MINIREVIEW

Disintegrins: A Family of Integrin Inhibitory Proteins from Viper Venoms(43129B)

ROBERT J. GOULD,* MARK A. POLOKOFF,* PAUL A. FRIEDMAN,* TUR-FU HUANG,† JOHN C. HOLT,‡ JACQUELYNN J. COOK,‡,§ AND STEFAN NIEWIAROWSKI‡,§,1

Department of Biological Chemistry, Merck, Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486; Department of Pharmacology, National Taiwan University,† Taipei, Taiwan, Republic of China; and Department of Physiology‡ and Thrombosis Research Center,§ Temple University School of Medicine, Philadelphia, Pennsylvania 19140*

Abstract. **Disintegrins represent a new class of low molecular weight, RGD-containing, cysteine-rich peptides isolated from the venom of various snakes. They interact with the β_1 and β_3 families of integrins and their potency is at least 500–2000 times higher than short RGDX peptides. Analysis of the amino acid sequences of 14 different disintegrins suggests that the RGD sequence, in the spatial configuration determined by the appropriate pairing of the cysteine residues, functions as a cell recognition site. However, certain nonconserved amino acids appear to modify the activity of disintegrins, their specificity for various receptors, and their ability to compete specifically with various ligands.** [P.S.E.B.M. 1990, Vol 195]

Cytoadhesive receptors expressed on cell surfaces are essential for cell-cell interaction and cell adhesion to the extracellular matrix. These receptors, referred to as integrins, comprise a superfamily of transmembrane heterodimeric molecules (1). Examples of members of this family include the platelet fibrinogen receptor (glycoprotein IIb/IIIa) and the vitronectin and fibronectin receptors on endothelial cells and fibroblasts. A number of high molecular weight protein ligands, such as fibronectin, vitronectin, osteopontin, collagens, thrombospondin, laminin, fibrinogen, von Willebrand factor, and complement component C3bi, contain the tripeptide sequence arginine-glycine-aspartic acid (RGD) which represents a common integrin recognition site (2). However, other sequences in these high molecular weight proteins also function as cell recognition sites (3–5).

Recently, a number of low molecular weight, RGD-containing, cysteine-rich peptides have been isolated from the venom of various vipers. Early studies by Taiwanese investigators demonstrated that the venom of *Agkistrodon halys* (6), *Agkistrodon rhodostoma* (7), *Trimeresurus gramineus* (8), and *Echis carinatus* (9) contains peptides which are potent inhibitors of platelet aggregation. Huang *et al.* (7) suggested that the peptide obtained from the *A. rhodostoma* venom inhibits the initiation of the intercellular linkages of platelets. Further studies resulted in the purification and amino acid sequencing of trigramin, which appeared to be a potent inhibitor of platelet aggregation and fibrinogen binding to ADP-stimulated platelets (10, 11). Trigramin also bound to glycoprotein IIb/IIIa on the platelet surface (10). Subsequently, the amino acid sequences of other viper venom peptides (molecular mass, 5400–9000 daltons), including echistatin (12), bitistatin 3 (13), applagin (14), albolabrin (15), elegantin (15), flavoridin (16, 17), and kistrin (18), have been reported. These peptides all contain the RGD sequence, are rich in cysteine, and bind with high affinity to integrins on the surface of platelets and other cells. On a molar basis, the concentrations of viper venom peptides required to cause 50% inhibition of ADP-induced platelet aggregation in platelet-rich plasma was 3,000 to 30,000 times lower than the required concentration of RGDS (16). In other experimental systems, such as

[1] To whom all correspondence and reprint requests should be addressed at Department of Physiology, Temple University School of Medicine, Philadelphia, PA 19140.

0037-9727/90/1952-0168$2.00/0

suspensions of washed platelets, the potency of disintegrins was 500–2000 times higher than that of short RGDX peptides. The snake venom peptides are potent inhibitors of fibrinogen binding to glycoprotein IIb/IIIa (10–14, 18) and of the adhesion of cultured cells to fibronectin. Additionally, human melanoma cells and avian fibroblasts adhere to these peptides when immobilized; this reaction is blocked by an excess of RGDS and antibodies to β_1 integrins (19, 20).

We have compared the amino acid sequences of 14 peptides purified from viper venoms and believe that RGD is a cell recognition site of these peptides. However, the potency of these molecules in inhibiting integrin-ligand interaction is probably a function of both the specific conformation of the RGD sequence and the amino acids which are adjacent to it. Since the amino acids flanking the RGD sequence in small peptides are known to contribute to the specificity of those peptides for different integrins (2), nonconserved residues within the viper-derived peptides may also contribute to integrin selectivity. Along with monoclonal antibodies, these peptides are the most potent known inhibitors of integrin function and we therefore propose that they be named "disintegrins."

Individual disintegrins were purified initially from lyophilized venoms via a three-step procedure of gel filtration, ion exchange chromatography, and reverse phase C18 high-performance liquid chromatography (10–12). Alternatively, purification from crude venom can be achieved by two cycles of reverse phase high-performance liquid chromatography (15). Fractions containing active disintegrins were identified by their ability to inhibit ADP-induced platelet aggregation. The amount of purified disintegrins varied from 1 to 10 mg/g of lyophilized venom (10–13, 15).

The primary sequences of 14 members of the disintegrin family are shown in Figure 1. All disintegrins were isolated from the Viperidae family of snakes, and the names of these integrin inhibitor proteins were modified from the genera of the snakes from which the venoms were obtained. It is interesting to note that the vipers from which the disintegrins have thus far been purified are found on every continent except Australia and include desert-, water-, and forest-dwelling snakes. The disintegrins fall into three subfamilies: a short group containing 48–49 amino acids (echistatin and eristostatin); a medium group which contains 70–73 amino acids (trigramin, albolabrin, elegantin, agkistrostatin, applagin, batroxostatin, flavoridin, and rhodostomin), and a long group with 83–84 residues (bitistatins). The sequence of rhodostomin is identical to that of kistrin reported by Dennis *et al.* (18). In Figure 1, and throughout this report, the numbering of amino acids refers to the numbering of the longest member sequenced to date, bitistatin 3. It is interesting that applagin, recently isolated from the venom of *A. piscivorous piscivorous* (14), has significant differences in amino acid sequence as compared with agkistrostatin, which was isolated from the venom of the same snake (Fig. 1). This raises the possibility that one viper can contain more than one disintegrin in its venom. In addition, Dennis *et al.* (18) isolated four variants of trigramin from the venom of *T. gramineus.* Similarly, bitistatins 1, 2, 3, and 4 (Fig. 1) and bitan α (18) were all isolated from the venom of *Bitis arietans*, the puff adder found in sub-Saharan Africa. Bitan α differs from bitistatin 2 by four amino acids substituted at positions 28, 45, 53, and 58 (18). Since the lyophilized venom from which these proteins are purified is collected and pooled from a population of snakes, we do not know if these variants reflect multiple genes for bitistatin or population variation.

Invariant amino acids for the disintegrin family are shown in Figure 1. Notable is the conservation of cysteine residues at positions 43, 48, 49, 52, 61, 73, and 80; the conservation of the RGD tripeptide at positions 65–67; and the conservation of glycine at position 46, phenylalanine at position 54, aspartic acid at position 70, and proline at position 81. Although not present in the short peptides, cysteine residues at positions 17, 19, 25, 29, 30, and 35 are conserved in the medium and long disintegrins. All disintegrins contain a high proportion of cysteine in disulfide linkages (8 cysteine residues in short disintegrins, 12 residues in medium disintegrins, and 14 residues in bitistatin). In the short disintegrins, an additional cysteine appears at position 78, presumably to maintain disulfide pairing. It may also be significant that the most variable amino acids in these molecules are those close to the RGD sequence and the cysteines in the C-terminal region. A number of amino acids are conserved in the NH_2 terminal domains of the medium and long disintegrins. However, these domains are deleted in short disintegrins.

Disintegrins also show some similarities with other proteins; however, these homologies are limited to only a few members of the disintegrin family. For instance, a region of homology, the tetrapeptide sequence PRNP, is found in the α chain of fibrinogen at positions 267–270 (21) and at the C terminus of trigramin, albolabrin, and echistatin (Fig. 1). The deletion of this sequence from synthetic echistatin (22) reduces its ability to inhibit platelet aggregation. The homology of medium disintegrins, specifically trigramin, with human von Willebrand factor precursor, collagen α_1 (I) and laminin B_1 has been previously reported (11). This comparison cannot be made with echistatin and eristostatin, since the portion corresponding to the N-terminal region of medium-range disintegrins is deleted in these two molecules.

We believe that the RGD sequence represents a cell recognition site of disintegrins, since it is conserved in all molecules and since alterations of these amino

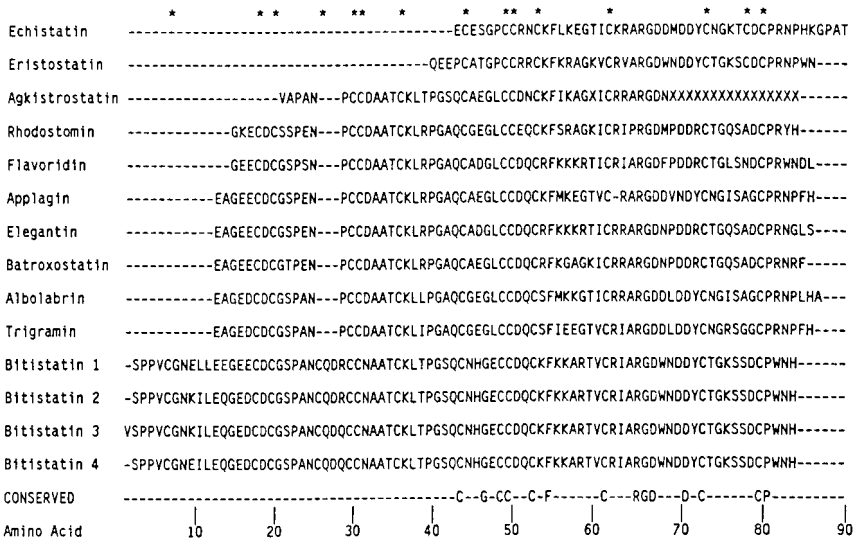

Figure 1. The primary sequences of 14 members of the disintegrin family isolated from the venoms of *E. carinatus* (echistatin; 2); *Eristocophis macmahoni* (eristostatin), *Agkistrodon piscivorous* (agkistrostatin, applagin; 14); *A. rhodostoma* (rhodostomin), *Trimeresurus flavoviridis* (flavoridin; 16, 17); *Trimeresurus elegans* (elegantin; 15); *Bothrops atrox* (batroxostatin; 20); *Trimeresurus albolabris* (albolabrin; 15), *T. gramineus stejnegeri formosensis* (trigramin; 11); *B. arietans* (bitistatin; 13). The disintegrins are arranged in length, with the tripeptide sequence RGD aligned. Cysteine residues are indicated with an asterisk. Sequences were determined as described previously (10–12). Undetermined amino acids are indicated with an X. Numbering refers to the longest member found, bitistatin 3. Amino acids conserved in all disintegrins are shown at the bottom. The amino acid sequence of rhodostomin determined in our laboratory is identical to that of kistrin (18).

acids change the activity of the peptide. For example, we have demonstrated that the replacement of the arginine with ornithine or alanine results in greatly diminished activity of echistatin (22). The spatial configuration of RGD may depend on the tertiary and secondary structures of the molecule as determined by the appropriate pairing of cysteine residues, since reduction and alkylation of the disulfide bridges results in an almost complete disappearance of activity (10–12, 19). This is in agreement with the theory that the appropriate conformation of the RGD sequence is essential for attachment of adhesive proteins to integrins (2, 23). We further propose that although the activity of disintegrins resides primarily with the RGD sequence, other sequences may modify their activity, their specificity for various receptors, and their ability to compete specifically for various ligands. For instance flavoridin (IC_{50} = 40 nM) is about five times more active than trigramin (IC_{50} = 200 n*M*) in blocking ADP-induced platelet aggregation (10, 16). Perhaps this is because of the substitution of Asp 66 (trigramin) with Phe 66 (flavoridin), since it has been reported that RGDF is one of the most potent tetrapeptides at blocking ADP-induced platelet aggregation and fibrinogen binding to platelets (24). Therefore, it appears that both the spatial configuration established by the disulfide linkages and the amino acids adjacent to the RGD sequence contribute to the high potency of disintegrins as compared with that of the linear tetrapeptides. That is, disintegrins are active at nanomolar concentrations whereas the activity of the short RGDX peptides is seen at the micromolar level. In addition, amino acid differences between albolabrin and echistatin may contribute to the differential effects of these peptides on platelet adhesion to artificial surfaces and on platelet aggregation (16). Similarly, the occurrence in trigramin of homologous sequences with von Willebrand factor and the absence of these sequences in echistatin may result in the stronger inhibitory activity of trigramin as compared with that of echistatin in blocking the binding of von Willebrand factor to activated platelets (Lukasiewicz *et al.*, unpublished data).

Recently, echistatin, a short disintegrin, has been obtained by chemical synthesis (22) and by DNA recombinant technology (25, 26). At present, the biological significance of disintegrins in the venom is not known but they do represent a powerful tool for basic and clinical research. Experiments with hamsters (27) and dogs (13, 28, 29) demonstrated that disintegrins prevent the *in vivo* formation of platelet aggregates. In another experimental model, simulated extracorporeal circulation of human blood, we found that disintegrins prevent the loss of platelets by inhibiting their adhesion to silicone rubber surfaces (16). These experiments also demonstrate that disintegrins have a short half-life and are apparently nontoxic (13, 27). In addition, albolabrin showed a potent anti-thrombotic effect due to reduced platelet accumulation in mouse lungs (28). Albolabrin, at the dose of 10 μg per mouse, significantly inhibited the metastases of B-16 melanoma cells in mouse lungs (Soszka *et al.*, unpublished data). Finally, it has been reported that echistatin inhibits bone resorption by interfering with the RGD-dependent activity of the osteoclasts (31). Disintegrins and their analogs may

have potential utility in a variety of clinical situations where the inhibition of integrin function is desired.

Note added in proof. Most recently, Savage *et al.* (32) have confirmed that disintegrins bind to the RGD recognition site on platelet glycoprotein IIa/IIIb complex.

This work was supported in part by NIH Grants HL15226 (S. N.), HL36579 (S. N.), and F05TW03682 (T.-F. H.).

1. Hynes RO. Integrins: A family of cell surface receptors. Cell **48**:549–554, 1987.
2. Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion: RGD and integrins. Science **238**:491–497, 1987.
3. Graf J, Iwamoto Y, Sasaki M, Martin GR, Kleinman HK, Robey FA, Yamada Y. Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis and receptor binding. Cell **48**:989–996, 1987.
4. Tashiro K, Sephel GC, Weeks B, Sasaki M, Martin GR, Kleinman HK, Yamada Y. A synthetic peptide containing the IKVAV sequence from the A chain of laminin mediates cell attachment, migration and neurite outgrowth. J Biol Chem **264**:16174–16182, 1989.
5. Kloczewiak M, Timmons S, Lukas TJ, Hawiger J. Platelet receptor recognition site on human fibrinogen. Synthesis and structure-function relationship of peptides corresponding to the carboxy-terminal segment of the gamma chain. Biochemistry **23**:1767–1779, 1984.
6. Ouyang C, Yeh H-I, Huang T-F. A potent platelet aggregation inhibitor purified from *Agkistrodon halys* (Mamushi) snake venom. Toxicon **21**:797–804, 1983.
7. Huang T-F, Wu Y-J, Ouyang C. Characterization of a potent platelet aggregation inhibitor from *Agkistrodon rhodostoma* snake venom. Biochim Biophys Acta **925**:248–257, 1987.
8. Ouyang C, Huang T-F. Platelet aggregation inhibitor from *Trimeresurus gramineus* snake venom. Biochim Biophys Acta **757**:332–341, 1983.
9. Ouyang C, Ma YJ, Jih HC, Teng CM. Characterization of the platelet aggregation inducer and inhibitor from *Echis carinatus* snake venom. Biochim Biophys Acta **841**:1–7, 1985.
10. Huang T-F, Holt JC, Lukasiewicz H, Niewiarowski S. Trigramin: A low molecular weight peptide inhibiting fibrinogen interaction with platelet receptors expressed on glycoprotein IIb/IIIa complex. J Biol Chem **262**:16157–16163, 1987.
11. Huang T-F, Holt JC, Kirby EP, Niewiarowski S. Trigramin: Primary structure and its inhibition of von Willebrand factor binding to glycoprotein IIb/IIIa complex on human platelets. Biochemistry **28**:661–666, 1989.
12. Gan Z-R, Gould RJ, Jacobs JW, Friedman PA, Polokoff MA. Echistatin. A potent platelet aggregation inhibitor from the venom of the viper, *Echis carinatus*. J Biol Chem **263**:19827–19832, 1988.
13. Shebuski RJ, Ramjit DR, Bencen GH, Polokoff MA. Characterization and platelet inhibitory activity of bitistatin, a potent arginine-glycine-aspartic acid containing peptide from the venom of the viper, *Bitis arietans*. J Biol Chem **264**:21550–21556, 1989.
14. Chao BH, Jakubowski JA, Savage B, Ping Chow E, Marzec UM, Harker LA, Maraganore JM. *Agkistrodon piscivorus piscivorus* platelet aggregation inhibitor: a potent inhibitor of platelet activation. Proc Natl Acad Sci USA **86**:8050–8054, 1989.
15. Williams J, Rucinski B, Holt JC, Niewiarowski S. Elegantin and albolabrin purified peptides from vipor venoms: Homologies with the RGDS domain of fibrinogen and von Willebrand factor. Biochim Biophys Acta **1039**:81–89, 1990.
16. Musial J, Niewiarowski S, Rucinski B, Stewart GJ, Cook JJ, Williams JA, Edmunds LH Jr. Inhibition of platelet adhesion to surfaces of extracorporeal circuits by disintegrins: RGD-containing peptides from viper venoms. Circulation **82**:261–273, 1990.
17. Rucinski B, Niewiarowski S, Holt JC, Musial J, Knudsen K. Flavoridin, a potent inhibitor of platelet aggregation and cell adhesion from venom of *T. flavoridis*. Presented at the International Scientific Symposium on Fibrinogen, Thrombosis, Coagulation and Fibrinolysis. Taipei, Taiwan, 1989.
18. Dennis MS, Henzel WJ, Pitti RM, Lipari MT, Napier MA, Deisher TA, Bunting S, Lazarus RA. Platelet glycoprotein IIb/IIIa protein antagonists from snake venoms: Evidence for a family of platelet aggregation inhibitors. Proc Natl Acad Sci USA **87**:2471–2475, 1990.
19. Knudsen KA, Tuszynski GP, Huang T-F, Niewiarowski S. Trigramin, an RGD-containing peptide from snake venom, inhibits cell-substratum adhesion of human melanoma cells. Exp Cell Res **179**:42–49, 1988.
20. Rucinski B, Niewiarowski S, Holt JC, Soszka T, Knudsen KA. Batroxostatin, a RGD-containing peptide from *B. atrox* is a potent inhibitor of platelet aggregation and cell interaction with fibronectin. Biochim Biophys Acta **1054**:257–262, 1990.
21. Doolittle RF, Watt KWK, Cottrell BA, Strong DD, Riley M. The amino acid sequence of the α chain of human fibrinogen. Nature **280**:464–468, 1979.
22. Garsky VM, Lumma PK, Freidinger RM, Pitzenberger SM, Randall WC, Veber DF, Gould RJ, Friedman PA. Chemical synthesis of echistatin, a potent inhibitor of platelet aggregation from *Echis carinatus*: synthesis and biological activity of selected analogs. Proc Natl Acad Sci USA **86**:4022–4026, 1989.
23. Reed J, Hull WE, von der Lieth C-W, Kubler D, Suhaj S, Kinzel W. Secondary structure of the Arg-Gly-Asp recognition site in proteins involved in cell-surface adhesion. Evidence for the occurrence of nested β-bends in the model hexapeptide GRGDSP. Eur J Biochem **178**:141–154, 1988.
24. Plow EF, Pierschbacher MD, Ruoslahti E, Marguerie G, Ginsberg MH. Arginyl-glycyl-aspartic acid sequences and fibrinogen binding to platelets. Blood **70**:110–115, 1987.
25. Gan Z-R, Condra JH, Gould RJ, Zivin RA, Bennett CD, Jacobs JW, Friedman PA, Polokoff MA. High level expression in *Escherichia coli* of a chemically synthesized gene for (Leu-28) echistatin. Gene **79**:159–166, 1989.
26. Jacobson MA, Forma FM, Buenaga RF, Hofmann KJ, Schultz LD, Gould RJ, Friedman PA. Expression and secretion of biologically active echistatin in *Saccharomyces cerevisiae*. Gene **85**:511–516, 1989.
27. Cook JJ, Huang T-F, Rucinski B, Strzyzewski M, Tuma RF, Williams JA, Niewiarowski S. Inhibition of platelet hemostatic plug formation by trigramin, a novel RGD-peptide. Am J Physiol **266**:H1038–H1043, 1989.
28. Shebuski RJ, Stabilito IJ, Sitko GR, Polokoff MA. Acceleration of recombinat tissue type plasminogen activator induced thrombolysis and prevention of reocclusion by the combination of heparin and the RGD containing peptide bitistatin in a canine model of coronary thrombosis. Circulation **82**:169–177, 1990.
29. Shebuski RJ, Ramjit DR, Sitko GR, Lumma PK, Garsky VM. Prevention of canine coronary artery thrombosis with echistatin, a potent inhibitor of platelet aggregation from the venom of the viper, *E. carinatus*. Thromb Haemost (in press).
30. Poggi A, Rossi C, Soszka T, Beviglia L, Niewiarowski S. Albolabrin, a RGD containing peptide from viper venom is a potent antithrombotic agent in mice. Presented at the International Symposium on Biotechnology of Plasma Proteins. Florence, Italy, April 1990.
31. Sato M, Sardana MK, Grasser WA, Garsky VM, Murray JM, Gould RJ. Echistatin is a potent inhibitor of bone resorption in culture. J Cell Biol (in press).
32. Savage B, Marzec UM, Chao BH, Harker LA, Maraganore JM, Ruggeri ZM. Binding of the snake venom-derived proteins applagin and echistatin to the arginine-glycine-aspartic acid recognition site(s) on platelet glycoprotein IIa-IIIb complex inhibits receptor function. J Biol Chem **265**:11766–11772, 1990.