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CHARACTERIZATION OF THE CROSS-LINKING SITE OF DISINTEGRINS ALBOLABRIN, BITISTATIN, ECHISTATIN, AND ERISTOSTATIN ON ISOLATED HUMAN PLATELET INTEGRIN GPIIb/IIIa

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Summary Disintegrins, a family of low molecular weight, RGD-containing peptides found in snake venoms prevent the binding of adhesive ligands to a number of integrin receptors. Albolabrin, bitistatin, echistatin, and eristostatin bind to the platelet fibrinogen receptor (GPIIb/IIIa) acting thus as potent inhibitors of platelet aggregation. Here, we have determined the cross-linking of these disintegrins on isolated GPIIb/IIIa. The cross-linking site of all of them was within GPIIIa 217-302, a domain that has been implicated in a number of receptor functions including heterodimer association, activation-dependent conformational changes, and fibrinogen binding.

Albolabrin (1), bitistatin (2), echistatin (3), and eristostatin (4) belong to the large family of peptides (5-9 kDa) naturally occurring in venoms of viper and rattlesnake species, called "disintegrin", and act as potent platelet aggregation antagonists (4-6). The inhibitory activity of disintegrins is largely determined by an (arginine/lysine)-glycine-aspartic acid (R/KGD) sequence (7,8) within the appropriate disulphide bond-dependent peptide conformation (3,9-11). Structural studies of disintegrins (see ref. 12 and references therein) have shown that their overall structure consists of overlapping loops and loops within loops, and that the RGD sequence is located at the most exposed end of a mobile loop (13) protruding 14-17 Å from the protein core.

On the other hand, the complementary surface of integrin receptors where disintegrins bind has not been defined. The platelet fibrinogen receptor, GPIIb/IIIa or integrin $\alpha_{IIb}\beta_3$, is the most thoroughly studied human integrin (14). Current evidence indicates that its ligand-binding domain is discontinous and located at the subunit interface (15-17). Though soluble fibrinogen (340 kDa) binding to platelet GPIIb/IIIa (225 kDa) requires previous activation of the receptor via a measurable conformational change (18), the RGD-binding site on resting GPIIb/IIIa can be

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reached by both disintegrins (4-6) and peptides that extend out 11-32 Å from the surface of polyacrylonitrile beads (19). Thus, disintegrins are potential molecular probes for exploring the ligand binding site's topology of GPIIb/IIIa or other RGD-dependent integrin receptors.

Here, we have investigated the cross-linking site of disintegrins albolabrin, bitistatin, echistatin, and eristostatin on isolated human platelet GPIIb/IIIa. Our results show that all four disintegrins have their cross-linking site(s) on GPIIIa between residues 217-302. Interpretation of our cross-linking results in the context of an emerging structural model of GPIIb/IIIa is discussed.

Materials and Methods

- GPIIb/IIIa was isolated from outdated human platelets as described (20).
- Disintegrins albolabrin, bitistatin, echistatin, and eristostatin were purified by reverse-phase from the lyophilized venoms of Trimeresurus albolabris (1), Bitis arietans (2), Echis carinatus (3) (Sigma, St.Louis, Mo), and Eristocophis macmahoni (4) Active peptides were identified as described (11). Their purity was assessed by SDS-polyacrylamide electrophoresis and by amino acid analysis (after hydrolysis with 6 N HCl at 110° C for 24 h).
- Purified disintegrins were radiolabeled with ^{125}Iodine using Iodobeads (Pierce Chemical Co., Rockford, IL).Specific activities were: albolabrin, 0.8 μCi/μg (1055940 cpm/μg); bitistatin, 0.21 μCi/μg (278823 cpm/μg); echistatin, 0.28 μCi/μg (376224 cpm/μg); eristostatin, 0.34 μCi/μg (448800 cpm/μg). ^{125}I-Disintegrins (1 mg/ml in 20 mM phosphate, 135 mM NaCl, pH 7.4 (PBS): dimethylformamide, 10:90, v/v) were derivatized at NH_2 groups with the heterobifunctional photoreactive crosslinker N-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA) (Pierce). For cross-linking to GPIIb/IIIa, 50 μg derivatized disintegrin were incubated for 1 h at 37° C with 0.5 mg GPIIb/IIIa in 0.5 ml of 20 mM phosphate, 135 mM NaCl, 1 mM $CaCl_2$, 1% octyl ß,D-glucopyranoside, pH 7.4), following by irradiation of the reaction mixture for 5 min at 340 nm using a 1-kW ultraviolet lamp (17). As a control, the same binding experiment was carried out including 1 mM GRGDS peptide in the buffer. Following SDS-polyacrylamide gel electrophoresis detection of radioactive material was effected by autoradiography using X-Omat films (Kodak) at -70° C.
- Dissociation of the GPIIb/IIIa-disintegrin complexes was done by addition of 12 mg SDS/mg protein, and the glycoprotein-peptide dimers were isolated by size-exclusion chromatography (21). Limited proteolytic digestion of isolated GPIIIa-^{125}I-disintegrin complexes (5 mg/ml in 50 mM ammoniumhydrogen carbonate, 1% octylglucoside, pH 8.0 was performed with trypsin at 1/250 enzyme/substrate ratio as described (22).

Results and Discussion

Sheu et al. (23) have reported that disintegrin triflavin (flavoridin) cross-links exclusively to GPIIIa on ADP-stimulated platelets, but no characterization of the cross-linking site was performed. Here, we examined the cross-linking of four ^{125}I-disintegrins to isolated GPIIb/IIIa. Figure 1 shows that all four disintegrins cross-linked only to GPIIIa. This interaction was RGD-specific as it was completely abolished by including 1 mM GRGDS peptide in the reaction buffer. To analyze the region of GPIIIa where disintegrins albolabrin, bitistatin, echistatin, and eristostatin became cross-linked, the GPIIIa-disintegrin complexes were isolated and subjected to limited tryptic digestion. Figure 2A shows the typical degradation pattern of isolated GPIIIa (22). Only intact GPIIIa and its 120 kDa degradation product (containing fragments 1-216 and 217-(725) disulphide-bonded) had radioactivity. The same result was obtained when each of the four GPIIIa-disintegrin complexes were analyzed. Since a further degradation product of 90 kDa (1-151 disulphide-bonded to 303-725) (22) did not contain bound disintegrin, we concluded that the cross-linking site for disintegrins resides between residues 152 and 302 of GPIIIa. When the

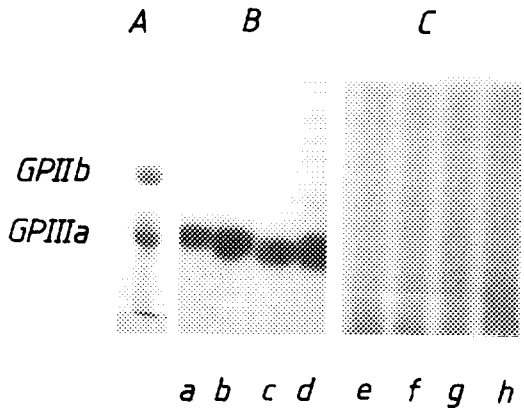

Fig. 1. Analysis of the cross-linking of ASA-^{125}I-disintegrins to isolated GPIIb/IIIa. A) SDS-polyacrylamide (7%) gel electrophoresis of isolated GPIIb/IIIa. B) Autoradiography of complexes of GPIIb/IIIa cross-linked with radiolabeled a) albolabrin; b) bitistatin; c) echistatin; and d) eristostatin. C) Lanes e-h, the same as lanes a-d when the cross-linking experiment was carried out in the presence of 1 mM GRGDS.

tryptic digestion pattern of GPIIIa-^{125}-disintegrins was analyzed under reducing conditions (Fig. 2B) the bulk of the radioactivity was associated with a 95 kDa product. This product corresponds to the large peptide found in the unreduced 120 kDa GPIIIa fragment (residues 217-725) (22). This narrows down the cross-linking site for disintegrins to GPIIIa 217-302.

The GPIIIa region 217-302 is linearly close to sequences that have been involved in GPIIb/IIIa function. Thus, synthetic peptides encompassing residues 211-222 (24) and 217-231 (25) block the binding of adhesive proteins. Peptide 217-230, in addition, also inhibits the binding of radiolabeled albolabrin (26) to GPIIb/IIIa. Furthermore, the mutation GPIIIa Arg^{214}-> Gln results in thrombasthenic platelets with reduced ability to bind RGD peptide ligands (27). GPIIIa 208-231 also contains an activation-dependent epitope (28) and the epitope for monoclonal antibody P40, whose expression is dependent on subunit dissociation, has been mapped to the region 262-302 (29). In addition, regions within GPIIIa 217-298 form part of the subunit interface (15,16). Interestingly, using a cross-linking approach, we have previously showed that some segments of GPIIIa 303-350 and 151-191 are very close (~10 Å) to each other and to the integrin's ligand binding site (17). This latter region is close to the polypeptide stretch 109-171 which contains the cross-linking site of peptide KYGRGDS on GPIIb/IIIa in activated platelets (30), Asp^{119}, whose mutation for Tyr produces a mutant αIIbß3 integrin that lacks ligand recognition (31), and the activation-dependent epitopes recognized by monoclonal antibodies $P_{23}7$ (29) and AC7 (32). It has been proposed that Asp^{119} forms part of a Ca^{2+}-binding site involved in ligand recognition through coordination to the asparte residue of the RGD sequence (33,34). Altogether, these data indicate that the ligand-binding pocket on GPIIb/IIIa may be discontinous and reside at, or near, the subunit interface.

In albolabrin, bitistatin, echistatin and eristostatin all lysine residues, except one in echistatin, are located outside the RGD loop and facing the same side of the molecule. Hence, interpretation of our cross-linking results in the context of the above structural data indicate that GPIIIa 217-302 contains one or more cross-linking site for disintegrins, which are be located some

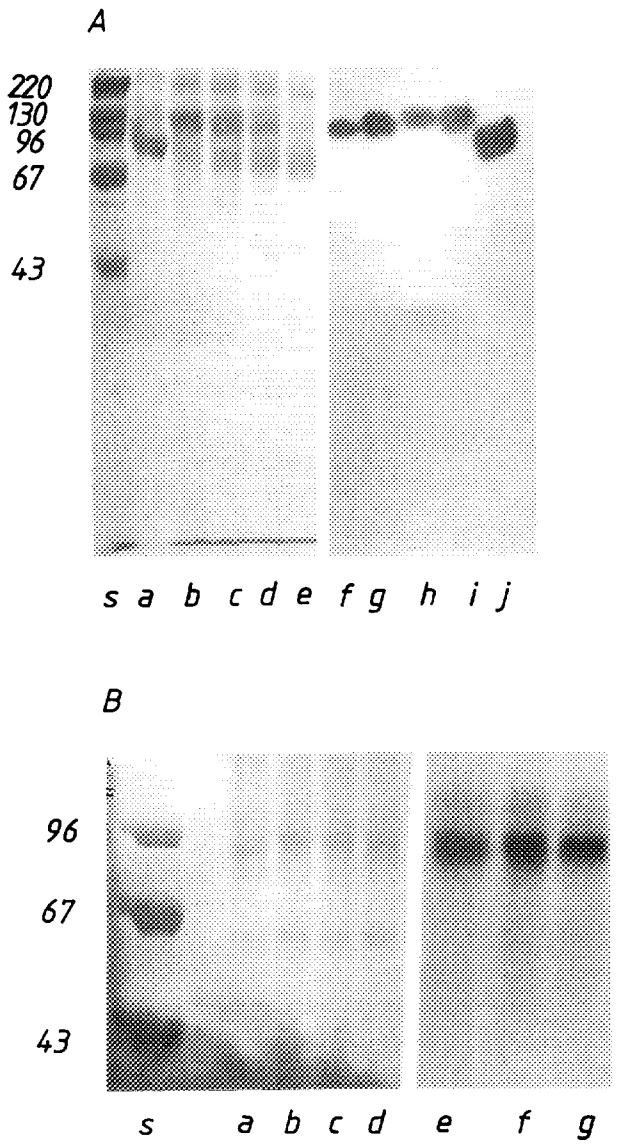

Fig.2. A) Non-reduced SDS-polyacrylamide (12.5%) gel electrophoretic analysis of the time-course digestion of GPIIIa-^{125}I-albolabrin (2 mg/ml in 10 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1% ß,D-octylglucopyranoside, pH 7.4) with TPCK-trypsin (100:1, w/w) at 37° C for a) 0 min; b) 2 min; c) 10 min; d) 15 min, and e) 25 min. Lane s, molecular weight standard. Lanes f-j, autoradiography of lanes e, d, c, b, and a, respectively. B) Lanes a-d, SDS-polyacrylamide (10%) gel electrophoresis of the same samples shown in A) (lanes b-e) analyzed under reducing conditions; lanes e-g, autoradiography of the samples shown in lanes a-c, respectively. Lane s, molecular weight markers.

15-20 Å from the RGD contact site (Fig.3). Our results do not rule out the possibility that GPIIb also possesses part of the complementary surface for binding disintegrins which was, however, not available for cross-linking in our system. Our present data may help define the RGD binding site on GPIIb/IIIa when its three-dimensional structure becomes available.

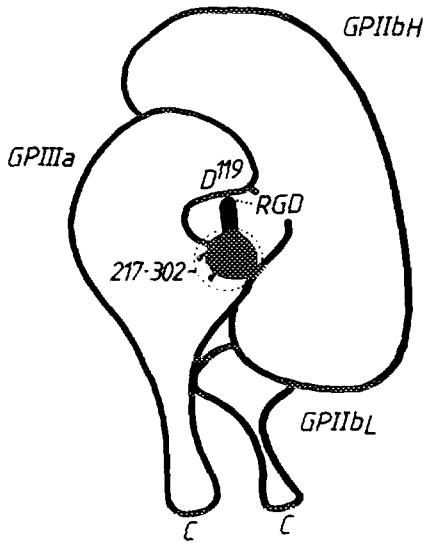

Fig.3. Schematic drawing of the proposed cross-linking of disintegrins to GPIIb/IIIa. It is hypothesized that disintegrins bind to the interface between both subunits of the heterodimer, though only GPIIIa is accesible to the cross-linker(s) (thin lines). The proposed geometrical relationship between cross-linking site(s) (GPIIIa 217-302) and the putative RGD-contact site (around GPIIIa Asp^{119}) is shown. C, C-terminus.

Acknowledgments

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