Labeled protein was separated from free Na^{125}I on a Sephadex G-25 column. The extent of labeling was determined by precipitation of protein with 10% trichloroacetic acid. After purification, the labeled proteins showed less than 5% unbound ^{125}I. Labeled AL-B contained approximately (1–3) × 10^9 cpm/mg.

Binding of ^{125}I-AL-B to Platelets. Platelets were incubated at 37 °C for at least 10 min prior to doing binding studies. ^{125}I-AL-B was diluted in Tris–saline buffer containing 1

mg/mL BSA and preincubated with either buffer or the sample to be tested for inhibitory activity. The platelet suspension was added and incubated for 5 min at 37 °C. Platelets were separated from free ligand by centrifugation in an Eppendorf centrifuge equipped with a swinging-bucket rotor for 4 min at 12000*g*. Supernatants were removed with Pasteur pipets that had been drawn out to a fine capillary tip. Supernatants and pellets were counted in an Intertechnique γ counter to determine the amount of free and bound AL-B, respectively. Nonspecific binding (2–4% of total added radioactivity) was determined in the presence of excess unlabeled AL-B. Since nonspecific binding was such a small fraction of the total binding, correction for nonspecific binding was not generally done, except where exact quantitation was essential. Scatchard analysis was performed with the radioligand binding analysis programs (LIGAND) by G. A. McPherson, published and distributed by Elsevier–BIOSOFT (Cambridge, U.K.).

Biological Characteristics of AL-B. (a) Stability of AL-B to Heat Treatment. Aliquots of AL-B (100 μL) were heated at different temperatures from 22 to 75 °C for 20 min. At the end of heating, 20 μL of heated AL-B was tested for its ability to induce agglutination of fixed platelets.

(b) Effect of pH on AL-B Function. Concentrated fixed platelets were diluted 1:10 in a buffer of 0.025 M NaH_2PO_4, 0.025 M Tris, and 0.1 M NaCl, at different pHs. The final pH of the platelet suspensions was varied from 5.0 to 9.2. Platelet agglutination induced by AL-B and binding of ^{125}I-AL-B to platelets were then determined.

(c) Effect of Ionic Strength on AL-B Function. Concentrated fixed platelets were diluted in Tris–saline buffer containing varied concentrations of Na_2SO_4 (0.012–0.24 M). Platelet agglutination induced by AL-B and binding of ^{125}I-AL-B to platelets were then measured.

(d) Binding of ^{125}I-AL-B to Erythrocytes. Human erythrocytes were isolated by centrifugation of blood at 500*g* for 15 min. The erythrocytes were used as a 3% suspension in Tris–saline buffer. Binding of ^{125}I-AL-B to erythrocytes was measured by the same procedure as the binding of ^{125}I-AL-B to platelets. Binding of ^{125}I bovine vWF was employed as a negative control. Agglutination of erythrocytes by AL-B and by bovine vWF was determined macroscopically on a microtiter plate and compared to that induced by wheat germ lectin (83.3 μg/mL).

Monoclonal Antibodies. Antibody 6D1 was a gift from Dr. Barry Coller (Department of Medicine, SUNY—Stony Brook, NY). AP1 was kindly supplied by Dr. Thomas J. Kunicki (Milwaukee Blood Center, Milwaukee, WI). AK1, AK2, AK3, and FMC25 were gifts from Dr. Michael C. Berndt (Research Centre for Thrombosis and Cardiovascular Disease, University of Sydney, Sydney, Australia). Antibody G10 directed against GPIIb/IIIa was from Dr. Elizabeth Kornecki (Department of Anatomy and Cell Biology, State University of New York, Brooklyn, NY). Antibody IC3 to bovine serum albumin was a gift from Dr. David Benjamin (University of Virginia School of Medicine, Charlottesville, VA). Monoclonal antibodies to bovine vWF (Mascelli et al., 1986) were from our laboratory. Lyophilized ascites fluids containing monoclonal antibodies AP1, AK1, AK2, AK3, and FMC25 were dissolved in distilled water and the antibodies purified as described by Newman et al. (1985). Monoclonal antibody solutions were acidified with 0.06 M acetic acid to pH 4.0 under moderate stirring at room temperature. Caprylic acid was added in the volume ratio of 1:30 over a 5-min period and stirring continued for another 30 min. Precipitated material was removed by centrifugation at 5000*g* for 30 min. Supernatants were filtered through Whatman #1 paper and dialyzed against Tris–saline buffer.

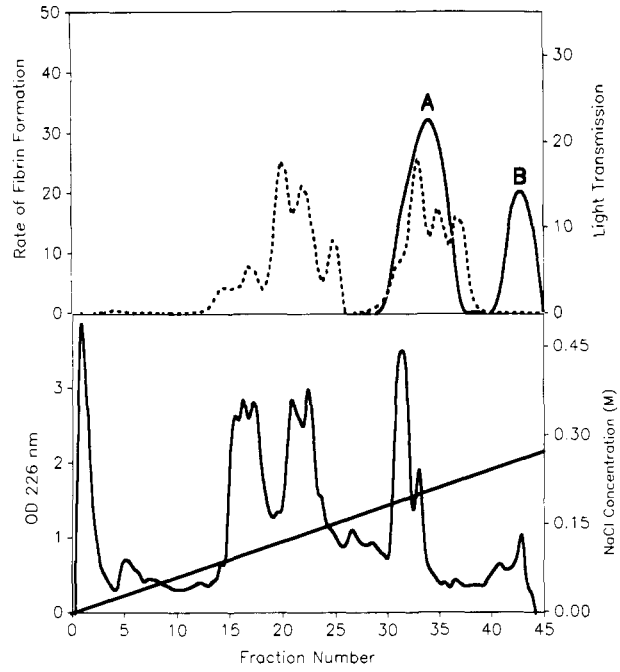

FIGURE 1: Purification of alboaggregins by chromatography on Mono-Q. Elution was performed with a 0–0.7 M NaCl gradient in 0.05 M Tris buffer, pH 8.0. Fractions were assayed for fibrinogen clotting activity (···) and for agglutination of fixed platelets (—) in the upper panel. Crude AL-A and AL-B eluted at about 0.25 and 0.28 M NaCl, respectively.

Proteolytic Digestion of Platelet Membrane Proteins. Fixed platelet suspensions (3×10^8/mL) were incubated with different concentrations of elastase for 5 min at 37 °C, followed by the addition of 10 μL of α_1-antitrypsin (5-fold excess w/w) to terminate the digestion. Digestion with *Serratia marcescens* protease (0.9 μg/mL) was at 37 °C for different periods of time and then stopped by the addition of EDTA (11 mM).

RESULTS

Purification and Physical Characteristics of AL-B. Chromatography of *T. albolabris* venom on Mono-Q (Figure 1) yielded two peaks of platelet agglutinating activity. The first peak, which we have termed alboaggregin-A (AL-A), was contaminated with a thrombin-like enzyme which could be removed by further chromatography. The second peak (alboaggregin-B, AL-B) had somewhat different physical properties from AL-A and is the primary focus of this paper.

Crude AL-B was eluted from the column at about 0.28 M NaCl. In most cases, crude AL-B was contaminated with a small amount of fibrinogen clotting activity which could be removed by rechromatography under the same conditions. AL-B comprised about 0.6% of the total venom protein. It retained its activity at 4 °C for up to several months.

On SDS–PAGE, AL-B showed an apparent molecular mass of about 23 kDa in the absence of DTT and two bands of 17 and 14 kDa in the presence of DTT (Figure 2). This suggests that the AL-B molecule is a dimer. Gel filtration chromatography on Sephacryl S-200 in the absence of DTT gave a single peak with an elution volume consistent with a globular protein of M_r 19K. Isoelectric focusing showed a single band of protein with a p*I* of 5.3.

AL-B agglutinated both fixed platelets and washed platelets in a dose-dependent manner (Figure 3A,B). Once platelets were maximally agglutinated by AL-B, addition of bovine vWF gave no further response (Figure 3C). AL-B also caused

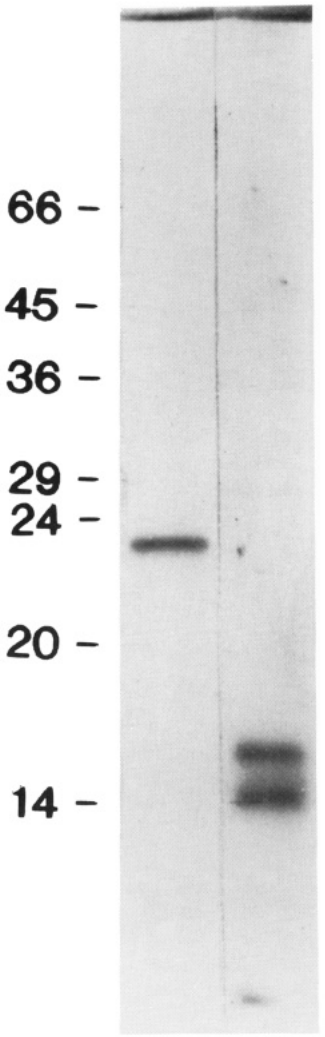

FIGURE 2: SDS–PAGE of AL-B. Samples were electrophoresed on a 12% polyacrylamide gel under nonreducing (NR) and reducing (R) conditions. The gel was stained with Coomassie Blue. The molecular mass standards were bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa).

aggregation in PRP without causing platelet shape change or the release reaction. Platelet shape change was estimated from the decreased transmittance and loss of base-line oscillation seen in the aggregometer, and by microscopic examination of the platelet suspension. Release of [^{14}C]serotonin was determined according to Ardlie and Han (1974). Platelet agglutination induced by AL-B was not inhibited by EDTA (11 mM), soybean trypsin inhibitor (0.5 mg/mL), *N*-ethylmaleimide (10 mM), or PGE_1 (0.35 μM). Heating of AL-B at 70 °C for 20 min caused complete loss of its platelet agglutinating activity.

Measurement of the Binding of ^{125}I-AL-B to Platelets. AL-B was labeled with ^{125}I without loss of its platelet agglutinating activity. Labeled AL-B comigrated with unlabeled AL-B on SDS–PAGE. Labeled AL-B bound directly to platelets in an unstirred system. The half-time for binding was less than 15 s, and the binding was more than 90% complete by 1 min (Figure 4). Binding was rapidly reversible as shown by the ability of excess unlabeled AL-B to remove previously bound AL-B. Binding to fixed platelets was saturable (Figure 5). The observed 2–4% nonspecific binding

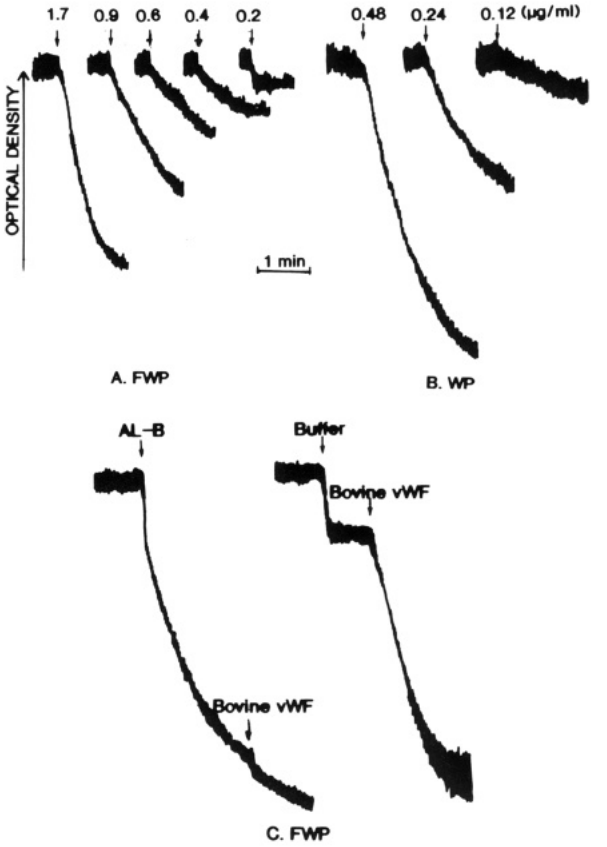

FIGURE 3: Agglutination of platelets by AL-B. AL-B was added to suspensions of (A) fixed washed platelets (FWP) or (B) washed platelets (WP) at the final concentrations (micrograms per milliliter) indicated. The platelet concentration was approximately 3×10^8 platelets/mL in each suspension. (C) Fixed washed platelets (3×10^8/mL) were agglutinated by the addition of AL-B (1.7 μg/mL) and/or bovine vWF (2.7 μg/mL) as indicated at the arrows.

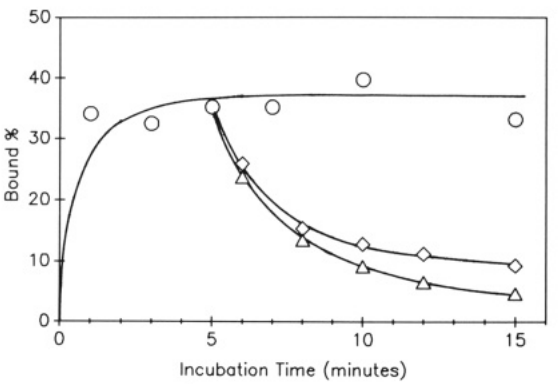

FIGURE 4: Time course of AL-B binding to platelets. ^{125}I-AL-B (25 ng/mL) was added to a suspension of fixed platelets (1.9×10^8/mL) and incubated at 37 °C for different periods of time. Unlabeled AL-B, 1.8 (◇) or 8.6 μg/mL (△), was added to duplicate suspensions after 5 min of incubation with ^{125}I-AL-B. Samples were centrifuged to determine the amount of labeled AL-B bound.

probably represented a combination of trapping of the AL-B within the platelet pellet and adherence of some of the labeled AL-B solution to the platelet tube, as well as some nonspecific binding of labeled AL-B to the platelets. The size of the platelet pellet from 0.37 mL of platelet suspension was approximately 3 μL which was only $^1/_{120}$th of the total assayed volume. There was no significant difference in the estimates of binding when platelets were separated by centrifugation through silicone oil; 50% of the maximal specific binding occurred at an AL-B concentration of about 0.3 μg/mL (Figure 5). Scatchard binding analysis revealed one class of

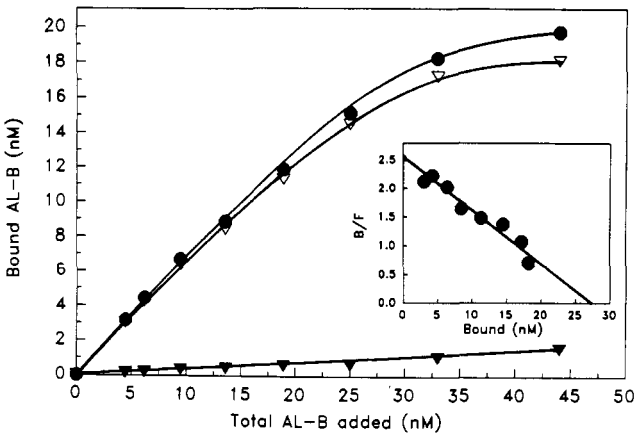

FIGURE 5: Saturable binding of ^{125}I-AL-B to formalin-fixed platelets. Labeled AL-B was diluted to different concentrations with Tris–saline buffer containing BSA (1 mg/mL). The assay mixture was composed of 300 μL of platelet suspension (3.2 × 10^8 platelets/mL), 20 μL of labeled AL-B at varied concentration (specific activity 3.2 × 10^8 cpm/mg), and 50 μL of buffer. Nonspecific binding was determined in the presence of 50 μL of unlabeled AL-B (9.5 μg/mL = 411 nM). After 5 min of incubation at 37 °C, the platelet mixtures were centrifuged to determine the amount of AL-B bound. Total binding (●), specific binding (△), and nonspecific binding (▼). (Inset) Scatchard plot of AL-B binding to fixed platelets. Data have been corrected for nonspecific binding. Points are averages of duplicate determinations. The results are representative of six different experiments performed with fixed platelets.

binding sites for AL-B on fixed platelets (inset in Figure 5). Estimates of K_d and the number of binding sites from six comparable experiments were 13.6 ± 9.3 nM and 30 800 ± 14 300 molecules per platelet, respectively.

Effects of Incubation Conditions on Agglutination and Binding. Variation of pH in the range of pH 5 to pH 9 had little effect either on ^{125}I-AL-B binding to platelets or on platelet agglutination. This is in contrast to agglutination induced by bovine vWF, which was quite pH-dependent (Kirby, 1982). Below pH 5 or above pH 9, platelet agglutination estimates were unreliable because the platelets tended to flocculate by themselves. Increasing the ionic strength of the buffers by addition of sodium sulfate inhibited AL-B binding and platelet agglutination (data not shown). The IC_{50} for sodium sulfate was approximately 0.08 M.

In order to determine whether AL-B functioned as a lectin, we examined the effects of eight different sugars (lactose, galactose, galactosamine, mannosamine, glucosamine, *N*-acetylglucosamine, methyl α-glucoside, and methyl α-mannoside) at concentrations up to 5 mg/mL. None of these sugars showed any effect on the binding of AL-B to platelets. AL-B did not agglutinate red blood cells, in contrast to several other platelet agglutinating agents isolated from snake venoms (Ogilvie et al., 1989), nor did ^{125}I-AL-B bind to red blood cells, suggesting that AL-B is probably not a lectin.

Competition between AL-B and vWF for Binding to Platelets. Unlabeled AL-B and bovine vWF were analyzed for their ability to compete with either labeled AL-B or labeled bovine vWF for binding to platelets. Figure 6A shows that it required approximately 0.6 μg/mL unlabeled AL-B for 50% inhibition of binding of ^{125}I-AL-B to platelets. In contrast, bovine vWF showed a very weak inhibition of the binding of ^{125}I-AL-B to platelets. Approximately 0.2 μg/mL unlabeled AL-B caused 50% inhibition of binding of vWF to platelets (Figure 6B), while it required about 10 μg/mL unlabeled bovine vWF for 50% inhibition of binding of labeled vWF. Human vWF (24 μg/mL) in the presence of ristocetin (1.5 mg/mL) showed only slight competition with labeled AL-B

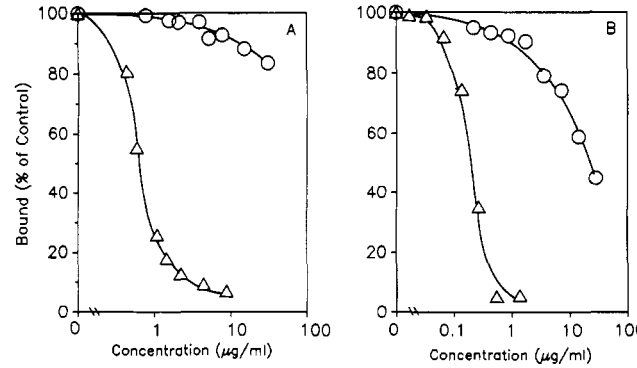

FIGURE 6: Competition between AL-B and bovine vWF for binding to platelets. (A) ^{125}I-AL-B (6.7 ng/mL) was mixed with varied concentrations of either unlabeled AL-B (△) or unlabeled bovine vWF (○). (B) ^{125}I-labeled bovine vWF (0.57 μg/mL) was mixed with either unlabeled AL-B (△) or unlabeled bovine vWF (○). Binding of radioactivity to fixed platelets (3.0 × 10^8 platelets/mL) was measured by centrifugation and expressed as a percent of the control value obtained in the absence of any unlabeled protein.

Table I[a]

	concentration (mg/mL)	binding of AL-B (% of control)
dextran sulfate	0.09	94.6
	0.93	62.8
heparin	0.05	86.2
	0.98	80.7
ristocetin	0.61	93.1
	1.63	101.9
	2.44	115.0

[a] These reagents were mixed with ^{125}I-AL-B and then formalin-fixed platelets (1.9 × 10^8 platelets/mL) added. The mixtures were incubated at 37 °C for 10 min. Samples were centrifuged to determine the amount of radioactivity bound to the platelets. The concentrations indicated are final concentrations of the reagents.

for binding. The binding of human vWF to platelets was not studied extensively, because of the artifacts which might arise due to the unknown mechanism of action of ristocetin and its tendency to bind nonspecifically to many proteins.

The presence of ristocetin (0.61–2.4 mg/mL) or porcine mucosal heparin (0.05–0.98 mg/mL) did not influence the binding of labeled AL-B to platelets (Table I). Dextran sulfate (molecular weight = 500 000) at up to 0.9 mg/mL also had little effect on AL-B binding (Table I).

Protease Susceptibility of AL-B Binding Sites. Cleavage of GPIb from platelets by *S. marcescens* protease has been reported by several authors (McGowan & Detwiler, 1985; Cooper et al., 1981). Increasing protease treatment caused progressive loss of GPIb with appearance of a major fragment (glycocalicin, 140 kDa) and was accompanied by loss of platelet susceptibility to agglutination by bovine vWF or by human vWF plus ristocetin (Cooper et al., 1977). Treatment of fixed platelets with *S. marcescens* protease (0.9 μg/mL) caused a parallel decrease in the binding of labeled AL-B and bovine vWF to the platelets (Figure 7A) and in the susceptibility of these platelets to agglutination (Figure 7B). Agglutination induced by bovine vWF seemed to be somewhat more sensitive to *Serratia* protease digestion than that induced by AL-B.

Elastase cleaves GPIb to release a 45-kDa peptide from the N-terminal of GPIb which has been proposed to contain the binding domain for vWF (McGowan & Detwiler, 1985; Cooper et al., 1981). Figure 8A shows that the binding of both ^{125}I-AL-B and ^{125}I-labeled bovine vWF to platelets was inhibited as a function of elastase treatment. Platelet agglu-

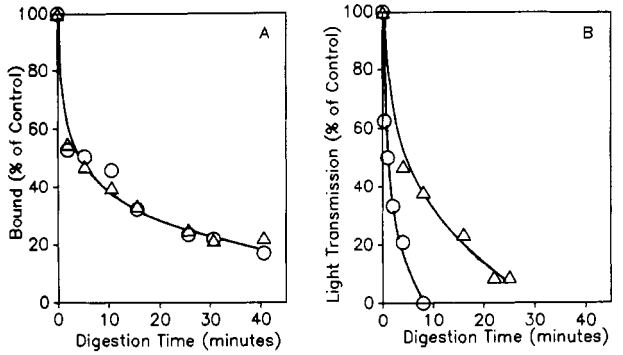

FIGURE 7: Effect of *S. marcescens* protease. Platelet suspensions (3.0×10^8 platelets/mL) were preincubated with *S. marcescens* protease (0.9 μg/mL) at 37 °C for different periods of time, and the digestions were then stopped by adding EDTA (final concentration 11 mM). (A) The binding of 3.45 ng/mL ^{125}I-AL-B (Δ) or of 0.37 μg/mL ^{125}I-labeled bovine vWF (O) was determined. (B) The extent of platelet agglutination after 2 min by 1.7 μg/mL AL-B (Δ) or 3.4 μg/mL bovine vWF (O) was measured. Data were plotted as the percent of untreated control. The data are representative of three replicated experiments.

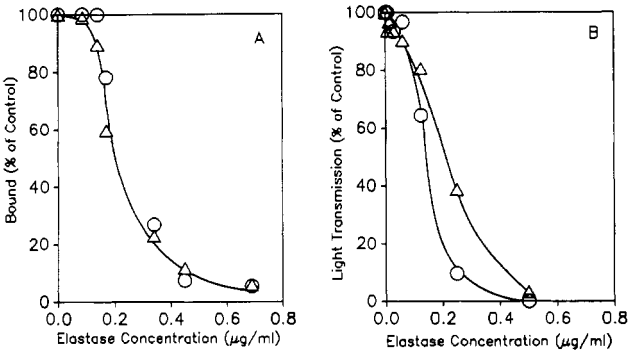

FIGURE 8: Effect of elastase. Elastase was diluted with Tris–saline buffer and added to fixed platelets at different concentrations (0.008–0.7 μg). Platelet suspensions (3×10^8/mL) were incubated with the elastase for 5 min at 37 °C. Digestion was stopped by the addition of a 5-fold excess of α_1-antitrypsin. Binding of labeled proteins (A) and platelet agglutination (B) were measured as described in Figure 9. (Δ) AL-B: (O) bovine vWF.

tination was inhibited in a similar manner (Figure 8B).

Effects of Monoclonal Antibodies. Monoclonal antibodies AP1 and AK2 against the 45-kDa N-terminal peptide region of GPIb strongly inhibited ^{125}I-AL-B and ^{125}I-labeled bovine vWF binding to platelets at essentially identical concentrations (Figure 9A,C). They also blocked platelet agglutination induced by AL-B and bovine vWF (Figure 9B,D). Antibody 6D1 had a similar effect as AP1 and AK2 (data not included). On the other hand, monoclonal antibodies AK3 and AK1 (directed against epitopes on the macroglycopeptide region of GPIb and the membrane-associated region of the GPIb–IX complex, respectively), FMC 25 (directed against GPIX), or G10 (directed against GPIIb/IIIa) had no effect on binding of AL-B or on platelet agglutination induced by AL-B. Control antibodies against BSA or bovine vWF also had no effect. Our results are in agreement with the observations of Berndt et al. (1988) that ristocetin-dependent binding of vWF to platelets and to beads coated with the GPIb–IX complex is inhibited by monoclonal antibodies directed against the 45-kDa N-terminal region of GPIb but not by monoclonal antibodies directed against other regions of the GPIb–IX complex.

Discussion

AL-B is distinct from other platelets agonists isolated from snake venoms. Teng et al. (1984) reported a protease, isolated

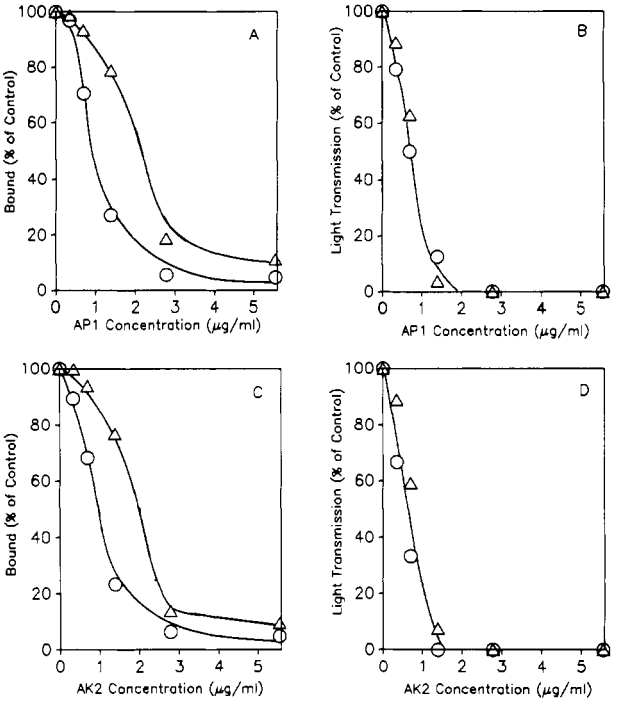

FIGURE 9: Effect of monoclonal antibodies. Fixed platelet suspensions (3.0×10^8 platelets/mL) were preincubated with the indicated concentrations of monoclonal antibodies AP1 (A and B) or AK2 (C and D) for 10 min at room temperature. The suspensions were then warmed to 37 °C, and binding of 3.24 ng/mL ^{125}I-AL-B (Δ) or 0.61 μg/mL ^{125}I-bovine vWF (O) to platelets was determined (A, C). Platelet agglutination (B, D) was measured with 1.25 μg/mL AL-B (Δ) or 1.74 μg/mL bovine vWF (O).

from *Vipera russellii* venom, which possessed procoagulant activity, activating factor X in the presence of calcium and leading to platelet aggregation in PRP. Thrombocytin, a thrombin-like enzyme from *Bothrups atrox* venom (Niewiarowski et al., 1979), and crotalocytin, from *Crotalus horridus horridus* venom (Schmaier & Colman, 1980), aggregate platelets directly, probably by a mechanism similar to thrombin. Purified AL-B did not contain any proteolytic or fibrin-clotting activity, and several proteolytic enzyme inhibitors including EDTA, soybean trypsin inhibitor, and *N*-ethylmaleimide, did not inhibit AL-B-induced platelet agglutination. This suggests that AL-B is not functioning as a protease to activate platelets.

Trigramin, a peptide purified from *Trimeresurus gramineus* venom, blocks the binding of fibrinogen and human vWF to the glycoprotein IIb/IIIa complex in thrombin-activated platelets (Huang et al., 1989). Albolabrin, isolated from *Trimeresurus albolabris* venom (Williams et al., 1990), showed a similar inhibitory activity in platelet aggregation. The biological activities of trigramin and albolabrin appear to depend upon the presence of an RGD sequence. The effects of 20 Australian snake venoms (19 elapid and a hydrophiid) and 4 crotalid venoms on human fresh and fixed platelets have been examined by Marshall and Herrmann (1989). All venoms except the hydrophiid venom, which required the presence of a plasma cofactor, directly caused fresh platelets to aggregate irreversibly. This was associated with degranulation as evidenced by electron microscopy. The response of fixed platelets to all of these venoms was much less, suggesting that metabolically active platelets were necessary for activation by these venoms.

Botrocetin, a purified protein from the venom of the South American pit viper *Bothrops jararaca*, has previously been shown to cause vWF-dependent agglutination of platelets

(Andrews et al., 1989). Both vWF and a 52/48-kDa dimeric fragment of vWF bound specifically and saturably to botrocetin-coupled beads. However, glycocalicin, a proteolytic fragment of the α chain of GPIb that contains the vWF binding domain, did not bind to immobilized botrocetin (Andrews et al., 1989). This agrees with the observation by Read et al. (1989) that botrocetin appears to act in a two-step manner, first binding with vWF to form a complex, which then binds to GPIb to cause agglutination. In our study, AL-B bound directly to platelets and produced agglutination without the need for vWF. Monoclonal antibodies to vWF did not inhibit AL-B activity. It is clear that AL-B agglutinates platelets in a manner distinct from that of botrocetin.

Ristocetin is an antibiotic which has been shown to induce platelet agglutination in the presence of human vWF (Howard & Firkin, 1971; Jenkins et al., 1979). It is not clear whether ristocetin promotes the binding of vWF to platelet GPIb by interacting with vWF or with the receptor. Ristocetin promoted the binding of labeled bovine vWF to platelets (Howard & Firkin, 1971), although it had no effect on bovine vWF-induced platelet agglutination (Kirby, 1982). In contrast, we found that ristocetin did not influence AL-B-induced platelet agglutination or the binding of labeled AL-B to platelets.

Five snake venom lectins have been reported by Ogilvie et al. (1989) which agglutinate red cells and stimulate the aggregation of human platelets. Lactose, a specific inhibitor of hemagglutination mediated by these lectins, and monoclonal antibodies to GPIIb/IIIa were potent inhibitors of this lectin-induced platelet aggregation (Ogilvie et al., 1989). Lectins from other sources such as lentil lectin (Ganguly & Fossett, 1980a), phytohemagglutinin (Majerus & Brodie, 1972), and wheat germ lectin (Ganguly & Fossett, 1980b) also bind to the platelet surface and can cause agglutination. Unlike these lectins, AL-B neither agglutinated red cells nor was its platelet agglutinating activity inhibited by sugars, suggesting that it is not functioning like a lectin.

Electrostatic effects may play a role in AL-B function, since salts of divalent ions inhibited AL-B binding and platelet agglutination. This is consistent with the observations of Cooper et al. (1977) and Kirby (1982) that platelet agglutination induced by bovine vWF and by ristocetin is ionic strength dependent (Kao et al., 1979). Polyanions, such as heparin and dextran sulfate, which strongly inhibited bovine vWF-induced platelet agglutination (Kirby & Mills, 1975), however, had no effect on AL-B-induced agglutination.

Unlabeled bovine vWF did not compete efficiently with AL-B for binding, but unlabeled AL-B strongly competed with labeled bovine vWF for binding to platelets (Figure 6), perhaps because of its much higher affinity. This suggests that the binding domain for AL-B on platelet GPIb is close to or identical with the one for vWF. Microscopic observation of platelets agglutinated by AL-B showed tiny clumps of about 20–30 platelets. Bovine vWF caused much more extensive platelet agglutination, forming significantly larger clumps than AL-B did. Once the binding sites on platelet GPIb were occupied by AL-B and subsequent platelet agglutination occurred, addition of bovine vWF did not produce further agglutination (Figure 3C). Platelet agglutination by bovine vWF involves the binding of multivalent vWF to multiple GPIb molecules on the platelet surface, allowing the formation of extensive interplatelet bridges. The molecular weight of AL-B is 10-fold less than that of one subunit of bovine vWF, so the formation of interplatelet bridges may be much less extensive, although the binding affinity of platelets for AL-B is much greater than for bovine vWF. Therefore, the extent of platelet agglutination probably depends on the extent of bridge formation.

GPIb is one of the major membrane glycoproteins on the platelet surface. Antibodies against the 45-kDa fragment from the N-terminal domain of GPIb inhibit the response of platelets to vWF (Wicki & Clemetson, 1985; Handa et al., 1986). We have tested the effects of five monoclonal antibodies to GPIb or to the GPIb–IX complex on the binding of AL-B or bovine vWF to platelets and on platelet agglutination induced by AL-B and bovine vWF. Those antibodies directed against the 45-kDa N-terminal domain of GPIb (6D1, AP1, and AK2) inhibited the binding and agglutinating activity of both AL-B and vWF, suggesting that the binding domain for AL-B is also in the 45-kDa N-terminal domain of GPIb. Additionally, since AL-B can compete efficiently with bovine vWF for binding to platelets, this suggests that bovine vWF and AL-B have either the same or closely adjacent binding sites on GPIb. Since AL-B is a strong inhibitor of the binding of vWF to platelets and it is a much weaker stimulator of platelet agglutination than vWF is, studies on the relationship between structure and function of AL-B could aid in designing peptide analogues which may retain the unique high-affinity binding capacity of AL-B, but may not cause platelet agglutination. These analogs could act as inhibitors of platelet adhesion and be valuable in preventing formation of platelet microthrombi in vivo.

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Registry No. vWF, 109319-16-6.

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Interaction of Microtubule-Associated Proteins with Microtubules: Yeast Lysyl- and Valyl-tRNA Synthetases and τ 218-235 Synthetic Peptide as Model Systems†

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ABSTRACT: The respective contributions of electrostatic interaction and specific sequence recognition in the binding of microtubule-associated proteins (MAPs) to microtubules have been studied, using as models yeast valyl- and lysyl-tRNA synthetases (VRS, KRS) that carry an exposed basic N-terminal domain, and a synthetic peptide reproducing the sequence 218-235 on τ protein, known to be part of the microtubule-binding site of MAPs. VRS and KRS bind to microtubules with a K_D in the 10^{-6} M range, and τ 218-235 binds with a K_D in the 10^{-4} M range. Binding of KRS and τ 218-235 is accompanied by stabilization and bundling of microtubules, without the intervention of an extraneous bundling protein. τ 218-235 binds to microtubules with a stoichiometry of 2 mol/mol of assembled tubulin dimer in agreement with the proposed binding sequences α[430-441] and β[422-434]. Binding stoichiometries of $2/\alpha\beta_S$ tubulin and $1/\alpha_S\beta_S$ tubulin were observed following partial or complete removal of the tubulin C-terminal regions by subtilisin, which localizes the site of subtilisin cleavage upstream residue α-441 and downstream residue β-434. Quantitative measurements show that binding of MAPs, KRS, VRS, and τ 218-235 is weakened but not abolished following subtilisin digestion of the C-terminus of tubulin, indicating that the binding site of MAPs is not restricted to the extreme C-terminus of tubulin.

Microtubules are dynamic cytoskeletal polymers involved in a variety of motile phenomena. Elucidating the mechanism of regulation of microtubule dynamics is a crucial issue in cell motility. The irreversible hydrolysis of tubulin-bound GTP that accompanies microtubule assembly is known to be at the origin of the dynamic instability behavior (Mitchison & Kirschner, 1984; Carlier, 1989). However, how dynamic instability is regulated in vivo is not understood yet. Accessory proteins (MAPs) that bind tightly to microtubules are known to stabilize the polymer. The most extensively studied of these MAPs are the brain proteins MAP-2 and τ (Olmsted, 1986;

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