A Novel Viper Venom Metalloproteinase, Alborhagin, is an Agonist at the Platelet Collagen Receptor GPVI *

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RUNNING TITLE: Alborhagin, a collagen-like viper venom protein

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SUMMARY

The interaction of platelet membrane glycoprotein (GP) VI with collagen can initiate (patho)physiological thrombus formation. Viper venom C-type lectin family proteins, convulxin and alboagreggin-A, activate platelets by interacting with GPVI. In this study, we isolated from white lipped tree viper (Trimeresurus albolabris) venom, alborhagin, which is functionally related to convulxin in that it activates platelets, but is structurally different, and related to venom metalloproteinases. Alborhagin-induced platelet aggregation (EC₅₀, <7.5 μg/ml) was inhibitable by an anti-αIIbβ3 antibody, CRC64, and the Src family kinase inhibitor, PP1, suggesting alborhagin activates platelets leading to αIIbβ3-dependent aggregation. Additional evidence suggested that, like convulxin, alborhagin activated platelets by a mechanism involving GPVI: (1) alborhagin- and convulxin-treated platelets showed a similar tyrosine phosphorylation pattern, including a similar level of PLCγ2 phosphorylation, (2) alborhagin induced GPVI-dependent responses in GPVI-transfected K562 and Jurkat cells, and (3) alborhagin-dependent aggregation of mouse platelets was inhibited by the anti-GPVI monoclonal antibody, JAQ1. Alborhagin had minimal effect on convulxin binding to GPVI-expressing cells, indicating these venom proteins may recognize distinct binding sites. Characterization of alborhagin as a GPVI agonist that is structurally distinct from convulxin demonstrates the versatility of snake venom toxins and provides a novel probe for GPVI-dependent platelet activation.
INTRODUCTION

In both normal hemostasis and thrombotic disease, platelet adhesion and aggregation may be initiated by engagement of specific membrane receptors that leads to platelet activation and \( \alpha_{IIb}\beta_3 \)-dependent aggregation (1-5). At high shear stress, platelet adhesion is dependent on binding of the platelet membrane glycoprotein (GP)\(^1\) Ib-IX-V complex to its ligand, von Willebrand Factor (vWF) and is supported by collagen receptors \( \alpha_2\beta_1 \) integrin (platelet GPIa-IIa) and/or GPVI (3, 5). At low shear, \( \alpha_2\beta_1 \) and/or GPVI support platelet adhesion and activation without the requirement for vWF (5).

Viper venom proteins that activate platelets have played a significant role in elucidating mechanisms of platelet activation. Several groups have described a 50-kDa protein of the C-type lectin family, alboaggregin-A, from venom of the white-lipped tree viper, *Trimeresurus albolabris*, which binds to GPIb-IX-V (6-9) and GPVI (10, 11), and activates platelets by a mechanism that may involve one or both receptors. A related C-type lectin-like protein, aggrekin from the Malayan pit viper, *Calloselasma rhodostoma*, has been reported to activate platelets by a mechanism that involves GPIb and GPIa-IIa, but not GPVI (12). An analogous ~85-kDa, C-type lectin-like protein, convulxin, from the venom of the tropical rattlesnake, *Crotalus durissus terrificus*, activates platelets by a mechanism involving GPVI (5, 13-15).

In the present study, we have isolated a protein termed alborhagin from *Trimeresurus albolabris* venom that is functionally related to convulxin in that it is a potent agonist at GPVI but which is a member of the metalloproteinase family and binds to a distinct site on GPVI. Evidence is presented to suggest that alborhagin binds to
GPVI at a distinct site to convulxin. Alborhagin therefore represents a novel tool to further characterise GPVI.

MATERIALS AND METHODS

**Materials**—Lyophilized venom from *Trimeresurus albolabris* was purchased from Sigma, St Louis, MO, U.S.A. or Venom Supplies, Tanunda, South Australia. Bovine α-thrombin was from Sigma, St Louis, MO, U.S.A. PP1 was purchased from Calbiochem-Novabiochem (Nottingham, U.K.). Convulxin isolated from the venom of *Crotalus durissus terrificus* (15) was kindly provided by Drs M. Leduc and C. Bon (Unite des Venens, Institut Pasteur, Paris, France). The cobra venom metalloproteinase, mocarhagin, was purified from *Naja mocambique mocambique* venom (Sigma, St Louis, MO, U.S.A) as previously described (16, 17).

**Antibodies**—The anti-αIIbβ3 monoclonal antibody, CRC64, was the gift of Dr A. Mazurov (Moscow, Russia) and has been described elsewhere (18). An anti-murine GPVI monoclonal antibody, JAQ1, has also been described elsewhere (19). Rabbit polyclonal antiserum against mocarhagin or convulxin was prepared using standard methods (10, 20). The anti-phosphotyrosine monoclonal antibody, 4G10, was from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). The anti-PLCγ2 and anti-Syk polyclonal antibodies were a gift from Dr M. G. Tomlinson (DNAX Research Institute, CA, U.S.A.).

**Purification of Alborhagin**—Throughout the purification procedure, fractions were assayed for their ability to induce platelet aggregation of citrated platelet-rich plasma and analyzed by SDS-polyacrylamide gel electrophoresis as previously described.
Lyophilized *Trimeresurus albolabris* venom (20 mg) was dissolved in 4 ml of TS buffer (0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4) and loaded at 30 ml/h onto a 1.5 x 20-cm heparin-agarose (Bio-Rad, Richmond, CA, U.S.A.) column and washed with TS buffer. Bound protein was eluted with a linear 0.15–1.0 M NaCl gradient in 0.01 M Tris-HCl, pH 7.4. Fractions containing an ~60-kDa protein (reduced and nonreduced) that eluted from the heparin-agarose column were dialyzed into 5 mM NaH$_2$PO$_4$, pH 6.8, and loaded at 25 ml/h onto a 10 x 1-cm hydroxylapatite column equilibrated in the same buffer. Bound protein was eluted with a linear 5–200 mM NaH$_2$PO$_4$, pH 6.8, gradient. Fractions containing alborhagin were pooled, concentrated using an ultrafiltration device (Amicon, Danvers, MA, U.S.A.) fitted with a YM30 membrane, and dialyzed into TS buffer. The concentration of purified protein was estimated using the BCA method with BSA as standard according to the manufacturer's instructions (Pierce, Rockford, IL, U.S.A.).

**Sequence Analysis**—N-terminal sequence analysis was performed as previously described (7, 17). For internal sequence analysis, alborhagin (0.58 mg) was dialyzed into distilled water and digested with trypsin (0.1 mg trypsin/mg protein) overnight at 37 °C. Tryptic fragments were separated by RP-HPLC eluted by a linear 0-60% [v/v] acetonitrile gradient and sequenced as previously described (17).

**Alborhagin-Dependent Digestion of Fibrinogen**—Digestion of human fibrinogen (Kabivitrum, Stockholm, Sweden) at a final concentration of ~100 µg/ml in TS buffer by alborhagin (10 µg/ml, final concentration) for 30 min at 22 °C was carried out in the presence of EDTA (10 mM, final concentration) or CaCl$_2$ (10 mM, final concentration) according to a published method (22).

**Platelet Aggregation**—Platelet aggregation of human platelet-rich plasma (0.32% citrate, final concentration) was performed using a whole blood Lumiaggregometer
(Chronolog, Havertown, PA, U.S.A.) as previously described (7, 17, 21). Alborgin was added to platelets at a final concentration of 2.5-25 µg/ml. In some assays, the anti-αIIbβ3 antibody, CRC64, at 20 µg/ml, or mocarhagin at 10 µg/ml, were pre-incubated with the platelets for 5 min or 6 min, respectively, at 37 °C prior to the addition of alborgin. Other assays included EDTA at a final concentration of 10 mM.

The effect of PP1 (10 µM) on alborgin-induced aggregation of washed human platelets, isolated as previously described (23), was performed at 37 °C in the presence of indomethacin (10 µM) and apyrase (2 units/ml). Alborgin-dependent aggregation of mouse platelets (1x10^8/ml) in the absence or presence of the monoclonal antibody, JAQ1, at a final concentration of 10 µg/ml, was carried out essentially as previously described (19).

Effect of Alborgin on Platelet Protein Phosphorylation—The incorporation of carrier-free [32P]orthophosphate (~8750 Ci/mmol; NEN Life Sciences, Boston, MA, U.S.A.) into platelet proteins was assessed using previously described methods (7). Briefly, washed platelets at 10^9/ml in 0.01 M HEPES, 0.15 M NaCl, 5 mM EDTA, pH 7.4 (EHS buffer), were labeled with 1 mCi [32P]orthophosphate for 1 h at 22 °C, washed in the same buffer, and resuspended in a Tyrode’s buffer containing 138 mM NaCl, 29 mM KCl, 12 mM NaHCO3, 0.36 mM Na2PO4, 5.5 mM glucose, 1.8 mM CaCl2, 0.49 mM MgCl2, pH 7.4. Stirred 32P-labeled platelets (2 x 10^8/ml) were treated with a final concentration of 10 µg/ml collagen or 10 µg/ml alborgin for 0.25, 1, 2 or 5 min at 37 °C. Samples were quenched by electrophoresis sample buffer, electrophoresed on SDS–polyacrylamide gels under reducing conditions and analyzed by autoradiography.

For immunoblotting, washed platelets (2 x 10^8/ml) isolated as previously described (23) were treated with 1-15 µg/ml alborgin or 1 µg/ml convulxin for
various times at 37 °C. Reactions were terminated by adding SDS-containing sample buffer, electrophoresed on SDS–8-18%-polyacrylamide gels under reducing conditions, electrotransferred to polyvinylidene difluoride (PVDF) membranes, and immunoblotted with anti-phosphotyrosine antibody, 4G10. Blots were visualized using a horseradish peroxidase-coupled second antibody (Silenus, Hawthorn, Victoria, Australia) and the ECL detection system (Amersham International, Cardiff, Wales, U.K.).

For immunoprecipitation studies, platelets (2 x 10^8/ml) were pre-incubated with vehicle (0.1% DMSO) or 10 μM PP1 for 5 min at 37 °C prior to the addition of 3 μg/ml alborhagin or 1 μg/ml convulxin for 90 s or 30 s, respectively. Platelets were then lysed by adding an equivalent volume of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.3, 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2% [v/v] Nonidet P-40, 2 mM Na3VO4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A), centrifuged at 15000g for 10 min to remove detergent-insoluble material, and pre-cleared with 30 μl of a 50% suspension of protein A-Sepharose in TBS-T buffer (20 mM Tris-HCl, 137 mM NaCl, 0.1% [v/v] Tween 20, pH 7.3). Samples were then immunoprecipitated with monoclonal anti-PLCγ2 antibody and 30 μl of protein A-Sepharose for 2 h at 4 °C. The beads were washed once with lysis buffer and three times with TBS-T buffer, then solubilized in electrophoresis sample buffer and boiled for 10 min. Immunoblotting with anti-phosphotyrosine antibody, 4G10, was carried out as described above. Membranes were then stripped and re-probed with anti-PLCγ2 antibody.

**Alborhagin-Dependent Syk Phosphorylation in GPVI-Expressing K562 Cells**—K562 human erythroleukaemia cells stably co-transfected with empty pRc plasmid and pMG-FcRγ-chain (FcRγ) (pRc/γ-chain), or pRc-GPVI and pMG-FcRγ (GPVI/FcRγ) (10, 23), were treated with PMA for 24 h to increase surface expression of GPVI, then
stimulated with alborhagin for 90 s. Cells were lysed and immunoprecipitated with anti-Syk antibody as described above. Proteins were electrophoresed on SDS-polyacrylamide gels, transferred to PVDF membranes and immunoblotted with 4G10. Filters were stripped and re-probed with anti-Syk antibody. The affect of alborhagin (0.1-100 µg/ml, final concentration) on binding of convulxin (1 µg/ml) to GPVI-expressing K562 cells was assessed by flow cytometry using an anti-convulxin IgG and a secondary FITC-labeled anti-rabbit IgG.

Alborhagin-Dependent Ca\(^{2+}\) Mobilization in GPVI-Transfected Jurkat Cells–GPVI-transfected Jurkat cells were transfected with GPVI using similar methods to those described above for K562 cells (10, 23). Jurkat cells are a human T lymphocytic cell line that do not express endogenous GPVI or FcRγ. Ca\(^{2+}\) flux in response to alborhagin (10 µg/ml) in untransfected or GPVI-transfected cells was measured as previously described (24). The affect of alborhagin (0.1-100 µg/ml, final concentration) on binding of convulxin (1 µg/ml) to GPVI-expressing Jurkat cells was assessed using methods described above for K562 cells.

RESULTS

Purification and Characterization of Alborhagin and its Autolytic Fragment–In the course of our purification of the platelet agonist, alboaggregin-A, from the venom of the white-lipped tree viper, *Trimeresurus albolabris* (7), we identified a structurally distinct protein termed alborhagin that was also a potent platelet agonist. Alborhagin had an apparent molecular weight on SDS-polyacrylamide gels of ~60 kDa under reducing (Fig. 1A) and nonreducing (not shown) conditions, and bound strongly to
heparin (eluted at >0.5 M sodium chloride). Several lines of evidence suggested alborhagin was a member of the metalloproteinase-disintegrin family. Firstly, it was immunoreactive towards an antibody against the cobra venom metalloproteinase-disintegrin, mocarhagin (Fig. 1B). This antibody is also cross-reactive with jararhagin (data not shown). Secondly, like other venom metalloproteinase-disintegrins (22, 25), alborhagin showed metalloproteinase activity towards the fibrinogen α-chain in the presence of Ca²⁺, but there was no significant digestion in the presence of EDTA (Fig. 1C). Thirdly, amino acid sequence analysis of tryptic fragments corresponding to a total of 36 residues (Fig. 2A) were analogous to sequences within the metalloproteinase domain of the venom metalloproteinase-disintegrins, jararhagin (26), HR1B (27) and MT-d (28). Fourthly, we obtained sequence from the disintegrin domain by N-terminal sequencing of a 42-kDa autolytic fragment of alborhagin (Fig. 3A). Autolytic digestion of alborhagin was inhibited in the presence of EDTA (Fig. 3B), suggesting it involved the metalloproteinase activity of alborhagin. The 42-kDa fragment did not bind heparin (data not shown), and was isolated in the flow-through of a heparin-agarose column to allow separation of any undigested alborhagin. The N-terminal sequence of the 42-kDa fragment (Fig. 2B) was similar to the region at the boundary between the metalloproteinase and disintegrin domains of jararhagin (26). This sequence was conserved in MT-d (Fig. 2B) which also undergoes autodigestion (28).

**Alborhagin-Dependent Platelet Aggregation**—Alborhagin induced aggregation in platelet-rich plasma with maximal activity at ~7.5 µg/ml (Fig. 4A). Aggregation was strongly inhibited by the anti-αIIbβ3 antibody, CRC64, that blocks ligand binding to αIIbβ3 (18) and by EDTA (Fig. 4A), suggesting that aggregation was αIIbβ3-dependent and involved alborhagin-induced platelet activation. In the presence of EDTA, alborhagin still induced a platelet shape change (Fig. 4A) suggesting that alborhagin
could induce platelet activation independently of its metalloproteinase function. In contrast to intact alborhagin, however, the ~42-kDa alborhagin fragment corresponding to the disintegrin/C-terminal region minus the metalloproteinase domain (refer above) did not induce platelet aggregation at a final concentration of 10 µg/ml in platelet-rich plasma (data not shown). Together, these results suggested an intact metalloproteinase domain, but not proteolysis, is necessary for alborhagin-induced platelet activation.

Pre-treating platelets with the cobra venom metalloproteinase, mocarhagin, had no effect on alborhagin-dependent platelet aggregation (data not shown). Mocarhagin has previously been shown to cleave the GPIbα chain between Glu-282 and Asp-283 and abolish binding of vWF to the GPIb-IX-V complex on platelets and vWF-dependent platelet aggregation (17). Lack of effect of mocarhagin on alborhagin activity therefore suggests alborhagin is not acting through GPIb-IX-V on platelets. However, alborhagin-induced aggregation of washed human platelets was strongly inhibited by PP1 (Fig. 4B). PP1 is a Src family kinase inhibitor previously shown to abolish GPVI-dependent platelet aggregation (29).

Alborhagin-Induced Signaling in Platelets—Treating [32P]orthophosphate-loaded platelets with alborhagin at 10 µg/ml resulted in phosphorylation of the previously characterized protein kinase C substrates, p47/pleckstrin (30) and myosin light chain (31) on a time scale comparable to that induced by collagen (data not shown). Additionally, alborhagin stimulated tyrosine phosphorylation of a range of proteins, measured using an anti-phosphotyrosine antibody (Fig. 5A). This platelet phosphorylation profile resembled that induced by collagen or convulxin (Fig. 5B), a venom protein of the C-type lectin family previously shown to activate platelets by an interaction with GPVI (13, 14). For both alborhagin and convulxin stimulation, prominent phosphorylated bands were observed at ~135, ~72, ~36, and ~12 kDa. The
time course for convulxin-induced phosphorylation peaked at 15 sec before declining. In contrast, alborhagin stimulated a slower, sustained increase in tyrosine phosphorylation reminiscent of responses to collagen and collagen related peptide. The transient nature of the increase in tyrosine phosphorylation induced by convulxin is thought to be related to its more powerful stimulatory action (32). PLCγ2 was identified as being specifically phosphorylated in response to both alborhagin and convulxin, a response that was blocked by PP1 (Fig 6) along with the increase in whole cell phosphorylation (not shown). Of the other phosphorylated bands, it is probable that the 12, 36 and 72 kDa bands phosphorylated both by alborhagin and convulxin corresponds to the FcRγ, LAT and Syk/SLP76 respectively, based on previous studies (13, 14, 32).

Interaction of Alborhagin with GPVI-Expressing Cells–The similar pattern of tyrosine phosphorylation and sensitivity to PP1 in platelets treated with alborhagin implicated GPVI in the mechanism of alborhagin activity. We therefore examined the activity of alborhagin on two cell lines transfected with GPVI. In FcRγ-expressing K562 cells transfected with GPVI (10) alborhagin induced concentration dependent GPVI-dependent phosphorylation of Syk whereas there was no response in mock transfected cells (Fig. 7A). K562 cells contain no endogenous GPIb-IX-V or GPVI expression as assessed by flow cytometry (data not shown), suggesting GPVI was specifically required for alborhagin-induced Syk phosphorylation in this system. Alborhagin also induced elevation of cytosolic Ca^{2+} specifically in GPVI-transfected Jurkat cells but not in untransfected cells (Fig. 7B). Jurkat cells do not express GPVI or FcRγ, although the role of the latter is taken by the T cell receptor ζ chain (Tulasne, Bori and Watson, unpublished). These results provide additional evidence that alborhagin is targeting GPVI.
Effect of Alborhagin on Convulxin Binding to GPVI—Binding of convulxin (1 µg/ml) to GPVI-expressing K562 cells was partially inhibited by alborhagin in a dose-dependent manner. There was ~40% inhibition at a final concentration of 10 µg/ml alborhagin, with no increased inhibition up to 100 µg/ml (Fig. 8A). Similar experiments using GPVI-expressing Jurkat cells showed there was only minimal displacement of convulxin binding by 10 µg/ml alborhagin, with no further inhibition at 100 µg/ml (Fig. 8A). Together, these data suggest that while alborhagin and convulxin may both bind GPVI, they may recognize separate binding sites. This is supported by studies on mouse platelets using a rat monoclonal antibody that binds GPVI, JAQ1. JAQ1 has previously been shown to specifically inhibit aggregation of mouse platelets by collagen related peptide (CRP) and low concentrations of collagen but not by convulxin or high concentrations of collagen (19, 33, 34). JAQ1 inhibited alborhagin-induced shape change and aggregation of mouse platelets (Fig. 8B) suggesting that it binds to a site that is the same or located close to that used by collagen related peptide but which is distinct from that used by convulxin. Convulxin binding is not blocked by JAQ1 (34) implying that alborhagin is binding to a distinct epitope. In this regard, the effect of JAQ1 is consistent with alborhagin binding to the CRP binding site, whilst convulxin may bind to the site used by the second binding site in collagen (33).

DISCUSSION

In the present study, we isolated a novel protein termed alborhagin from Trimeresurus albolabris venom that is functionally related to alboaggreggin-A and
convulxin in that it potently activated platelets by an interaction with GPVI, but in contrast to these C-type lectin-like proteins, alborhagin was structurally different. Several lines of evidence suggested alborhagin may be a member of the metalloproteinase-disintegrin family. Firstly, the molecular mass of alborhagin on SDS-polyacrylamide gels (~60 kDa, nonreduced and reduced), and its metalloproteinase activity (EDTA inhibitable) towards fibrinogen α-chain, was consistent with other metalloproteinase-disintegrins (17, 22, 25, 26, 35). Secondly, alborhagin was immunoreactive towards an antibody against the cobra venom metalloproteinase, mocarhagin (17). Thirdly, the sequences of four peptides throughout the length of alborhagin revealed similarity to jararhagin, HR1B and MT-d of the metalloproteinase-disintegrin family (26-28). This combined evidence suggests alborhagin is very likely to be a metalloproteinase-disintegrin, although in the absence of the full sequence, we cannot exclude the possibility that it is a closely related protein. Like MT-d, a venom metalloproteinase-disintegrin from Agkistrodon halys brevicaudus (28), alborhagin underwent autolysis yielding an ~42-kDa fragment. Autolysis was prevented by the presence of EDTA, suggesting it involved the metalloproteinase activity of alborhagin. The N-terminal sequence of the 42-kDa fragment corresponded to sequences at the N-terminus of two naturally occurring jararhagin-derived fragments, one-chain botrocetin (36) and jaracetin (37). This result supports the possibility that autodigestion might be a general mechanism for processing venom metalloproteinases.

Several lines of evidence demonstrated that alborhagin was a potent platelet agonist, and that like convulxin, it appeared to be targeting GPVI. Firstly, alborhagin induced platelet aggregation that was αIIbβ3-dependent, since it was inhibitable by a blocking anti-αIIbβ3 monoclonal antibody (CRC64). Secondly, alborhagin activated protein kinase C (PKC) as shown by phosphorylation of p47/pleckstrin and myosin light
Thirdly, alborhagin induced a tyrosine phosphorylation profile similar to that induced by convulxin (this study; 12-14, 38-40). One of these tyrosine phosphorylated proteins PLC<sub>γ2</sub> was identified as being phosphorylated in response to both convulxin and alborhagin. PLC<sub>γ2</sub> phosphorylation induced by either agonist was inhibitable by the Src kinase family inhibitor, PP1. Importantly, PP1 also blocked both alborhagin- and convulxin-dependent platelet aggregation.

Additional evidence that alborhagin could target GPVI was provided by studies showing that alborhagin, like convulxin, induced GPVI-dependent phosphorylation of Syk in FcRγ-expressing K562 cells co-transfected with GPVI, but not in untransfected cells. Alborhagin also induced elevation of cytosolic Ca<sup>2+</sup> in Jurkat cells expressing recombinant GPVI, but had no effect in untransfected cells. Interestingly, although both alborhagin and convulxin appeared to target GPVI, alborhagin only partially inhibited (by ~40%) convulxin binding to recombinant GPVI expressed on K562 cells, had a negligible effect on binding to Jurkat cells, suggesting the proteins may recognize separate sites. This was supported by the observation that alborhagin dependent aggregation of mouse platelets was inhibited by an anti-mouse GPVI monoclonal antibody, JAQ1. This antibody also blocks aggregation to collagen related peptide (CRP) and low concentration of collagen but not convulxin (34).

Finally, the observation that structurally distinct viper venom proteins, convulxin and alboaggrecin-A of the C-type lectin family (10, 11, 13, 14) and alborhagin (this study) may target distinct sites at the same receptor (GPVI) parallels other viper venom proteins that target distinct sites on vWF. Two-chain botrocetin of the C-type lectin family, and jararhagin of the metalloproteinate-disintegrin family interact with vWF at distinct sites enabling it to bind GPIb-IX-V (35). These latter observations may be
related to recent evidence suggesting C-type lectin-like proteins and metalloproteinase-disintegrins might be derived from a common precursor (41).

In conclusion, we have identified a novel viper venom protein, alborhagin, that is a potent platelet agonist. Alborhagin is functionally related to convulxin in that it appears to target GPVI, but is structurally different and appears to target the collagen receptor at a distinct site thereby making it a novel probe for analysis of GPVI-dependent platelet activation.

Acknowledgments—We gratefully acknowledge the excellent technical assistance of Ms Carmen Llerena and Ms Mary Matthew.
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FOOTNOTES

* The National Health & Medical Research Council of Australia and the British Heart Foundation supported parts of this work. S.P.W. is a British Heart Foundation Senior Research Fellow.

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\(^1\)The abbreviations used are: BSA, bovine serum albumin; CRP, collagen related peptide; Da, Dalton; FeR\(_\gamma\), Fc receptor \(\gamma\) chain; GP, glycoprotein; PLC\(_\gamma\)2, phospholipase C\(_\gamma\)2; PKC, protein kinase C; vWF, von Willebrand Factor.
FIG. 1. **Characterization of alborhagin.** (A) SDS–5-20% polyacrylamide gel of purified alborhagin electrophoresed under reducing conditions and stained with Coomassie blue. (B) Western blot of mocarhagin (5 µg) and alborhagin (5 µg) electrophoresed on SDS–5-20% polyacrylamide gels under reducing conditions, transferred to nitrocellulose, probed with rabbit anti-mocarhagin antibody, and visualized using a peroxidase-coupled goat anti-rabbit IgG and the ECL reagent. (C) Digestion of fibrinogen (100 µg/ml) by 10 µg/ml alborhagin at 22 °C for 80 min in the presence of either 10 mM EDTA or 10 mM Ca²⁺.

FIG. 2. **Amino acid sequence analysis of alborhagin.** (A) Amino acid sequences of tryptic peptides of alborhagin compared with sequences from jararhagin (26) (residues 4-14, 50-61 and 67-80), HR1B (27) (residues 3-12, 48-59 and 65-78) and MT-d (28) (residues 193-202, 239-250 and 256-269). (B) N-terminal sequence of the 42-kDa fragment of alborhagin compared with jararhagin residues 206-223 (26) and MT-d residues 393-410 (28). The boundary between the metalloproteinase and disintegrin domains of jararhagin is indicated. Identical residues or conservative substitutions are boxed.

FIG. 3. **Autodigestion of alborhagin.** (A) Time course for autodigestion of alborhagin at 4 °C. Samples at each time point were electrophoresed under reducing conditions on SDS–5-20% polyacrylamide gels and stained with Coomassie blue. (B) Alborhagin treated under the same conditions as in panel A, except in the presence of 10 mM EDTA.
FIG. 4. Alborhagin-induced platelet aggregation. (A) Aggregation of platelet-rich plasma at 37 °C induced by alborhagin (arrow) at the indicated concentrations, or by 15 µg/ml alborhagin after pretreating platelets for 5 min with anti-αIIbβ3 monoclonal antibody, CRC64 (final concentration, 20 µg/ml) or EDTA (final concentration, 10 mM). (B) Aggregation of washed human platelets (2x10^8/ml) that were pre-incubated with vehicle (0.1% DMSO) or 10 µM PP1 prior to stimulation with a final concentration of 7.2 µg/ml alborhagin.

FIG. 5. Platelet phosphorylation in response to alborhagin or convulxin. Washed platelets (2 x 10^8/ml) were treated with (A) 3 µg/ml alborhagin or (B) 1 µg/ml convulxin for the time indicated. Reactions were terminated by addition of SDS-PAGE sample buffer and electrophoresed on SDS–8-18%-polyacrylamide gels under reducing conditions and visualized using an anti-phosphotyrosine antibody, 4G10.

FIG. 6. Effect of PP1 on alborhagin- or convulxin-induced PLCγ2 phosphorylation. Washed platelets (2 x 10^8/ml) were pre-incubated with vehicle (0.1% DMSO) or 10 µM PP1 for 5 min, then treated with 3 µg/ml alborhagin for 90 s, or 1 µg/ml convulxin for 30 s. Lysates were immunoprecipitated with anti-PLCγ2 monoclonal antibody, and analyzed by immunoblotting with either the anti-phosphotyrosine antibody 4G10 (top panel), or following stripping of the membrane, the anti-PLCγ2 antibody (lower panel).

FIG. 7. Interaction of alborhagin with GPVI-transfected cells. (A) K562 cells stably co-transfected with either empty pRc plasmid and pMG-FcRγ (pRc/γ-chain), or
pRe-GPVI and pMG-FcR γ-chain (GPVI/γ-chain), were untreated or stimulated with 2, 5, or 15 µg/ml alborhagin for 90 s. Cells were lysed and subjected to Syk immunoprecipitation, then immunoblotted with the anti-phosphotyrosine antibody, 4G10. Membranes were stripped and blotted again with an anti-Syk antibody. The results are representative of two experiments. (B) Mobilization of cytosolic Ca\(^{2+}\) in Jurkat cells either untransfected or transfected with GPVI.

FIG. 8. Alborhagin binding to GPVI in competition with convulxin or JAQ1. (A) Effect of alborhagin on binding of convulxin (1 µg/ml) to GPVI-expressing K562 (solid circles) or Jurkat (open circles) cells. Convulxin binding was assessed using FITC-labeled anti-convulxin IgG, and results are expressed relative to maximal binding measured in the absence of alborhagin. (B) Aggregation of mouse platelets (1 x 10^8/ml) that were pre-incubated with buffer only or 10 µg/ml JAQ1 prior to stimulation with a final concentration of 7.2 µg/ml alborhagin.
Figure 2

A

alborgan: ...R/K Y I P Y D Q E D V K...
jararhagin: ...R/Y D P Y K Y L E F F...
HR1B: ...R - E P R Y I K L A...
MT-d: ...R F - P Q R Y I E L V...

alborgan: ...R/K Q M - V A A I G I X D...
jararhagin: ...L Y M H V A L V G L E I...
HR1B: ...L N I L V A L V Y L E I...
MT-d: ...L N V R V S L T E L E I...

alborgan: ...R/K N T V T R A N Y Y T L D L...
jararhagin: ...K I T V K P D V D Y T L N S...
HR1B: ...K I T V Q S A S N V T L D L...
MT-d: ...L I N V Q S A A D T L E A...

B

42-kDa albo: X I D T V T P D V N R N E L K E K...
jararhagin: ...L G T D I S P P V C G N E L L E V...
MT-d: ...L R T D T V S T P V S G N E L L E A...

metalloprot. ↔ Disint.
Figure 3
Figure 4

A

25 µg/ml  
15 µg/ml  
7.5 µg/ml  
2.5 µg/ml  
15 µg/ml + CRO64  
15 µg/ml + EDTA

1 min

B

vehicle

PP1
Figure 5
Figure 6
**Figure 7**

**A** Syk Phosphorylation (K562)

| IP: Syk | pRc/\(\gamma\)-chain | GPVI/\(\gamma\)-chain |
|---------|-----------------------|-----------------------|
| Blot: 4G10 |  |  |
| Blot: Syk |  |  |

Alborhagin (\(\mu g/ml\))

**B** Ca\(^{2+}\) Mobilization (Jurkat)

![Graph showing Ca\(^{2+}\) mobilization](image)
Figure 8
A novel viper venom metalloprotease, alborhagin, is an agonist at the platelet collagen receptor GPVI

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J. Biol. Chem. published online May 8, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011352200

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