An echistatin C-terminal peptide activates GPIIbIIIa binding to fibrinogen, fibronectin, vitronectin and collagen type I and type IV

Paul S. WRIGHT,* Vladimir SAUDEK, Thomas J. OWEN, Scott L. HARBESON and Alan J. BITONTI
Marion Merrell Dow Research Institute, Cincinnati, OH 45215-6300, U.S.A.

Integrin binding to proteins often involves recognition of domains containing the arginine-glycine-aspartate (RGD) motif. Different binding affinities and specificities of the integrin–ligand protein interactions involve additional protein domains. The n.m.r. structure of the snake-venom protein echistatin suggested that the C-terminal portion of the molecule might be important, in addition to the RGD domain, in binding to the integrin glycoprotein IIbIIIa (GPIIbIIIa) [Saudek, Atkinson and Pelton (1991) Biochem. **30**, 7369–7372]. The synthetic C-terminal peptide, echistatin-(40–49), PRNPHKGPAT, (1) inhibited binding of GPIIbIIIa to immobilized echistatin (IC_{50} 3–6 mM), but did not inhibit binding of GPIIbIIIa to immobilized fibrinogen (up to 5 mM peptide), (2) activated GPIIbIIIa binding to fibronectin and vitronectin, usual ligands for the activated integrin, (3) activated binding of GPIIbIIIa to collagen type I and type IV, proteins not usually regarded as ligands for the integrin, and (4) stimulated ^{125}I-fibrinogen binding by human platelets. These findings argue for an interaction of this non-RGD domain in echistatin with GPIIbIIIa, leading to activation of the integrin and extension of the ligand specificity to include immobilized collagen.

INTRODUCTION

Integrins are receptors consisting of α- and β-subunits which can mediate a wide variety of cell–cell and cell–substrata adhesive interactions (Hynes, 1987). The arginine-glycine-aspartate (RGD) sequence is an important recognition element in many of these interactions (Ruoslahti and Pierschbacher, 1987). Additional sequence elements in the protein ligands, besides the RGD element, are important in generating different binding specificities to the various integrins (Yamada, 1991). For instance, the CS1 region of fibronectin accounts for about 40% of the binding activity of the whole molecule and contains a critical LDV sequence, which, when truncated, leads to a 20–25-fold loss of binding activity of the CS1 domain to $\alpha_4\beta_1$ integrin (Humphries et al., 1987; Komoriya et al., 1991).

Glycoprotein IIbIIIa (GPIIbIIIa), also designated $\alpha_{IIb}\beta_3$, is one of the best characterized integrins. It comprises approx. 1–2% of the total protein in human platelets (Jennings and Phillips, 1982). GPIIbIIIa mediates binding of resting platelets to immobilized fibrinogen (Savage and Ruggeri, 1991) and binding of activated platelets to soluble fibrinogen, fibronectin, vitronectin and von Willebrand factor (Phillips et al., 1988). The integrin can be activated both *in situ* in platelet cell membranes and *in vitro* (purified) for binding these soluble ligands by pretreatment with small RGD peptides (Kouns et al., 1990; Du et al., 1991; Phillips et al., 1991). The activation involves the cytoplasmic domain of the integrin in that truncation of that part of the α_{IIb} subunit increases the affinity of GPIIbIIIa for ligand in transfected CHO (Chinese-hamster ovary) cells (O'Toole et al., 1991). However, since RGD peptides can activate the purified integrin, modifications of the cytoplasmic domain are not required for this phenomenon (Du et al., 1991). In addition to its obvious role in haemostasis, activation of GPIIbIIIa may have other roles, because this integrin can also mediate ligand bridging with another fibrinogen-binding integrin, $\alpha_v\beta_3$, the vitronectin receptor (Gawaz et al., 1991). Those authors proposed that this type of interaction may mediate physiologically relevant heterotypic cell–cell adhesion between platelets with endothelial cells or tumour cells.

Echistatin, a small RGD-containing protein isolated from saw-scaled-viper (*Echis carinatus*) venom, binds with high affinity to GPIIbIIIa. The n.m.r. solution structure of this protein suggested that the C-terminal portion was important for specificity of the echistatin-integrin binding (Saudek et al., 1991). This peptide sequence (residues 40–49), PRNPHKGPAT, resembles a collagen-like polyproline helix. Here we show that this C-terminal echistatin-(40–49)-peptide inhibits echistatin–GPIIbIIIa binding and activates the integrin to bind immobilized vitronectin, fibronectin, collagen (type I and IV) and increases fibrinogen binding to peptide-treated platelets.

MATERIALS AND METHODS

GPIIbIIIa enzyme-linked immunoassay

Low-solubility fibrinogen, prepared as described previously (Lipinska et al., 1974) (human, KABI), fibronectin (human, Boehringer Mannheim), vitronectin (human, Telios), collagen type I (bovine, Collagen Corp.), and collagen type I and type IV (human, Collaborative Research) were immobilized at 2–5 μg/well on Immunolon 2 96-well plates (Dynatech). Echistatin (Sigma) was immobilized at 1.5 μg/well. Each protein was left overnight at 4 °C to bind to the wells. The wells were blocked with 0.5% BSA in buffer A [20 mM Tris/HCl (pH 7.5)/2 mM $CaCl_2$/120 mM NaCl/0.02% NaN_3] for 2 h at room temperature. All subsequent steps were carried out at room temperature. The wells were washed three times with buffer A plus 0.5% Tween 20 (Bio-Rad). Purified GPIIbIIIa (2 μg/well) plus synthetic peptides supplemented at the indicated concentrations were coincubated for 90–120 min (unless otherwise stated) with immobilized proteins. The wells were washed as described above, then the anti-GPIIbIIIa monoclonal antibody, CD41a, was added to each well and incubated for 60 min. After

Abbreviations used: GP, glycoprotein; BOC, t-butoxycarbonyl; Bzl, benzyl; Chx, cyclohexyl; Tos, tosyl; Cl-Z, chlorobenzyloxycarbonyl.
* To whom correspondence should be addressed.

another three washes, goat anti-mouse IgG–horseradish peroxidase conjugate was added to each well (60 min). The wells were washed three times with buffer A plus detergent, followed by two washes with buffer A minus detergent. The peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine (Kirkegaard and Perry Laboratories) was added to the wells for colour development. The reaction was stopped with 0.6 M H_2SO_4, and absorbances were measured at 450 nm using a microplate reader (Multiscan II; Flow-Titertek).

GPIIbIIIa purification

GPIIbIIIa was purified from human platelets as described by Fitzgerald et al. (1985), except that the final gel-purification step was performed with an HW55 size-exclusion column (Waters Advanced Protein Purification System) instead of Sephacryl S-300. The protein, greater than 90% pure as assessed by SDS/PAGE, was stored at −80 °C in small aliquots.

Peptide synthesis

The echistatin C-terminus peptides and the corresponding scramble peptide were synthesized at the Marion Merrell Dow Research Institute by solid-phase methods with an Applied Biosystems model 430A peptide synthesizer using the appropriate t-butoxycarbonyl (Boc-)amino acid Pam resin and protocols supplied by the manufacturer. Boc-amino acids were used with the following side-chain protections: Thr(Bzl), Asp(Chx), Arg(Tos), Ser(Bzl), Glu(Bzl), Lys(Cl-Z), His(π-benzyloxymethyl). All peptides were cleaved from the resins and deprotected using anhydrous HF containing 5% anisole at −5 °C for 40 min. The peptides were purified by preparative h.p.l.c. using a Dynamax C_{18} column (21.4 mm × 250 mm; Rainin) with various gradients of acetonitrile and aqueous trifluoroacetic acid. The purity and identity of the peptides were assessed by analytical h.p.l.c. (Vydac 218TP54 column; 4.6 mm × 250 mm), by amino acid analyses of the peptide hydrolysates and by fast-atom-bombardment m.s. Other peptides used in these studies were commercially available (Bachem Biosciences).

Preparation of human platelets

Human blood was collected by venipuncture in tubes containing 0.1 vol. of acid/citrate/dextrose as an anticoagulant. Platelet-rich plasma was prepared by centrifugation of the blood for 10 min at 500 *g* at room temperature. The platelet-rich plasma was decanted and 0.1 vol. of acid/citrate/dextrose was added, followed by centrifugation at 1000 *g* at room temperature for 10 min to sediment the platelets. The platelets were suspended in 2 ml of modified Tyrode's buffer (2 M NaCl/0.5 M dextrose/0.2 M $NaHCO_3$/0.1 M KCl/0.1 M $MgCl_2$/0.1 M NaH_2PO_4/0.1 M Hepes, pH 7.3), containing 0.35% BSA and then filtered on a 50 ml column of Sepharose 2B (Pharmacia) equilibrated with Tyrode's buffer. Finally, the platelets were counted in an automated haematology analyser.

Fibrinogen iodination

Human low-solubility fibrinogen (Kabi) was prepared as described previously (Lipinska et al., 1974). For ^{125}I labelling, fibrinogen (1 mg) was incubated with three Iodo-Beads (Pierce Chemical Co.) and 1 mCi of $Na^{125}I$ for 15 min, after which ^{125}I-fibrinogen was separated from free radioactivity by filtration through a PD-10 column (Pharmacia). The specific radioactivity was approx. (1–5) × 10^{17} c.p.m./mol of fibrinogen.

Fibrinogen binding assay

Binding of fibrinogen to human platelets was performed as described by Plow and Ginsberg (1981). Binding assay mixtures contained, in a volume of 0.2 ml, (1–2) × 10^7 platelets, 0.1 μM ^{125}I-fibrinogen, 2 mM $CaCl_2$ and 0.1 unit/ml thrombin or various concentrations of activator peptides as a stimulus. Incubations were carried out in 1.2 ml Eppendorf microcentrifuge tubes at room temperature for 30 min, and then duplicate 75 μl aliquots were layered on to 0.4 ml of 20% sucrose in Tyrode's buffer containing 1% BSA, and platelets were sedimented by centrifugation at 10000 *g* for 5 min in a Beckman microcentrifuge. Tips of the centrifuge tubes were cut off, and bound ^{125}I-fibrinogen was measured in a γ-radiation counter (LKB/Pharmacia). Non-specifically bound radioactivity was determined in incubations in which activators were excluded and these values were 1–5% of those obtained with thrombin activation.

RESULTS

In order to test the interaction of the C-terminal echistatin peptide with GPIIbIIIa we tested the ability of the purified integrin to bind to echistatin immobilized on microtitre plates. GPIIbIIIa bound specifically to echistatin presented in this manner, in that this interaction was competitively inhibited by echistatin in solution with an IC_{50} of 3–6 nM (Figure 1). This IC_{50} is approximately the same as that measured for inhibition by echistatin of GPIIbIIIa–fibrinogen binding (results not shown). The peptides, PRNPHKGPAT, representing the echistatin C-terminus, and an N-acetylated alanine-containing analogue of this peptide, Ac-APRNPHKGPAT, inhibited binding of GPIIbIIIa to echistatin (IC_{50} 3–6 mM; Figure 2), but did not inhibit the binding of GPIIbIIIa to fibrinogen at up to 5 mM. This lack of inhibition of the GPIIbIIIa–fibrinogen binding extended through a wide range of immobilized fibrinogen levels, whereas echistatin itself effectively inhibited the binding throughout the range of fibrinogen levels where detection of GPIIbIIIa binding was possible with the enzyme-linked immunoassay (10 ng of fibrinogen/well; results not shown). A 'scrambled' version of the echistatin peptide, HTPNAKPRPG, had no effect (up to 5 mM) on GPIIbIIIa binding to immobilized echistatin. Thus the echistatin C-terminal peptide appears specifically to inhibit interaction of GPIIbIIIa with echistatin, even though the IC_{50} for this inhibition is high (millimolar levels) when compared with

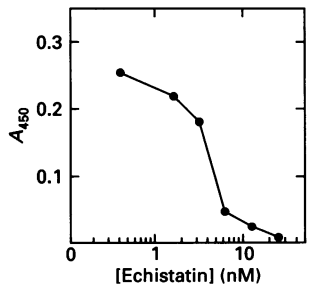

Figure 1 GPIIbIIIa binding to immobilized echistatin; inhibition by co-incubation with echistatin

The ability of GPIIbIIIa to bind immobilized echistatin was measured using the immunoassay described in the Materials and methods section. Echistatin was added at the indicated concentrations as a binding competitor during the GPIIbIIIa incubation step. The mean absorbance value for GPIIbIIIa binding to echistatin in the absence of competitor was 0.240 ± 0.021 (S.D., $n = 6$).

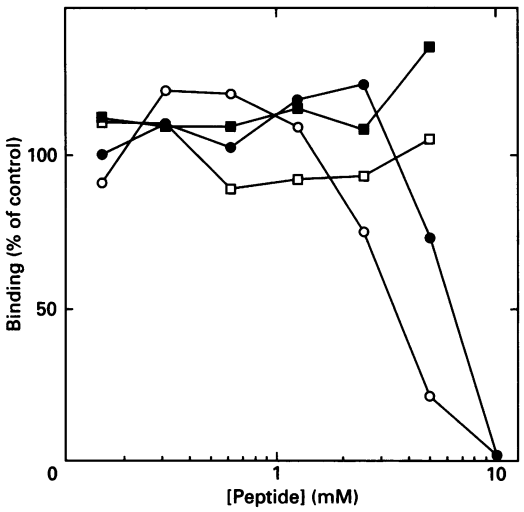

Figure 2 GPIIbIIIa binding to immobilized echistatin and fibrinogen: effect of echistatin C-terminal peptides

Echistatin-(40–49)-peptide (filled symbols) or [A^{39}]echistatin-(39–49)-peptide (open symbols) was added at the indicated concentrations as a binding competitor during the GPIIbIIIa incubation step in the immunoassay described in the Materials and methods section. The mean absorbance value for GPIIbIIIa binding to echistatin in the absence of competitor was 0.240 ± 0.021 (S.D., $n = 6$). The mean absorbance value for GPIIbIIIa binding to fibrinogen in the absence of the echistatin peptides was 0.429 ± 0.022 (S.D., $n = 8$). Each point is the average from duplicate wells coated with 1.5 μg/well echistatin (circles) or 5 μg/well fibrinogen (squares).

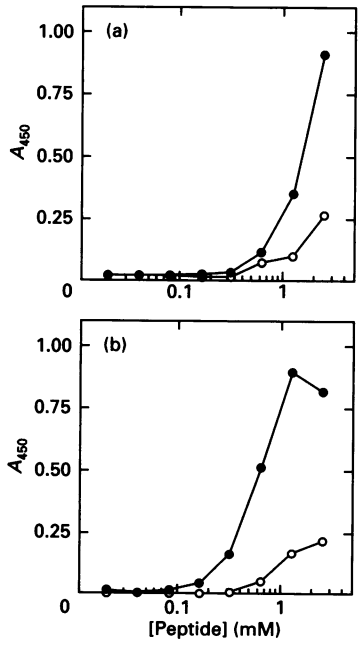

Figure 3 Concentration-dependence of activation of GPIIbIIIa binding to fibronectin and collagen (type I)

GPIIbIIIa was co-incubated with the echistatin-(40–49)-peptide (filled circles) or [A^{39}]echistatin-(39–49)-peptide (open circles) in the presence of immobilized (**a**) fibronectin or (**b**) collagen type I. The level of GPIIbIIIa binding was measured with the immunoassay described in the Materials and methods section.

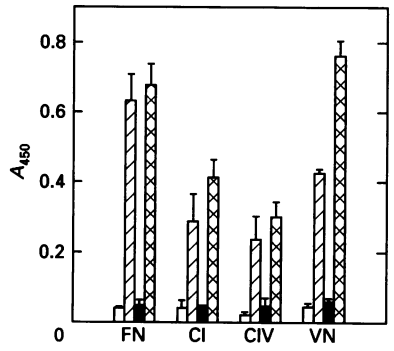

Figure 4 Activation of GPIIbIIIa binding to immobilized extracellular matrix proteins with echistatin peptides

GPIIbIIIa was co-incubated with the indicated immobilized proteins with no additions (□), with 2 mM echistatin-(40–49)-peptide (▨), with 2 mM echistatin peptide plus 20 mM EDTA (■) or with 2 mM echistatin peptide plus 200 μM GRGDTP peptide (⊠). The immobilized proteins (all at 4 μg/well) included fibronectin (FN), collagen type I (CI), collagen type IV (CIV) and vitronectin (VN). Each bar represents the mean plus or minus the standard deviation from three replicates using the immunoassay as described in the Materials and methods.

Table 1 Effect of RGD peptides on activated GPIIbIIIa binding to fibronectin and collagen type I

Purified GPIIbIIIa was activated with 2 mM echistatin-(40–49)-peptide in the presence of the indicated immobilized proteins (4 μg/well) plus the indicated levels of RGDS or GRGDTP peptides. Relative GPIIbIIIa binding to the immobilized proteins was assessed as described in the Materials and methods section.

Peptide	Concn. (mM)	Relative binding (% of control)*	
		Fibronectin	Collagen I
RGDS	0.1	90	104
	1.0	102	102
	10.0	57	48
GRGDTP	0.1	87	95
	1.0	125	136
	10.0	0	0

* Relative binding (% of control) = activated GPIIbIIIa bound (+RGD peptides)/activated GPIIbIIIa bound (−RGD peptides) × 100. Mean control values [$\pm$S.D. ($n = 4$)] for the activated binding (−RGD peptides) were 0.295 ± 0.013 for fibronectin and 0.194 ± 0.035 for collagen type I.

the concentration of RGD-related peptides necessary to compete for the integrin binding to fibrinogen.

GPIIbIIIa, as mentioned above, can be activated by small RGD peptides to bind RGD-containing proteins other than fibrinogen, including fibronectin, vitronectin and von Willebrand factor. The echistatin-(40–49)-peptide activated GPIIbIIIa binding to fibronectin maximally at approx. 1–2 mM (Figure 3a). The Ac-[A^{39}]echistatin-(39–49)-peptide was not as effective in activating GPIIbIIIa over the same concentration range. The concentration dependence for activation of GPIIbIIIa binding to collagen type I by the echistatin peptides was about the same as that measured for fibronectin (Figure 3b). The echistatin-(40–49)-peptide also activated GPIIbIIIa binding to immobilized vitronectin, and, to a lesser extent, collagen type I and type IV, in addition to fibronectin (Figure 4). The integrin binding to all of these proteins was blocked by removal of bivalent cations with

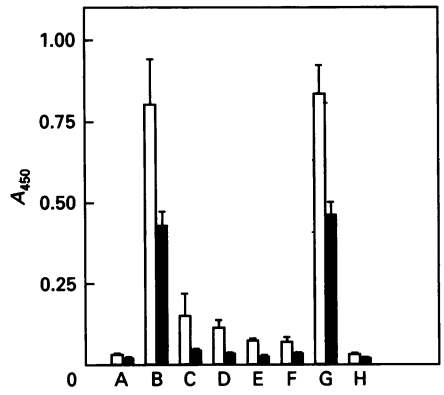

Figure 5 Effect of GPIIbIIIa binding inhibitors on activated binding to fibronectin and collagen type I

GPIIbIIIa was coincubated with immobilized fibronectin (□) or collagen type I (■), both at 5 μg/well, plus: A, no additions; B–H, 2 mM echistatin-(40–49)-peptide. The bars C–H additionally contain: C, 25 nM echistatin; D, 2 mM GPIIIa-(211–222)-peptide; E, 2 mM GPIIb-(296–306)-peptide; F, peptides from D and E mixed 1:1 (1 mM each); G, 200 μM GRGDTP; and H, 20 mM EDTA. Each bar represents the mean plus or minus the S.D. from three replicates using the immunoassay as described in the Materials and methods section.

Table 2 Relative effect of echistatin-(40–49)-peptide and control peptides on activation of GPIIbIIIa binding to fibronectin and collagen type I

Purified GPIIbIIIa was incubated with the indicated peptides in the presence of immobilized fibronectin or collagen type I (5 μg/well). The relative amount of GPIIbIIIa bound to duplicate wells after 2 h co-incubation was assessed as described in the Materials and methods section.

Peptide*	Concn. (mM)	GPIIbIIIa bound (A_{450}) Fibronectin	Collagen I
None	–	0.014	0.004
PRNPHKGPAT†	1.0	0.343	0.395
HTPNAKPRPG	2.5	0.032	0.022
APGPR	2.5	0.014	0.004
PGKAR	2.5	0.015	0.004
GPRP	2.5	0.010	0.003

* Peptide sequences are shown using the one-letter code for amino acids.
† PRNPHKGPAT is the echistatin-(40–49)-peptide.

EDTA. Surprisingly, the activated binding was not inhibited with the peptide GRGDTP (200 μM). In fact, the binding of GPIIbIIIa to vitronectin was enhanced in the presence of GRGDTP (Figure 4). The activated integrin binding to fibronectin or collagen type I was competitively inhibited by the peptides GRGDTP or RGDS at concentrations between 1 and 10 mM (Table 1). The interaction with fibronectin and collagen type I was competitively inhibited by 25 nM echistatin (Figure 5). Binding of the activated integrin to these proteins was also inhibited by either the GPIIIa-(296–306)-peptide, SVSRNRDAPEGG (Charo et al., 1991) or the GPIIb-(296–306)-peptide, TDVNGDGRHDL (D'Souza et al., 1991; Figure 5). These peptides comprise the RGD-binding domains within the α- and β-subunits of the integrin and provide another way to test the specificity of the activated GPIIbIIIa binding. The scrambled version of the echistatin-(40–49)-peptide, HTPNAKPRPG, did not activate GPIIbIIIa binding to fibronectin or collagen type I (Table 2). Other commercially available peptides with similar amino acid composition also failed to activate the integrin.

Table 3 Effect of the C-terminal echistatin peptide on ^{125}I-fibrinogen binding to human platelets

Expt.	Treatment	Radioactivity bound (c.p.m.)*	% Thrombin†
1	None	364	2.8
	Peptide‡ (2 mM)	3491	27.0
	Thrombin	12934	(100)
2	None	328	1.1
	Thrombin	29152	(100)
	Peptide (mM)		
	0.1	297	1.0
	0.2	438	1.5
	0.5	408	1.4
	1.0	1313	9.0
	2.0	6587	21.9

* Mean platelet-associated radioactivity for ^{125}I-fibrinogen from two determinations, measured as described in the Materials and methods section.
† '% Thrombin' equals (platelet-associated radioactivity for the different treatments/platelet-associated radioactivity for thrombin-activated cells) × 100.
‡ Peptide refers to the echistatin-(40–49)-peptide, PRNPHKGPAT.

The echistatin peptide also activated GPIIbIIIa *in situ*, in that human platelets were stimulated to bind ^{125}I-fibrinogen (Table 3). The extent of activation was not as great as that seen with thrombin stimulation (about 24% of thrombin controls), but was greater than that typically measured for ADP-stimulated platelets (about 10% of that for the thrombin controls; results not shown). The peptide GRGDTP inhibited ^{125}I-fibrinogen-specific binding to thrombin-stimulated platelets with an IC_{50} of 10–20 μM (results not shown). The activation of fibrinogen binding to platelets by the echistatin peptide showed about the same concentration-dependence as that observed for activation of purified GPIIbIIIa (maximal activation at about 2 mM; Figures 3a and 3b). The scrambled control peptide, HTPNAKPRPG, and the peptide APGPR failed to activate platelets to bind fibrinogen when added at 2 mM.

DISCUSSION

We tested in the present study a hypothesis that the C-terminal sequence, PRNPHKPAT, of echistatin might exhibit some biological activities. Our work was motivated by a striking observation that echistatin contains two mobile domains protruding from a very rigid disulphide- and hydrogen-bond-cross-linked core (Saudek et al., 1991). One of them is a loop containing the RGD sequence; the other, located in its close spatial proximity, is the C-terminus. Very similar conformational properties were demonstrated independently for kistrin, a homologous snake-venom disintegrin (Adler et al., 1991). Moreover, the inspection of the models of echistatin and kistrin reveals that the C-termini do not play any important role in the stabilization of the three-dimensional folding of the proteins. Yet the alignment of the amino acid sequences of C-termini from several disintegrins shows important sequence similarities (Gould et al., 1990; Figure 6). Such a high degree of conservation suggests that a special activity may reside in their sequences. In addition, a systematic sequential replacement of all amino acids in kistrin (alanine scan) revealed that the proline in the C-terminus is the third most important amino acid (after arginine and aspartic acid in RGD) for the full activity (Genentech, 1989). And finally, removal of the C-terminus from echistatin reportedly reduces its

Echistatin α1	38	C	P	R	N	P	H	K	G	P	A	T
Echistatin α2	38	*	*	*	*	*	*	*	*	*	–	–
Eristicophin	42	*	*	*	*	*	W	N	G	–	–	–
Eristostatin	42	*	*	*	*	*	W	N	–	–	–	–
Kistrin	63	*	*	*	Y	H	–	–	–	–	–	–
Flavoridin	63	*	*	*	W	N	D	L	–	–	–	–
Trigramin α	63	*	*	*	*	*	F	H	–	–	–	–
Trigramin β1	63	*	*	*	*	*	F	H	–	–	–	–
Albolabrin	63	*	*	*	*	*	L	H	A	–	–	–
Batroxostatin	63	*	*	*	*	R	F	–	–	–	–	–
Elegantin	63	*	*	*	*	G	L	Y	S	–	–	–
Applaggin	63	*	*	*	*	*	F	H	–	–	–	–
Bitan α	78	*	*	W	*	H	–	–	–	–	–	–
Bitastatin	78	*	*	W	*	H	–	–	–	–	–	–
Tergeminin	65	*	*	*	*	G	L	Y	*	–	–	–
Barbourin	65	*	*	*	*	G	L	Y	*	–	–	–
Halysin	65	*	*	*	*	*	F	–	–	–	–	–

Figure 6 Comparison of C-termini of snake-venom disintegrins with echistatin α1

Amino acids are abbreviated using the one-letter code. The numbers indicate sequence length preceding the conserved cysteine (C) residue among the snake-venom proteins listed (*, conserved residue; –, residue not present).

binding affinity for GPIIbIIIa (Garsky et al., 1989; Williams et al., 1990).

Echistatin inhibits GPIIbIIIa–fibrinogen binding at nM concentrations, whereas small linear RGD peptides are generally effective at μM concentrations. Our data suggest that this "proline tail" peptide of echistatin may be responsible for this additional modulation of binding affinity. The echistatin C-terminal peptides inhibited binding of the integrin to immobilized echistatin at mM concentrations. This inhibition was selective in that the echistatin peptides did not inhibit GPIIbIIIa binding to fibrinogen. This reduced ability to inhibit binding might be expected for a synergistic or helper protein sequence distinct from the primary RGD-binding site.

The present work also shows that the echistatin-(40–49)-peptide activates, and perhaps alters, the usual ligand specificity of the integrin, GPIIbIIIa. For instance, collagens are not usually considered to be ligands for the integrin; however, type I collagen is an RGD-containing protein. Collagen type IV contains the sequence, GVKGDKGNPGWPGAP, which can promote melanoma cell adhesion, spreading and motility (Chelberg et al., 1990). Recently, it has been shown that GPIIbIIIa can recognize a KGD sequence in the snake-venom peptide barbourin (Scarborough et al., 1991). This KGD sequence, as underlined in the collagen IV peptide shown above, is present in a domain of the protein shown to be important for integrin interaction. RGD peptides have also been shown to expose cryptic collagen receptors on human fibroblasts (Agrez et al., 1991). The exposed collagen receptors (α_v integrins) are normally accessible membrane proteins where the usual binding specificity has been altered to include collagen, as shown for activated-purified GPIIbIIIa in the present paper.

The echistatin-(40–49)-peptide PRNPHKGPAT likely interacts at a distinct site from the RGD-binding site, as GPIIbIIIa binding to fibrinogen is not inhibited, and binding to other RGD containing proteins is stimulated. The activated integrin binding is not inhibited with 200 μM GRGDTP peptide. In fact, GRGDTP (200 μM) appeared to have an additive effect on GPIIbIIIa–vitronectin binding upon co-incubation with the echistatin-(49–49)-peptide. Very high concentrations of RGD peptides were required to compete for the binding to fibronectin and collagen type I (between 1 and 10 mM; Table 1). The binding interaction was specific, however, as both echistatin and the GPIIbIIIa active-site peptides SVSRNRDAPEGG (Charo et al., 1991) and TDVNGDGRHDL (D'Souza et al., 1991) were effective as inhibitors of GPIIbBIIIa binding to fibronectin and collagen type I (Figure 5). The Ac-[A^{39}]echistatin-(39–49)-peptide was about equal to the non-acetylated peptide in its ability to inhibit GPIIbIIIa binding to echistatin, but was much less effective in activating the integrin for binding fibronectin and collagen type I. This suggests that these properties, GPIIbIIIa binding and activation, can be dissociated with peptides. Possibly, antagonists directed at this site on the protein may allow regulation of receptor activation without using the RGD recognition site commonly employed for many integrin interactions.

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