Binding of a Novel 50-kilodalton Alboaggregin from *Trimeresurus albolabris* and Related Viper Venom Proteins to the Platelet Membrane Glycoprotein Ib-IX-V Complex. Effect on Platelet Aggregation and Glycoprotein Ib-Mediated Platelet Activation†

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Received March 22, 1996; Revised Manuscript Received June 20, 1996⊗

ABSTRACT: Binding of the multimeric adhesive glycoprotein, von Willebrand Factor (vWF), to the platelet membrane glycoprotein (GP) Ib-IX-V complex mediates platelet adhesion and initiates signal transduction leading to platelet activation. Recently described viper venom proteins that bind to the GP Ib α-chain and inhibit vWF binding provide novel probes for studying receptor function. We have purified a 50-kDa form of alboaggregin from the white-lipped tree viper (*Trimeresurus albolabris*) and two 25-kDa proteins, CHH-A and CHH-B, from the timber rattlesnake (*Crotalus horridus horridus*) in addition to a previously described 25-kDa alboaggregin and echicetin. Complete or partial amino acid sequencing of CHH-A, CHH-B, and 50-kDa alboaggregin and cross-reactivity of these proteins with an anti-botrocetin antiserum confirmed that they were disulfide-linked heterodimers or higher multimers of the C-type lectin protein family. These proteins, together with 25-kDa alboaggregin and echicetin, specifically bound to GP Ibα within the N-terminal peptide domain, His-1−Glu-282, and inhibited vWF binding with comparable IC_{50} values (∼0.2 μg/mL). However, cross-blocking studies between these structurally related proteins and anti-GP Ibα monoclonal antibodies demonstrated that the venom protein binding sites were not congruent. Further, the 50-kDa alboaggregin, but not the other venom proteins, potently induced platelet activation as assessed by dense granule serotonin release or elevation of cytosolic ionized calcium. Treatment of platelets with the 50-kDa alboaggregin was associated with activation of protein kinase C and tyrosine kinase(s), resulting in a platelet protein phosphorylation profile similar to that seen on shear-stress-induced vWF binding to platelets. These results suggest that the 50-kDa alboaggregin induces cytoplasmic signaling coincident with its binding to the GP Ib-IX-V complex and provides a potentially useful probe for studying the mechanism of vWF-dependent platelet activation.

The glycoprotein (GP)[1] Ib-IX-V complex is a constitutive platelet membrane receptor for the multimeric adhesive glycoprotein, von Willebrand Factor (vWF), an interaction responsible for platelet adhesion to the vascular subendothelium under shear flow at the onset of thrombosis and hemostasis (Booth et al., 1990; López, 1994; Weiss, 1995). vWF binding to the GP Ib-IX-V complex also induces Ca^{2+}-dependent platelet activation (Kroll et al., 1991, 1993; Razdan et al., 1994; Ikeda et al., 1993). vWF has been demonstrated to bind to a globular domain (His-1 to Glu-282) at the N-terminus of the GP Ib α-subunit (Handa et al., 1986; Berndt et al., 1988; Andrews et al., 1989b; Vicente et al., 1990). An anionic sequence within this domain, Tyr-276−Glu-282, containing three sulfated tyrosine residues has been shown to constitute an important motif that, at least in part, mediates vWF binding (Dong et al., 1994; Marchese et al., 1995; Ward et al., 1996). This motif of GP Ibα also mediates binding of α-thrombin to the GP Ib-IX-V complex, an association that facilitates platelet activation (Berndt et al., 1986; Coughlin et al., 1992; Marchese et al., 1995; Ward et al., 1996). Recently, proteins purified from viper venoms have been reported that specifically bind to GP Ibα and inhibit vWF binding, providing unique and highly selective probes for analyzing the structure−function relationships of this receptor (Peng et al., 1991, 1992; Scarborough et al., 1991).

Echicetin, an ∼26-kDa heterodimeric protein from the venom of the saw-scaled viper, *Echis carinatus*, binds to GP Ibα and inhibits vWF-dependent platelet agglutination but has no effect on ADP-induced platelet aggregation (Peng et al., 1993). Alboaggregin-B is an ∼23-kDa heterodimeric protein from the venom of the white-lipped tree viper, *Trimeresurus albolabris*, which binds to GP Ibα and ag-

† This work was supported by the National Health and Medical Research Council of Australia and by the National Institutes of Health, U.S.A. M.H.K. is supported by grants from the Research Service of the Department of Veterans' Affairs and is an Established Investigator of the American Heart Association (HL 18584).

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⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1996.

[1] Abbreviations: BSA, bovine serum albumin; Da, dalton; GP, glycoprotein; IC_{50}, concentration of inhibitor giving 50% inhibition; vWF, von Willebrand Factor.

S0006-2960(96)00704-0 CCC: $12.00

glutinates washed or fixed platelets in the absence of vWF or calcium ion (Peng et al., 1991). Alboaggregin-B also inhibits bovine vWF binding to platelets, and the specific binding of alboaggregin-B to platelets is blocked by monoclonal antibodies directed against the 45-kDa N-terminal peptide domain of GP Ibα (Peng et al., 1991). In a later study, two additional proteins with nonreduced molecular weights of 52 kDa (alboaggregin-A) and 121 kDa (alboaggregin-C) each composed of ∼14- to ∼18-kDa subunits were purified from two different commercial sources of *T. albolabris* venom (Peng et al., 1992). Both were functionally similar to alboaggregin-B. Another GP Ib-binding protein termed "jararaca GP Ib-BP" from the South American pit viper, *Bothrops jararaca*, inhibits vWF-dependent shear-induced platelet agglutination (Fujimura et al., 1995). Flavocetin-A and -B, 149- and 139-kDa proteins from *Trimeresurus flavoviridis*, respectively, also inhibited vWF binding to platelets (Taniuchi et al., 1995). Amino acid sequence analysis of echicetin, alboaggregin-B, and jararaca GP Ib-BP revealed that all three proteins were members of the C-type lectin family consisting of disulfide-linked heterodimers of ∼14-kDa α and β subunits (Yoshida et al., 1993; Peng et al., 1994; Fujimura et al., 1995; Usami et al., 1996). Other members of this family include the viper venom protein from *B. jararaca*, botrocetin, that instead of binding GP Ibα specifically binds to discrete amino acid sequences on vWF, activating it and allowing it to bind to the GP Ib-IX-V complex on platelets (Sugimoto et al., 1991; Berndt et al., 1992).

In the present study, a comparison of echicetin, a 25-kDa alboaggregin, two 25-kDa timber rattlesnake venom proteins from *Crotalus horridus horridus* (CHH-A and CHH-B), and a novel 50-kDa form of alboaggregin revealed that all of the proteins specifically bound to GP Ibα within the N-terminal peptide domain and blocked vWF binding. In marked contrast to the other GP Ib-binding proteins, however, the 50-kDa alboaggregin also potently induced platelet activation as assessed by dense granule serotonin release, elevation of cytosolic ionized calcium and activation of platelet protein kinase C and tyrosine kinase.

MATERIALS AND METHODS

Materials. Crude lyophilized venom from *E. carinatus* and *T. albolabris* was purchased from Sigma, St. Louis, MO, and *C. h. horridus* venom was from Miami Serpentarium Labs, Miami, FL. Heparin-Sepharose CL-6B and DEAE-Sephacel were obtained from Pharmacia (Uppsala, Sweden), and heparin-AffiGel and Bio-Gel HT hydroxylapatite were obtained from Bio-Rad (Richmond, CA). ^{14}C-labeled serotonin and Na^{125}I were purchased from Amersham, Castle Hill, Australia, and imipramine and human α-thrombin were from Sigma. Ristocetin was purchased from Boehringer Mannheim, Germany. The collagen-related peptide, Gly-Arg-Gly-Asp-Thr-Pro (GRGDTP), was purchased from Auspep, Parkville, Australia. Botrocetin was prepared as previously described (Andrews et al., 1989a). Human Factor VIII concentrate was a kind gift of the Commonwealth Serum Laboratories, Melbourne, Australia. vWF was purified from Factor VIII concentrate and radio-iodinated as previously described (Andrews et al., 1989a). The cobra venom metalloproteinase, mocarhagin, was purified from *Naja mocambique mocambique* venom (Sigma) as previously described (Ward et al., 1996).

Monoclonal Antibodies. All murine monoclonal antibodies were of the immunoglobulin G (IgG) class and methods for their purification and characterization have been described in detail elsewhere (Ruan et al., 1987). AK2 (Andrews et al., 1989a; Ward et al., 1996), SZ2 (Ruan et al., 1987; Ward et al., 1996), Hip1 (Berndt et al., 1988), VM16d (Mazurov et al., 1991; Ward et al., 1996), AP1 (Berndt et al., 1988), and WM23 (Berndt et al., 1988) are all directed against the GP Ib α-chain and were radio-iodinated where appropriate using the chloramine T method (Berndt et al., 1985a).

Purification of Echicetin, the 50- and 25-kDa Alboaggregins, CHH-A and CHH-B. Throughout the purification procedures for the various venom proteins, fractions were routinely assayed for their ability to either induce platelet agglutination or to inhibit ristocetin-dependent agglutination of citrated platelet-rich plasma. In addition, fractions were also analyzed by SDS−polyacrylamide gel electrophoresis (Laemmli, 1970; Booth et al., 1984). Echicetin was purified from *E. carinatus* venom by heparin-affinity chromatography. Lyophilized venom (10 mg) was dissolved in 4 mL of 0.01 M Tris, 0.15 M sodium chloride, pH 7.4 (TS buffer) and applied to a 10 × 1 cm heparin-Sepharose CL-6B column at 22 °C at a flow rate of 30 mL/h. Echicetin was retarded on the heparin-Sepharose column and eluted after the main flow-through peak. Peak fractions containing a 25-kDa species (nonreduced) and an ∼14-kDa doublet (reduced) were pooled, dialyzed into 5 mM sodium phosphate, pH 6.8, and loaded at 25 mL/h onto a 10 × 1 cm hydroxylapatite column equilibrated with 5 mM sodium phosphate, pH 6.8. Bound protein was eluted with a 200-mL linear 5−200 mM sodium phosphate gradient, pH 6.8, dialyzed exhaustively against TS buffer, and concentrated against Aquacide resin (Calbiochem, San Diego, CA). The alboaggregins were purified from the same batch of *T. albolabris* venom on the basis of the method of Peng et al. (1991). Lyophilized venom was dissolved in TS buffer (20 mg/4 mL) and loaded at 30 mL/h onto a 1.5 × 20 cm DEAE-Sephacel column equilibrated with TS buffer at 22 °C. A 50-kDa form and a 25-kDa form of alboaggregin that co-eluted from the DEAE-Sephacel column were dialyzed into TS buffer, loaded at 25 mL/h onto a 1 × 10 cm heparin-Affigel column and washed with TS buffer. The 50-kDa alboaggregin was retarded on the heparin column and eluted after the flow-through peak. The 25-kDa alboaggregin bound to heparin-Affigel and was eluted with 10 mM Tris, 500 mM sodium chloride, pH 7.4, and dialyzed into TS buffer. CHH-A and CHH-B were purified from *C. h. horridus* venom (0.5 g) that was dissolved in 7 mL of 0.5 M acetic acid and applied at 25 mL/h to a 100 × 2.5 cm Sephadex G50 column at 22 °C. Peak fractions were freeze-dried, reconstituted in 5 mL of 0.01 M ammonium acetate, pH 4.5, applied to a 13 × 2.2 cm carboxymethyl-Sephacel column, and eluted by a 0.01−0.5 M ammonium acetate, pH 6.5, gradient. Two major peaks designated CHH-A and CHH-B were obtained by subsequent HPLC using a semi-preparative C4 column eluted by a 15% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid to 70% (v/v) acetonitrile gradient. Protein concentrations of the purified venom proteins dialyzed into TS buffer were estimated using the BCA method and radio-iodinated where appropriate using Iodobeads, both according to the manufacturer's instructions (Pierce, Rockford, IL). Purified proteins were analyzed by

Western blotting with a rabbit polyclonal anti-botrocetin antibody raised against purified botrocetin using previously described procedures (Berndt et al., 1985a,b), and blots were visualized using horseradish peroxidase-coupled second antibody (Silenus, Hawthorn, Australia) and the ECL detection system (Amersham, Buckinghamshire, U.K.).

Reduction and Alkylation, Subunit Separation, and Endoprotease Digestion of CHH-B. CHH-B (1 mg) was dissolved in 1.5 mL of 0.25 M Tris, 6 M guanidinium hydrochloride, 20 mM EDTA, 20 mM dithiothreitol, pH 7.5, for 8 h at 25 °C, and ~25 mg of iodoacetamide was then added for an additional 8 h. The reaction mixture was quenched with 1 mL of 1% (v/v) trifluoroacetic acid and loaded onto a C4 reverse-phase HPLC column (Vydac, 214TP510), and the α- and β-chains were separated using an 8%−40% acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid for 30 min then to 60% acetonitrile in 10 min. The earlier eluting subunit, CHH-B β-chain, and the later eluting subunit, CHH-B α-chain, where individually collected and dried. Portions of the carboxyamidomethylated subunits were subjected to N-terminal Edman degradation for sequence analysis, and the remainder was used to generate overlapping peptides. Endoprotease digestion of the subunits (100−200 μg) was performed for 12−18 h at 37 °C in 50 mM Tris, pH 8.0, for endoprotease Lys-C (Boehringer Mannheim) at an enzyme to substrate ratio of 1:100, and in 10 mM Tris, pH 8.0, for endoprotease Asp-N (Boehringer Mannheim) at an enzyme to substrate ratio of 1:100−200. Digestions were terminated by addition of 1 μL of trifluoroacetic acid and directly loaded onto a 0.46 × 25 cm C18 reverse-phase HPLC column (Vydac, 201TP5415) equilibrated in 2% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid, and the eluate was monitored at 214 nm. Peptide fragments were eluted with a 30-min 2%−32% (v/v) acetonitrile gradient followed by a 20-min 32%−60% (v/v) acetonitrile gradient. Fractions containing peptide were manually collected into 1.5-mL Eppendorf tubes, dried in a Savant Speed Vac concentrator, and stored at −10 °C prior to sequence analysis.

Sequence Analysis. Direct amino acid sequencing of carboxyamidomethylated subunits of CHH-B (1−5 nmol) was performed using an Applied Biosystems model 473A protein sequenator using the program and reagents supplied by the manufacturer. Released phenylthiohydantoin-amino acids were identified with the on-line HPLC system. N-terminal sequencing of 50-kDa alboaggregin was performed as previously described (Ward et al., 1996).

Platelet Aggregation. Platelet aggregation studies were performed in a Whole Blood Lumiaggregometer (Chrono-Log, Havertown, PA) stirred at 900 rpm using citrated platelet-rich plasma or washed platelets at 5×10^8/mL in TS buffer or Tyrodes' solution as previously described (Booth et al., 1984). Tyrodes' solution contained 0.1% (w/v) glucose, 0.8% (w/v) sodium chloride, 0.1% (w/v) sodium bicarbonate, 0.02% (w/v) potassium chloride, 0.005% (w/v) sodium dihydrogen orthophosphate, 0.02% (w/v) calcium chloride, and 0.01% (w/v) magnesium chloride, pH 7.4. Viper venom proteins were added alone or were pre-incubated with platelets for 3 min at 37 °C prior to the addition of other agonists [1.5 mg of ristocetin/mL, 50 μg of botrocetin/mL, 10% (v/v) bovine plasma (as a source of bovine vWF), 5 μM ADP (Chrono-Par, Havertown, PA), 2 μg of equine tendon collagen/mL (Hormon-Chemie, München), or 0.05 units of α-thrombin/mL]. In some assays, monoclonal antibodies at a final concentration of 50 μg/mL or mocarhagin at 10 μg/mL were pre-incubated with the platelets for 5 or 6 min, respectively, at 37 °C prior to the addition of the 50-kDa alboaggregin. Other assays included EDTA at a final concentration of 10 mM or collagen-related peptide (GRGDTP) at a final concentration of 0.1, 0.5, or 1.0 mM.

Binding of ^{125}I-Labeled vWF to Platelets. The effect of venom proteins on the binding of ^{125}I-labeled vWF to platelets in the presence of 1 mg of ristocetin/mL or 50 μg of botrocetin/mL as vWF modulators was measured using a previously described assay (Andrews et al., 1989a; Berndt et al., 1988, 1992). Viper venom proteins were incubated with washed platelets (5×10^8/mL) in TS buffer containing 0.1% (w/v) BSA for 5 min at 22 °C, prior to the addition of 1 μg of ^{125}I-labeled vWF/mL. After 30 min, samples were centrifuged at 8750*g* for 1 min, and label associated with the pellet was measured in a γ-counter after aspiration of the supernatant.

Binding of ^{125}I-Labeled 50-kDa Alboaggregin to Platelets. Binding of ^{125}I-labeled purified 50-kDa alboaggregin to platelets was performed using the same method described for ^{125}I-labeled vWF binding (Andrews et al., 1989a; Berndt et al., 1988, 1992). ^{125}I-labeled 50-kDa alboaggregin (0.1−2.0 μg/mL) was incubated with 5×10^7 washed platelets/mL in TS buffer containing 0.1% (w/v) BSA for 30 min at 22 °C and centrifuged at 8750*g* for 1 min, and label associated with the pellet was measured in a γ-counter after aspiration of the supernatant. Specific binding was calculated from total binding by substracting nonspecific binding measured in a parallel assay containing a 50-fold excess of unlabeled 50-kDa alboaggregin. Some assays also included unlabeled venom proteins (0.1−10 μg/mL) or monoclonal antibodies (50 μg/mL). Pretreatment of platelets in other assays with the cobra venom metalloproteinase, mocarhagin, for 6 min at 22 °C in the presence of either 10 mM calcium chloride or 10 mM EDTA prior to the addition of ^{125}I-labeled 50-kDa alboaggregin was based on previous studies (Ward et al., 1996).

Binding of ^{125}I-Labeled Monoclonal Antibodies to Platelets. Specific binding of ^{125}I-labeled anti-GP Ibα monoclonal antibodies, AK2, SZ2, and WM23, to washed platelets was performed as described elsewhere (Berndt et al., 1988; Ward et al., 1996). Briefly, ^{125}I-labeled antibody (1 μg/mL) was incubated with 5×10^7 washed platelets/mL in TS buffer containing 0.1% (w/v) BSA for 30 min at 22 °C. After incubation, duplicate samples were centrifuged at 8750*g* for 3 min, and ^{125}I-labeled antibody associated with the pellet was measured in a γ-counter. Nonspecific binding was measured in the presence of 50 μg of unlabeled antibody/mL in a parallel assay. To test the ability of venom proteins to inhibit binding of monoclonal antibodies to platelets, some assays included purified proteins at concentrations of 0.1−10 μg/mL.

Measurement of Serotonin Release and Elevation of Cytosolic Ca^{2+}. Dense granule secretion from ^{14}C-labeled serotonin-loaded platelets was measured essentially as described by van Willigen et al. (1994). Platelet-rich plasma prepared from ACD-anticoagulated blood (Booth et al., 1984) was incubated with ^{14}C-labeled serotonin (50 μCi/mL, 55 mCi/mmol) for 30 min at 37 °C. The serotonin-labeled platelets were then washed twice in citrate−glucose−saline

buffer (Booth et al., 1984) containing 20 ng of PGE_1/mL and resuspended at 5×10^8/mL in Tyrodes' solution containing 2.5 μM imipramine. To test the effect of venom proteins or α-thrombin on platelet secretion, agonists were incubated with 300 μL of labeled platelets at 37 °C, which were then stirred at 900 rpm for 5 min in an aggregometer. To stop the release reaction, 50 μL of 33% (v/v) formaldehyde, 150 mM sodium chloride, was added to each sample and the mixture stirred in the aggregometer for an additional 1 min. The samples were transferred to microfuge tubes, and the platelets were pelleted by centrifugation at 8750*g* for 1 min. ^{14}C-labeled serotonin release was quantitated by taking duplicate 100 μL aliquots of the supernatant, adding each to 3 mL of scintillation fluid and counting in a β-counter. Changes of cytosolic ionized calcium in fura 2-loaded platelets stirred at 900 rpm in Tyrodes' solution (5×10^8/mL) at 37 °C was measured as previously described (Kroll et al., 1991).

Effect of the 50-kDa Alboaggregin on Platelet Protein Phosphorylation. The incorporation of [^{32}P]orthophosphate (DuPont-New England Nuclear) into platelet proteins was assessed by previously reported methods (Kroll et al., 1993). Stirred ^{32}P-labeled platelets were either untreated or treated with 1 unit of α-thrombin/mL, 2 μg of 50-kDa alboaggregin/mL, or 2 μg of echicetin/mL for 1 min at 37 °C. Samples were quenched by addition of electrophoresis sample buffer, electrophoresed on 6%−16% SDS−polyacrylamide gels, and analyzed by autoradiography. Activation of tyrosine kinase was determined by immunoblotting of platelet samples with the anti-tyrosine phosphate monoclonal antibody, 4G10 (UBI, Lake Placid, NY), essentially as previously described (Razdan et al., 1994). Stirred platelets either untreated or treated with 1 unit of α-thrombin/mL, 2 μg of 50-kDa alboaggregin/mL, or 2 μg of echicetin/mL for 1 min at 37 °C were quenched by addition of electrophoresis sample buffer, electrophoresed on 6%−16% SDS−polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and immunoblotted with 4G10. Blots were visualized using an alkaline phosphatase-conjugated second antibody and a UBI detection kit (Razdan et al., 1994).

RESULTS

A number of viper venom proteins have recently been described that specifically bind to the GP Ib-IX-V complex on platelets and potently inhibit vWF binding (Peng et al., 1991, 1992, 1993; Fujimura et al., 1995; Taniuchi et al., 1995). We have purified a number of these proteins including a novel 50-kDa alboaggregin from the white-lipped tree viper, *T. albolabris*, and two 25-kDa proteins from the timber rattlesnake, *C. h. horridus*, in order to map their binding sites and study their effect on platelet activation. Two forms of alboaggregin were purified from one batch of *T. albolabris* venom. One form was ∼25-kDa (nonreduced) on SDS−polyacrylamide gels, reducing to an ∼14-kDa doublet (not shown), suggesting that, like alboaggregin-B (Peng et al., 1991), it was a disulfide-linked heterodimer. Another form of alboaggregin appeared as an ∼50-kDa species under nonreducing conditions, and a triplet of bands of ∼14 kDa appeared under reducing conditions (Figure 1). Two distinct rattlesnake proteins, termed CHH-A and CHH-B, appeared on SDS−polyacrylamide gels as ∼25-kDa species nonreduced and as a doublet of ∼14 kDa, reduced (Scarborough et al., 1991; this study, data not shown). The

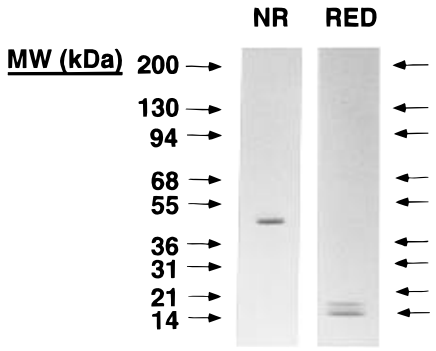

FIGURE 1: 5%−20% SDS−polyacrylamide gels stained with Coomassie Blue of the purified 50-kDa form of alboaggregin electrophoresed under nonreducing (NR) and reducing (RED) conditions. Molecular weight markers (arrows) in order of decreasing molecular weight are myosin (200 kDa), β-galactosidase (130 kDa), phosphorylase B (94 kDa), bovine serum albumin (68 kDa), glutamate dehydrogenase (55 kDa), lactate dehydrogenase (36 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa).

complete amino acid sequence of CHH-B was determined (Figure 2). Although CHH-A was not completely sequenced, the N-terminal sequences determined for both chains of CHH-A up to 25 residues were identical to CHH-B (not shown). The amino acid sequences of both chains of CHH-B are compared with the α- and β-chains of botrocetin (Usami et al., 1993) and the β-chain of echicetin (Peng et al., 1994) in Figure 3A, and compared with the α- and β-chains of alboaggregin-B (Usami et al., 1996) in Figure 3B. The β-chain of CHH-B displays 47% identity to the β-chain of echicetin, 49% identity to the β-chain of botrocetin, and 54% identity to the β-chain of alboaggregin-B. The α-chain of CHH-B displays 38% identity to the α-chain of botrocetin and 47% identity to the α-chain of alboaggregin-B. Although there was insufficient material for detailed structural analysis of the 50-kDa alboaggregin, three lines of evidence suggested that the 50-kDa alboaggregin was a member of the C-type lectin family. Firstly, the molecular mass of the subunits on SDS−polyacrylamide gels under reducing conditions (∼14-kDa; Figure 1) was consistent with other members of the C-type lectin family (Peng et al., 1991, 1993, 1994; Fujimura et al., 1995; Yoshida et al., 1993; Taniuchi et al., 1995; this study). Other alboaggregins described by Peng et al. (1992) with molecular masses under nonreducing conditions of 52-kDa (alboaggregin-A) and 121-kDa (alboaggregin-C) were composed of ∼14−18-kDa subunits, clearly demonstrating that alboaggregin subunits have the capacity to form higher multimers than the 23-kDa heterodimer, alboaggregin-B (Peng et al., 1991). Secondly, the major N-terminal sequence obtained for 50-kDa alboaggregin up to 18 residues (Figure 3C) was comparable to the published N-terminal sequences of alboaggregin-B and related proteins (Usami et al., 1993, 1996; Peng et al., 1994; this study). Finally, CHH-A, CHH-B, and the 50-kDa alboaggregin were all immunoreactive toward a rabbit polyclonal anti-botrocetin antiserum on Western blots (Figure 4), confirming that all of these proteins were members of the C-type lectin family.

CHH-A, CHH-B, and the 25-kDa alboaggregin inhibited ristocetin- and/or bovine vWF-induced agglutination in platelet-rich plasma (Figure 5A and B, respectively). Similarly, Peng et al. (1991, 1992, 1993) previously reported that echicetin and an ∼23-kDa alboaggregin-B inhibited vWF

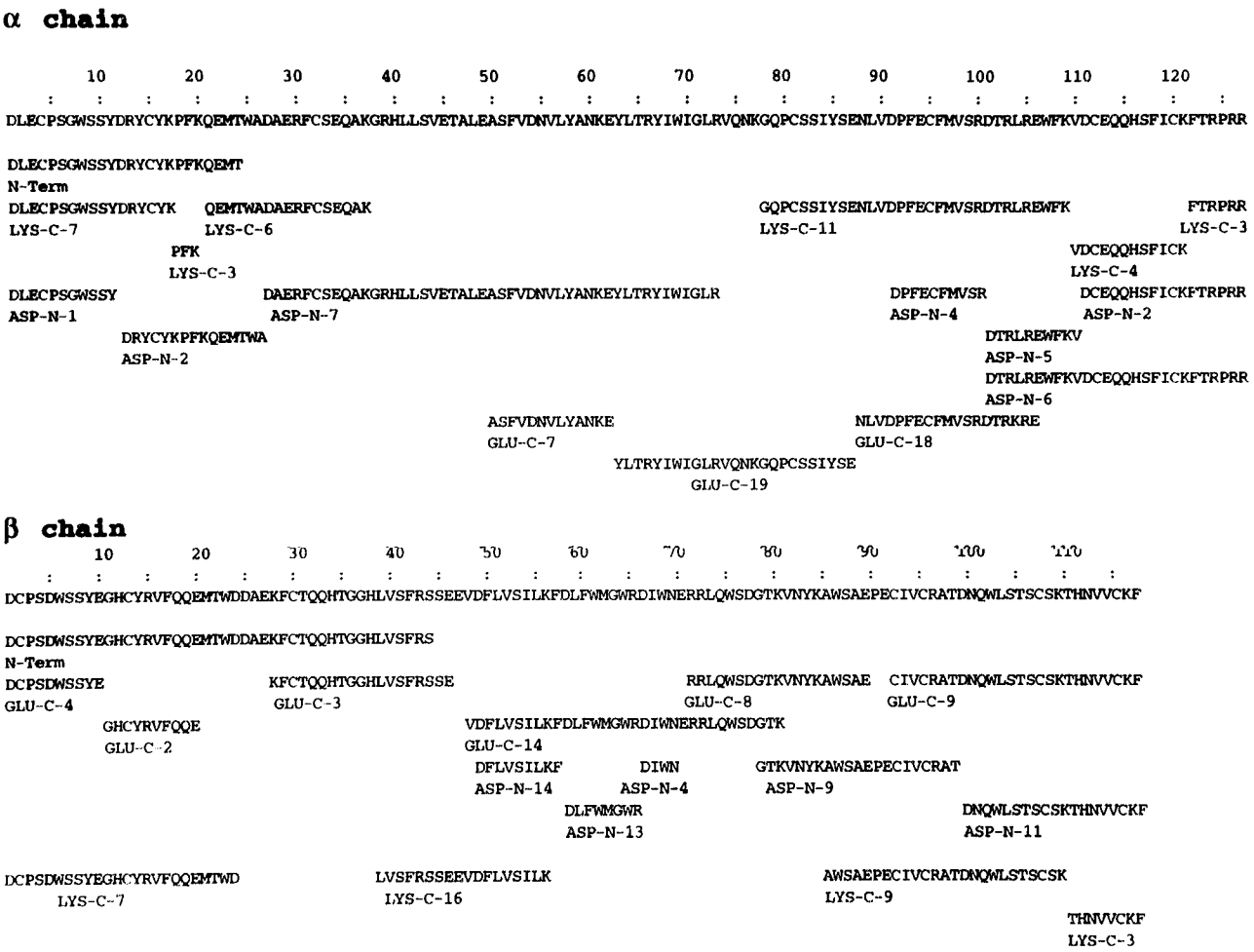

FIGURE 2: Amino acid sequence analysis of the α- and *β*-chains of CHH-B, showing the sequence of endoprotease Lys-C, Glu-C, and Asp-N fragments. The number of Edman cycles obtained for each of the N-terminal sequence determinations is indicated.

binding to platelets. In contrast, the 50-kDa alboaggregin was found to potently induce aggregation of platelet-rich plasma (Figure 5C). This aggregation was inhibitable by EDTA (Figure 5C) and by the collagen-related RGD-containing peptide, GRGDTP (Figure 5D). Pretreatment of platelet-rich plasma with the cobra venom metalloproteinase, mocarhagin, also abolished the 50-kDa alboaggregin-induced aggregation (Figure 5C). Mocarhagin selectively cleaves GP Ibα between residues Glu-282 and Asp-283 as the sole detectable substrate on the platelet surface and releases the N-terminal His-1−Glu-282 fragment (Ward et al., 1996). Aggregation of washed platelets in the presence of 50-kDa alboaggregin occurred in the absence of added vWF, and was inhibitable by the anti-GP Ibα monoclonal antibodies, AK2 and AP1, directed against the N-terminal peptide domain, His-1−Leu-275 (not shown). SZ2, another anti-GP Ibα monoclonal antibody directed against residues Tyr-276−Glu-282, caused only partial inhibition. The anti-GP Ibα macroglycopeptide domain monoclonal antibody, WM23, had no effect. Neither the 50-kDa alboaggregin, CHH-A, or CHH-B induced ^{125}I-labeled vWF binding to washed platelets (data not shown); however, both the 50- and the 25-kDa alboaggregins inhibited ristocetin-dependent binding of vWF to washed platelets with IC_{50} values of ∼0.2 μg/mL (data not shown). CHH-A and CHH-B gave qualitatively similar results (not shown).

^{125}I-labeled, purified 50-kDa alboaggregin bound to washed platelets in a specific and saturable manner (Figure 6). Analysis of binding by Scatchard analysis (Figure 6, inset) gave an apparent K_d of ∼3.0 nM. Binding was not affected by the presence of 10 mM EDTA (Figure 7). Three lines of evidence demonstrated that ^{125}I-labeled alboaggregin bound specifically to the N-terminal peptide domain of GP Ibα on platelets. Firstly, binding was completely blocked by the monoclonal antibodies AK2, Hip1, and AP1, all directed against the N-terminal peptide domain of GP Ibα (His-1−Leu-275), but not by another anti-GP Ibα monoclonal antibody, WM23, directed against the macroglycopeptide domain (Figure 7). Two other anti-GP Ibα monoclonal antibodies, VM16d and SZ2, with epitopes within His-1−Leu-275 and Tyr-276−Glu-282, respectively, were partially inhibitory (Figure 7). Secondly, pretreatment of platelets with mocarhagin, which selectively cleaves GP Ibα between residues Glu-282 and Asp-283 (Ward et al., 1996), abolished the ability of the platelets to bind ^{125}I-labeled 50-kDa alboaggregin (Figure 7). Treating platelets with mocarhagin in the presence of excess EDTA, which inhibits the activity of the protease (Ward et al., 1996), had essentially no effect on alboaggregin binding (not shown). Thirdly, binding of ^{125}I-labeled 50-kDa alboaggregin to platelets was competitively inhibited by an excess of unlabeled 50-kDa alboaggregin (Figure 8A), 25-kDa alboaggregin (Figure 8B, open circles), echicetin (Figure 8B, solid circles), and both CHH-A and CHH-B (Figure 8C, open and solid triangles, respectively), all with similar IC_{50} values in the range 0.2−0.5 μg/mL. These combined results demonstrate that all of the

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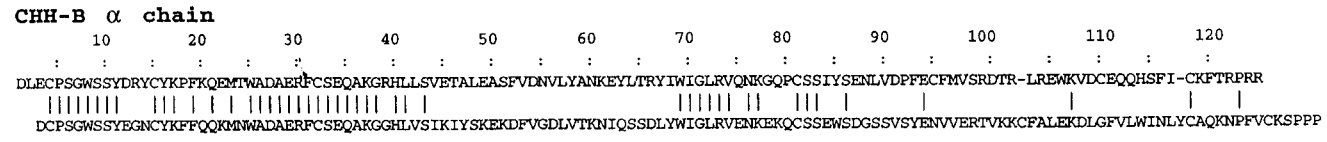
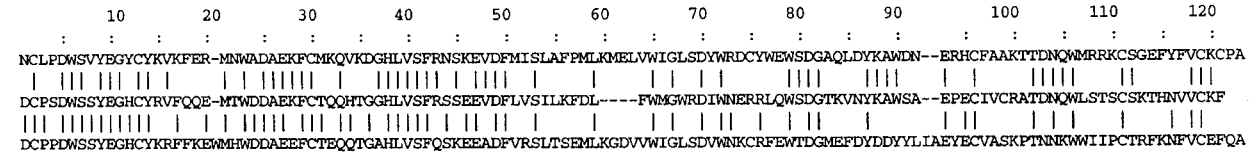


Botrocetin α chain 38% Identity

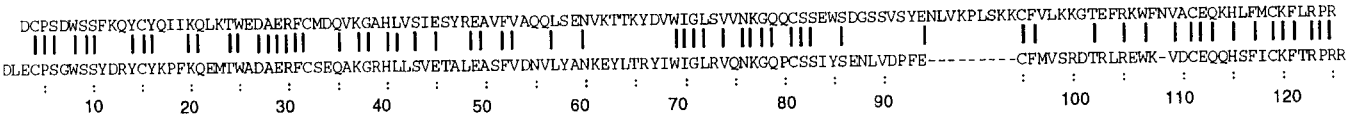
Echicetin β chain/CHH-B β 47% Identity



Botrocetin β chain/CHH-B β 49% Identity

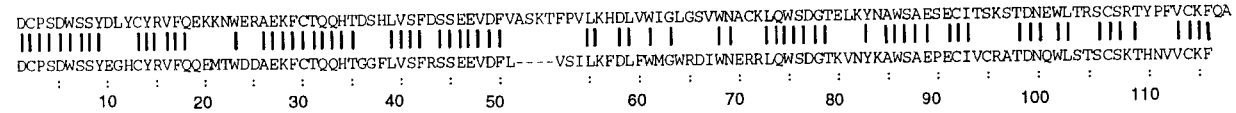
B

Alboaggregin-B α chain/CHH-B α 47% Identity



CHH-B α chain

Alboaggregin-B β chain/CHH-B β 54% Identity



CHH-B β chain

C

50-kDa Albo.:	D X P S D X S S Y D/E Q/Y Y X Y X V F V
Albo-B α:	D C P S D W S S F K Q Y C Y Q I F/I K
Albo-B β:	D C P S D W S S Y D L Y C Y R V F Q
CHH-B α:	D L E C P S G W S S Y D R Y C Y K P F K
CHH-B β:	D C P S D W S S Y E G H C Y R V F Q
Botro. α:	D C P S G W S S Y E G N C Y K F F Q
Botro. β:	D C P P D W S S Y E G H C Y K R F F
Echic. β:	N C L P D W S V Y E G Y C Y K V K F

FIGURE 3: (A) Amino acid sequence comparison of the α- and β-chains of CHH-B with the α- and β-chains of botrocetin (Usami et al., 1993) and the β-chain of echicetin (Peng et al., 1994). (B) Comparison of the α- and β-chains of CHH-B with the α- and β-chains of alboaggregin-B (Usami et al., 1996). (C) N-terminal sequence determined for the 50-kDa alboaggregin compared with the α- and β-chains of alboaggregin-B, botrocetin, CHH-B, and the echicetin β-chain (Usami et al., 1993, 1996; Peng et al., 1994; this study).

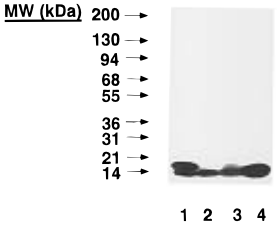


FIGURE 4: Western blots of purified 50-kDa alboaggregin (lane 1), CHH-A (lane 2), CHH-B (lane 3), and botrocetin (lane 4) electrophoresed on 5%−20% SDS−polyacrylamide gels under reducing conditions, electrotransferred to nitrocellulose and probed with rabbit anti-botrocetin antisera. Blots were visualized using peroxidase-coupled sheep anti-rabbit IgG and the ECL substrate. Molecular weight markers (arrows) are as described in the legend of Figure 1.

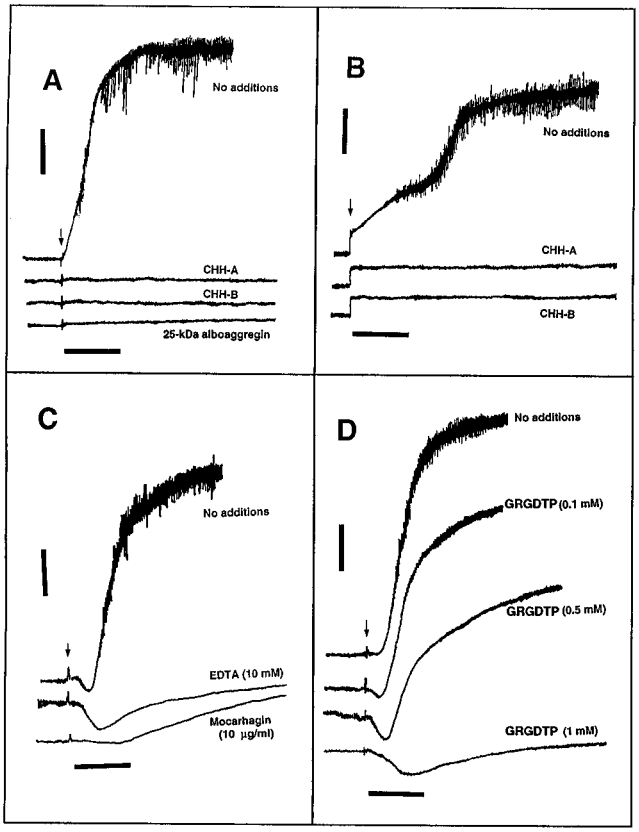


FIGURE 5: Aggregation of platelet-rich plasma induced by (A) ristocetin (1.5 mg/mL, final concentration), (B) bovine vWF, and (C and D) 50-kDa alboaggregin (0.5 μg/mL, final concentration). Platelet-rich plasma was incubated at 37 °C with 10 μg/mL (final concentration) of CHH-A, CHH-B, or 25-kDa alboaggregin for 3 min prior to the addition (arrows) of 1.5 mg/mL, final concentration, ristocetin (panel A), or one-tenth volume bovine plasma (panel B). In panels C and D, platelet-rich plasma was incubated with either EDTA (10 mM, final concentration) or collagen-related peptide GRGDTP (0.1, 0.5, or 1 mM, final concentration) or was pretreated with mocarhagin (10 μg/mL, final concentration) for 6 min at 37 °C prior to the addition of the 50-kDa alboaggregin (arrow). The vertical and horizontal bars represent 10% maximal transmittance and 1 min, respectively. Traces are representative of separate experiments with different platelet donors.

venom proteins tested had binding sites within the His-1−Glu-282 domain of GP Ibα.

Binding of ^{125}I-labeled anti-GP Ibα monoclonal antibodies, AK2 and SZ2, to platelets was differentially inhibited by the venom proteins. AK2 and SZ2 map into the N-terminal peptide domain of GP Ibα, with epitopes within the sequences His-1−Leu-275 and Tyr-276−Glu-282, respectively (Ward et al., 1996). The 50-kDa alboaggregin completely inhibited binding of ^{125}I-labeled AK2 to platelets (Figure 9A), but only partially inhibited ^{125}I-labeled SZ2 binding (Figure 9D). Both echicetin and the 25-kDa alboaggregin inhibited the binding to platelets of both ^{125}I-labeled AK2 and SZ2 (Figure 9B and E, respectively). The rattlesnake proteins, CHH-A and CHH-B, only inhibited ^{125}I-labeled AK2 binding (Figure 9C) and had no effect on ^{125}I-labeled SZ2 binding (Figure 9F). None of the snake venom proteins inhibited the binding of the anti-GP Ibα macroglycopeptide domain monoclonal antibody, WM23, to platelets (data not shown). Although the monoclonal antibodies and venom proteins variably cross-block binding to GP Ibα, the lack of detail regarding the AK2 epitope and information on GP Ibα tertiary structure as well as potential steric effects precludes precise identification of the venom protein binding sites. However, the combined results provide the first evidence that structurally-related venom proteins can bind to distinct sites on GP Ibα. This may, in part, explain functional differences between homologous snake venom proteins and supports the concept that the conformation of the GP Ib-IX-V receptor is a critical determinant of ligand binding (Roth, 1995; Ward et al., 1996).

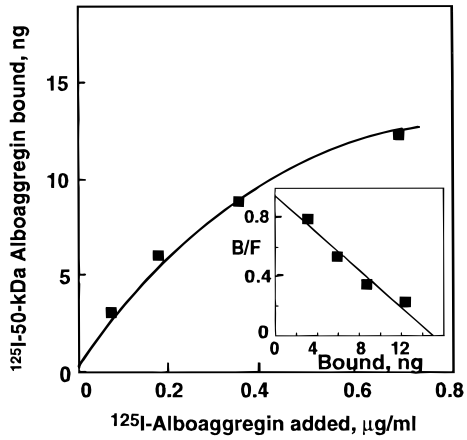


FIGURE 6: Specific binding of ^{125}I-labeled 50-kDa alboaggregin to washed platelets (5×10^7/mL) in 30 min at 22 °C. Specific binding was calculated from total binding by substracting nonspecific binding measured at low ^{125}I-labeled 50-kDa alboaggregin concentration in the presence of a 50-fold excess of unlabeled 50-kDa alboaggregin.

Since the 50-kDa alboaggregin not only bound to the GP Ib-IX-V complex on platelets but also potently induced platelet aggregation, we tested the ability of it and related snake venom proteins to stimulate granule release from ^{14}C-labeled serotonin-loaded platelets as a measure of platelet activation. α-Thrombin was used as a control agonist and resulted in ∼90% release of ^{14}C-labeled serotonin (Figure 10A). The 50-kDa alboaggregin was also a potent platelet agonist, with ∼65% release of the dense granule label (Figure 10A). However, none of the 25-kDa venom proteins stimulated platelet secretion (Figure 10A). The 50-kDa alboaggregin was also tested for its ability to induce elevations of cytosolic ionized calcium ($[Ca^{2+}]_i$) in fura 2-loaded platelets. As compared with α-thrombin (Figure 10B), the 50-kDa alboaggregin potently induced elevated $[Ca^{2+}]_i$ of platelets (Figure 10C). The $[Ca^{2+}]_i$ response induced by the 50-kDa alboaggregin lacked the initial rapid influx spike characteristic of α-thrombin (Figure 10B) and showed instead a slow initial rise similar to that observed

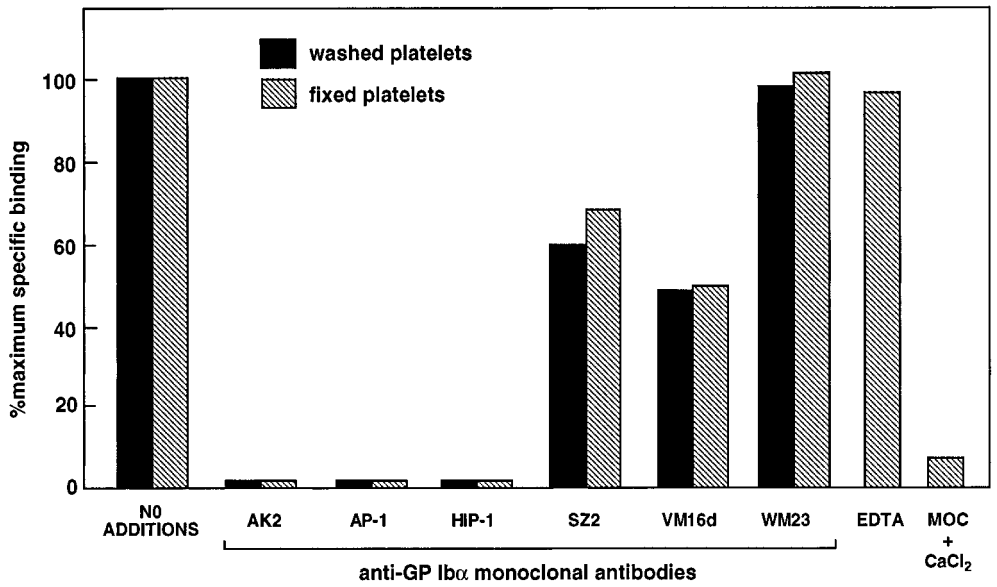


FIGURE 7: Effect of anti-GP Ibα monoclonal antibodies, EDTA, and the cobra venom metalloproteinase, mocarhagin, on binding of 50-kDa alboaggregin to platelets. Assays show the specific binding of ^{125}I-labeled 50-kDa alboaggregin (1 μg/mL) to washed platelets (solid bars) or paraformaldehyde-fixed platelets (hatched bars) at 5×10^8/mL. Platelets were incubated with TS buffer or EDTA (10 mM, final concentration) or were pretreated with the monoclonal antibodies (50 μg/mL, final concentration) for 5 min at 37 °C or with 75 μg/mL mocarhagin (MOC) in the presence of 10 mM calcium chloride for 6 min prior to the addition of alboaggregin.

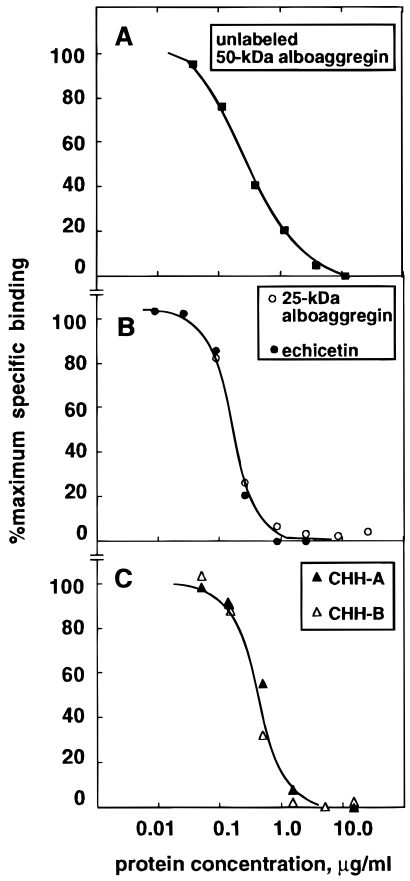


FIGURE 8: Effect of unlabeled 50-kDa alboaggregin (A), 25-kDa alboaggregin (B, open circles), echicetin (B, solid circles), CHH-A (C, solid triangles), and CHH-B (open triangles) on the specific binding of ^{125}I-labeled 50-kDa alboaggregin (1 μg/mL) to paraformaldehyde-fixed platelets (5×10^8/mL) in 30 min at 22 °C.

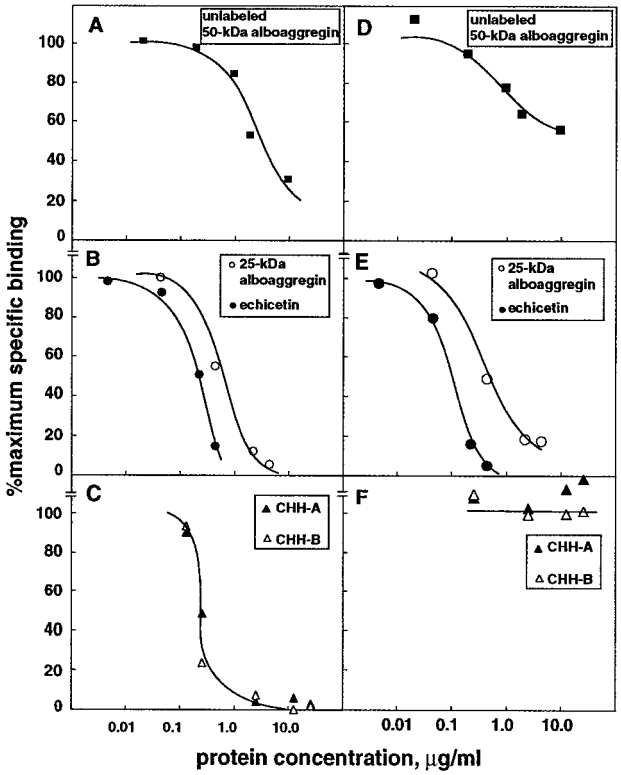


FIGURE 9: Effect of unlabeled 50-kDa alboaggregin (A and D), 25-kDa alboaggregin (B and E, open circles), echicetin (B and E, solid circles), CHH-A (C and F, solid triangles), and CHH-B (C and F, open triangles) on the specific binding of ^{125}I-labeled AK2 (1 μg/mL; A−C) or ^{125}I-labeled SZ2 (1 μg/mL; D−F) to paraformaldehyde-fixed platelets (5×10^8/mL).

with ristocetin-mediated vWF binding (Kroll et al., 1991). Echicetin had little, if any, effect on Ca^{2+} influx under the same conditions (data not shown).

Analysis of [^{32}P]phosphate incorporation into 50-kDa alboaggregin-treated platelets (Figure 11, upper panel) showed a prominent 47-kDa band consistent with phosphorylation of pleckstrin (p47), a previously defined protein kinase C substrate (Kroll et al., 1993), and an ~20-kDa band which represents myosin light chain (Scholey et al., 1980). Both of these bands were also seen in α-thrombin-treated platelets (Figure 11, upper panel). In contrast, treatment of platelets with echicetin showed no induction of the 47-kDa band. Interestingly, there was partial enhancement of the

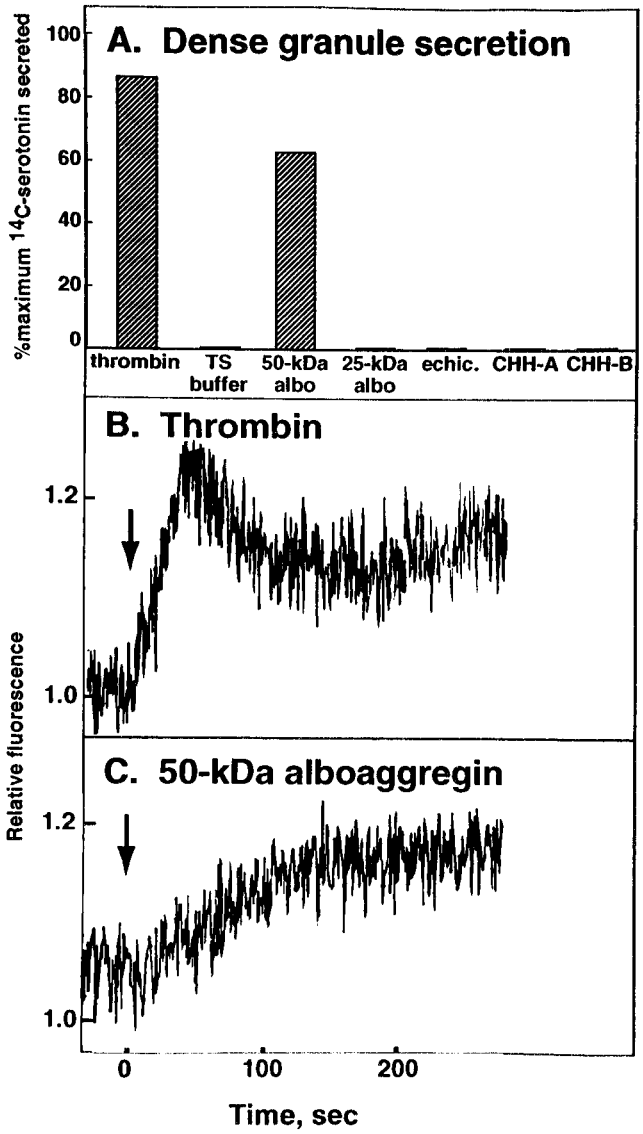


FIGURE 10: (A) Effect of α-thrombin (1.6 units/mL, final concentration) and the venom proteins 50-kDa alboaggregin (1.5 μg/mL), echicetin (1.4 μg/mL), CHH-A (2 μg/mL), CHH-B (2 μg/mL), and 25-kDa alboaggregin (1.5 μg/mL) on ^{14}C-labeled serotonin release from platelets (5×10^8/mL) in Tyrodes' solution containing 2.5 μM imipramine and incubated with agonists for 5 min at 37 °C in an aggregometer cuvette stirred at 900 rpm. Platelets were subsequently fixed with paraformaldehyde and microfuged, and the serotonin released into the supernatant measured in a β-counter. (B) Effect of α-thrombin (1 unit/mL) and (C) 50-kDa alboaggregin (1 μg/mL) on Ca^{2+} influx into fura 2-loaded platelets (5×10^8/mL) in Tyrodes' solution stirred at 900 rpm at 37 °C.

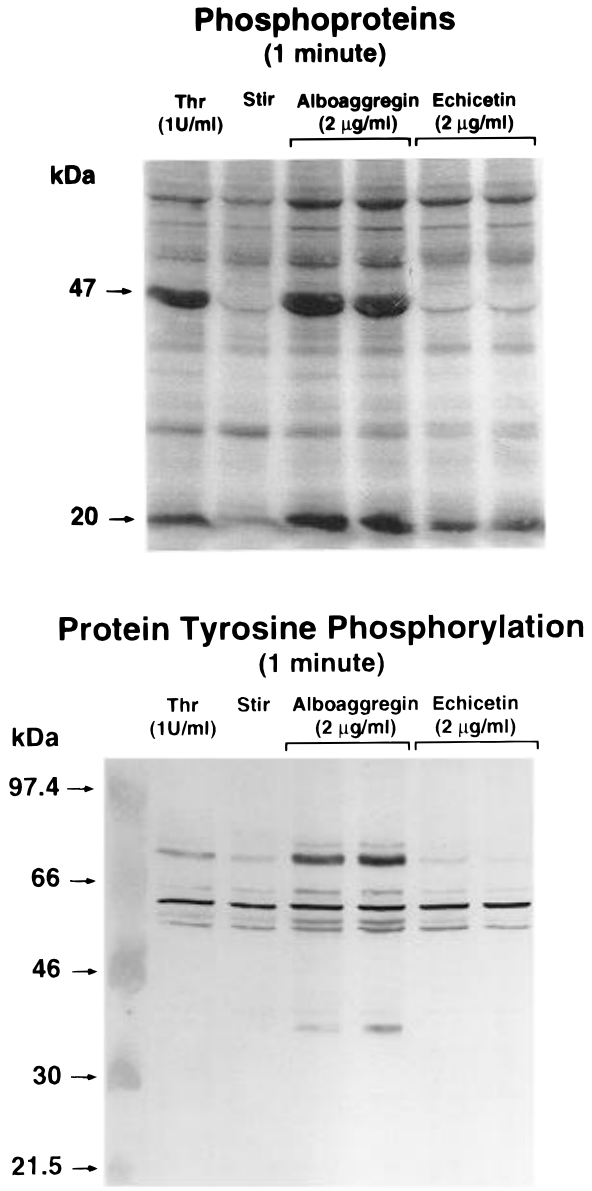


FIGURE 11: Effect of α-thrombin (1 unit/mL, final concentration), stirring only in the absence of agonist, 50-kDa alboaggregin (2 μg/mL) and echicetin (2 μg/mL) on protein phosphorylation in washed platelets (5×10^8/mL) in 1 min at 37 °C analyzed by incorporation of [^{32}P]orthophosphate (upper panel) or immunoblotting with the anti-tyrosine phosphate monoclonal antibody, 4G10 (lower panel). ^{32}P-labeled proteins were detected by electrophoresis on 5%−20% SDS−polyacrylamide gels and autoradiography.

~20-kDa band compared with the untreated platelet sample (Figure 11, upper panel). Platelets treated with 50-kDa alboaggregin also showed evidence for tyrosine kinase activation (Figure 11, lower panel) with induction of two major species with molecular weights of ~76 kDa and ~36 kDa. These bands were also observed associated with shear-stress-induced vWF binding to platelets (Razdan et al., 1994), whereas only the higher molecular weight band was weakly induced by α-thrombin (Razdan et al., 1994; this study, Figure 11, lower panel). There was no detectable difference between untreated platelets and platelets treated with echicetin under the same conditions (Figure 11, lower panel).

DISCUSSION

The platelet surface membrane glycoprotein (GP) Ib-IX-V complex plays an essential role in mediating the hemostatic response of platelets to blood vessel damage. GP Ib-IX-V functions as the primary receptor for von Willebrand Factor (vWF) bound to the subendothelial matrix, and this adhesive interaction is required for platelet adhesion to exposed matrix under conditions of high shear flow (Booth et al., 1990; López, 1994; Weiss, 1995). The GP Ib-IX-V complex is also involved in modulating the local effects of thrombin through a high-affinity binding site for α-thrombin present on the α-chain of GP Ib (Berndt et al., 1986). The extracellular portion of GP Ibα consists of the N-terminal peptide domain composed of the seven tandem leucine-rich

repeats and their disulfide-loop N- and C-terminal flanking sequences (His-1−Leu-275), the anionic/sulfated tyrosine region centered around the Tyr-276−Glu-282 sequence, and the highly glycosylated macroglycopeptide domain (López et al., 1987; López, 1994). Studies from our laboratory and others have demonstrated that binding sites for both vWF and α-thrombin can be localized to the globular N-terminal peptide domain of GP Ibα (His-1−Glu-282) (Handa et al., 1986; Berndt et al., 1988; Andrews et al., 1989b; Vicente et al., 1990; Ward et al., 1996). Further, structure−function analysis of GP Ibα using proteases and monoclonal antibodies has recently identified residues Tyr-276−Glu-282 as a recognition site for α-thrombin and for vWF complexed to its modulator, botrocetin (Ward et al., 1996). Residues Tyr-276−Glu-282 comprise a distinctive motif of negatively-charged amino acids and sulfated tyrosine residues (Dong et al., 1994; Marchese et al., 1995; Ward et al., 1996). The viper venom proteins first described by Peng and co-workers (1991, 1992, 1993) that bind to the N-terminal peptide domain of GP Ibα and inhibit vWF binding provide an additional means of investigating structure−function relationships of GP Ibα.

In the present work, we have purified and characterized a novel 50-kDa alboaggregin from the venom of the white-lipped tree viper, *T. albolabris*, and two 25-kDa timber rattlesnake venom proteins, CHH-A and CHH-B, from *C. h. horridus*. Three lines of evidence suggest that these proteins are all members of the C-type lectin protein family. Firstly, complete or N-terminal amino acid sequence analysis of the three proteins show homology to published sequences of alboaggregin-B, jararaca GP Ib-BP, flavocetin-A, and the GP Ib-binding *β*-chain of echicetin (Peng et al., 1994; Fujimura et al., 1995; Yoshida et al., 1995; Taniuchi et al., 1995; Usami et al., 1996). Secondly, all of these proteins appear on SDS−polyacrylamide gels as disulfide-linked heterodimers or higher multimers of ∼14−18-kDa subunits (Peng et al., 1991, 1993, 1994; Fujimura et al., 1995; Yoshida et al., 1993; Taniuchi et al., 1995; this study). Thirdly, CHH-A, CHH-B, and the 50-kDa alboaggregin are immunologically cross-reactive with polyclonal anti-botrocetin antisera (this study). Like the previously described proteins, echicetin from the saw-scaled viper *E. carinatus*, alboaggregin-B from *T. albolabris* and jararaca GP Ib-BP from the South American pit viper *B. jararaca*, the 50-kDa alboaggregin, CHH-A, and CHH-B all bound to the N-terminal peptide domain of GP Ibα and inhibited binding of vWF. Binding to this domain was confirmed since binding of purified radiolabeled 50-kDa alboaggregin to platelets was (i) blocked by anti-GP Ibα monoclonal antibodies, AK2, AP1, and Hip1, that map into the His-1−Leu-275 sequence, (ii) abolished by pretreatment of platelets with the cobra venom metalloproteinase, mocarhagin, which selectively cleaves GP Ibα between residues Glu-282 and Asp-283, and (iii) competitively inhibited to a similar extent by CHH-A and CHH-B, and by echicetin, previously shown to bind this domain (Peng et al., 1991, 1993). However, despite their structural similarity, cross-blocking studies of the venom proteins and two anti-GP Ibα monoclonal antibodies, AK2 and SZ2, suggest that the binding sites for these venom proteins are not identical. AK2 (His-1−Leu-275) and SZ2 (Tyr-276−Glu-282) have distinct epitopes within the N-terminal peptide domain of GP Ibα (Ward et al., 1996). Whereas a 25-kDa alboaggregin and echicetin completely blocked binding of AK2 and SZ2 to platelets, the 50-kDa alboaggregin strongly blocked AK2 and only partially blocked SZ2 binding, while CHH-A and CHH-B blocked only AK2 but not SZ2 binding. These results strongly suggest that structurally-related venom proteins are capable of binding distinct sites on GP Ibα. Single amino acid mutations within the leucine-rich repeats (Leu-57 to Phe and Ala-156 to Val) are known to cause dysfunctional vWF binding (Miller et al., 1992; Ware et al., 1993), even though vWF binding appears to involve the downstream sequences Tyr-276−Glu-282 (Ward et al., 1996), Asp-235−Lys-262 (Katagiri et al., 1990), and/or Ser-251−Glu-285 (Vicente et al., 1990). Roth (1995) has suggested that altered conformations of the N-terminal peptide domain may involve interactions between different leucine-rich repeats. It is possible that the structurally-related venom proteins interact with different leucine-rich repeat domains and that this may account for their observed functional difererences, although other explanations are tenable and this interesting question awaits further studies.

A major difference between the viper venom proteins was that only the 50-kDa form of alboaggregin, but not the other proteins, acted as a strong platelet agonist inducing rapid platelet dense granule secretion, elevation of cytosolic ionized calcium ($[Ca^{2+}]_i$) and protein phosphorylation. Platelet aggregation induced by the 50-kDa alboaggregin was inhibited by EDTA, even though EDTA had no significant effect on binding of ^{125}I-labeled 50-kDa alboaggregin to platelets. Aggregation was also inhibitable by the synthetic RGD-containing peptide, GRGDTP, an inhibitor of platelet aggregation in response to agonists (such as ADP or α-thrombin) that utilize the platelet fibrinogen receptor, the integrin αIIb*β*3 (GP IIb-IIIa) (Shattil, 1995). These results suggest that binding of the 50-kDa alboaggregin alone is not sufficient to induce agglutination, but that binding activates platelets allowing subsequent calcium-dependent platelet aggregation mediated by GP IIb-IIIa. Both aggregation and binding was blocked by pretreating platelets with mocarhagin, a protease that specifically cleaves off the His-1−Glu-282 GP Ibα fragment, showing that platelet activation induced by the 50-kDa alboaggregin was coincident with its binding to GP Ibα. In contrast, Peng et al. (1991, 1992) reported that the ∼23-kDa alboaggregin-B and ∼52-kDa alboaggregin-A supported calcium-independent agglutination of washed or fixed platelets. The reason for this discrepancy is not clear. It may simply reflect the common finding in venom studies that homologous proteins isolated either from different species, or from different commercial batches of venom from the same species, may have different properties (Peng et al., 1992; Fujimura et al., 1991).

Our results show that binding to the N-terminal peptide domain of GP Ibα is coincident with, and facilitative of, 50-kDa alboaggregin-dependent platelet activation, although the precise mechanism by which the 50-kDa alboaggregin activates platelets is presently unclear. The simplest, though not exclusive, mechanisms for this activation would be either (1) direct signaling through one or more GP Ib-IX-V complex(es) engaged by 50-kDa alboaggregin or (2) binding to GP Ib-IX-V and signaling through a vicinal, unidentified receptor. Currently, the mechanism for vWF-dependent platelet activation through the GP Ib-IX-V complex is also not clearly defined. Shear- or ristocetin-induced binding of multivalent native vWF to the GP Ib-IX-V complex activates platelets (Kroll et al., 1991, 1993; Razdan et al., 1994; Ikeda

et al., 1993). In contrast, a 39/34-kDa monomeric dispase fragment of vWF is able to bind to GP Ibα on platelets and block binding of native vWF but does not induce platelet activation (Andrews et al., 1989b). This would be consistent with a mechanism for vWF-dependent activation involving ligand valency. The multimeric 50-kDa alboaggregin has a similar potential to cross-link GP Ib-IX-V receptor complexes. Further study of this unusual platelet agonist therefore has the potential to clarify some of the unresolved questions concerning the role of the GP Ib-IX-V complex in platelet adhesion and activation.

ACKNOWLEDGMENT

We thank Carmen Llerena, Ketia Francis, and Mary Matthew for superb technical assistance.

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BI960704E