MINIREVIEW

Viper Venom Disintegrins and Related Molecules (44322)

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Abstract. **The term "disintegrin" was first used in 1990 to describe a group of viper venom–derived, nonenzymatic small proteins that shared numerous structural and functional properties. These proteins, which have been found in a great number of viper species studied since that time possess both a remarkable sequence homology and an equally notable variability in potency and selectivity in their interactions with integrin receptors. The discovery that small disintegrins may actually have been derived from much larger mosaic proteins possessing catalytic activity, and that species other than snakes (both plant and animal) produce proteins containing disintegrin-like domains, has led to much research related to both the proteins themselves and the receptors to which they bind. The purpose of this review is to discuss the literature and the authors' own data on the structure and function of disintegrins and their relevance to the studies on proteins containing disintegrin-like domains, such as hemorrhagins and ADAMs.** [P.S.E.B.M. 1998, Vol 219]

Venoms of various vipers contain three classes of proteins interfering with hemostasis and cell adhesion: GPIb/IX binding proteins, hemorrhagins, and disintegrins. GPIb/IX binding proteins inhibit von Willebrand Factor binding to platelets and may inhibit or induce platelet aggregation (see Ref. 1 for review). Hemorrhagins (hemorrhagic toxins) represent the family of snake venom metalloproteinases (SVMP or reprolysins) and can be divided into four classes: P-I, P-II, P-III and P-IV. The P-I class has only a metalloproteinase domain, whereas the P-II class has a disintegrin or disintegrin-like domain carboxyl to the proteinase domain. In addition, in the P-III class SVMP, the cysteine-rich domain, is adjacent to the carboxyterminal end of the disintegrin-like domain, and the P-IV class SMVP has a lectin domain at the carboxyl terminus (2, 3). Hemorrhagins are related to ADAMs (a disintegrin and metalloproteinase) or MDC (metalloproteinase/disintegrin/cysteine-rich protein) family occurring in mammalian tissues. In contrast to disintegrins and hemorrhagins, ADAMs are cell membrane–anchored proteins. They contain metalloproteinase, disintegrin-like, cysteine-rich, epidermal growth factor–like, transmembrane, and cytoplasmic domains (4, 5) (Fig. 1). ADAMs are believed to play important roles in the processes of fertilization, muscle development, and inflammatory responses.

Since the discovery of the first disintegrin, trigramin (18), this topic has been reviewed in several articles (15, 19, 20). The purpose of this review is to discuss the literature and the authors' own data on the structure and function of disintegrins and their relevance to the studies on proteins containing disintegrin domains, such as hemorrhagins and ADAMs.

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Figure 1. Comparison of the disintegrin-like domains from snake venom metalloproteinase and mammalian reproductive proteins to sequences of monomeric viper venom disintegrins. Cysteine residues are shaded to note conserved sites. The RGD tripeptide position is noted by asterisks. Ht-e (atrolysin E) (6); HR-1B (trimerelysin) (7); EAP-I (8); Jara = jararhagin (9); RVV-X (10): EAP-1 (8); PH-30β (fertilin β) (11); Cyri (cyritestin) (12); bitistat3 (bitistatin 3) (13); trigramin (14); rhodostomin (kistrin) (15); eristocophin (16); echistatin (17). Modified according to Bjarnason and Fox (2). Eristostatin is an analog of eristocophin, shorter by two amino acids.

Overview of RGD-Containing Monomeric Disintegrins

Disintegrins were originally defined as low-molecular-weight (49–84 amino acids), cysteine-rich proteins that contain an RGD/KGD loop maintained in an appropriate conformation by S-S bridges and as potent inhibitors of platelet aggregation. It has been proposed that the disintegrins are formed by proteolytic processing of the homologous precursors of the PII class of snake venom metalloproteinases composed of pre-, pro-metalloproteinase, and disintegrin domains (2, 21). However, it should be noted that in the disintegrin domain of hemorrhagic toxins and ADAMs, the RGD sequence is usually substituted with TDE, EDE, RDE or other sequences. Moreover, cysteine is inserted adjacent to the carboxy terminal end of a sequence corresponding to RGD (Fig. 1).

Originally viper venom disintegrins have been divided into three groups: 1) short disintegrins including echistatin, eristocophin, and eristostatin composed of 49–51 amino acids and 8 cysteines; 2) medium size disintegrins composed of about 70 amino acids and 12 cysteines (this group includes albolabrin, kistrin, trigramin, flavoridin, batroxostatin, elegantin, and barbourin); and 3) long disintegrins including bitistatin composed of 84 amino acids and 14 cysteines (19, 20). This classification does not include dimeric disintegrins such as contortrostatin (22, 23), EMF10 and EC3 (24, 25) that were discovered more recently. To date, more than 30 different disintegrins have been identified. All disintegrins show a high degree of homology with each other in the arrangement of cysteines, except for the extra cysteine found in the C-terminal portion of the molecules of short disintegrins, and the extra disulfide bridge Cys^5-Cys^{24} of bitistatin (26). It has been determined that an RGD sequence occurs in a hairpin loop composed of 13 amino acids, and it is maintained in an appropriate conformation by S-S bridges. NMR studies on the disintegrins albolabrin, echistatin, kistrin, and flavoridin revealed that they possess an RGD sequence at the apex of the mobile loop between two short β strands of the protein, protruding 14 Å–17 Å from the protein core (27–30). The RGD sequence is critical to the expression of biological activity of disintegrins since the substitution of Arg^{24} in echistatin (31) and Arg^{49} and Asp^{51} in kistrin (32) results in almost total loss of disintegrin activity. The disulfide bond pattern of disintegrins has been determined by chemical methods (26, 33) and by NMR spectroscopy (29, 30, 34). The appropriate pairing of cysteines is important for maintaining the RGD loop since the expression of biological activity is completely abolished after reduction and alkylation of these molecules. Calvete *et al.* (26, 33, 35) and McLane *et al.* (36) identified S-S bridges in five disintegrins: albolabrin, flavoridin, echistatin, eristostatin, and bitistatin (Fig. 2). This figure shows that disintegrins exhibit three different disulfide bridge (S-S) patterns: S-S (I), albolabrin; S-S (II), kistrin and flavoridin; S-S (III) echistatin and eristostatin. Our results show that the conserved cysteine residues of bitistatin adopt disulfide bridge pattern S-S (II) and that Cys^5 and Cys^{24}, which are found only in bitistatin, form an extra disulfide bridge. The S-S bond pattern in disintegrins has also been confirmed by NMR spectroscopy (28–30, 34). It should be emphasized that a characteristic pattern of conserved cysteine alignment defines the disintegrins. There are other low-molecular-weight proteins, like mambin (dendroaspin) from *Dendroaspis jamesonii* (37), and leech-derived ornatins (38) and decorsin (39), each of which contains an RGD hairpin loop and is a potent inhibitor of platelet aggregation. None of these proteins, however, fits the definition of a disintegrin since the cysteine residue alignment and primary structure are significantly different from those of disintegrins.

Disintegrins bind to the platelet fibrinogen receptor with much higher affinity than the natural ligand, fibrinogen, does. Over the last decade, the extensive research on RGD disintegrins focused on their interaction with platelet integrin αIIbβ3. This research led to the development of novel antiplatelet and antithrombotic drugs patterned on the disintegrin structure (40, 41). Early studies (42, 43) sug-

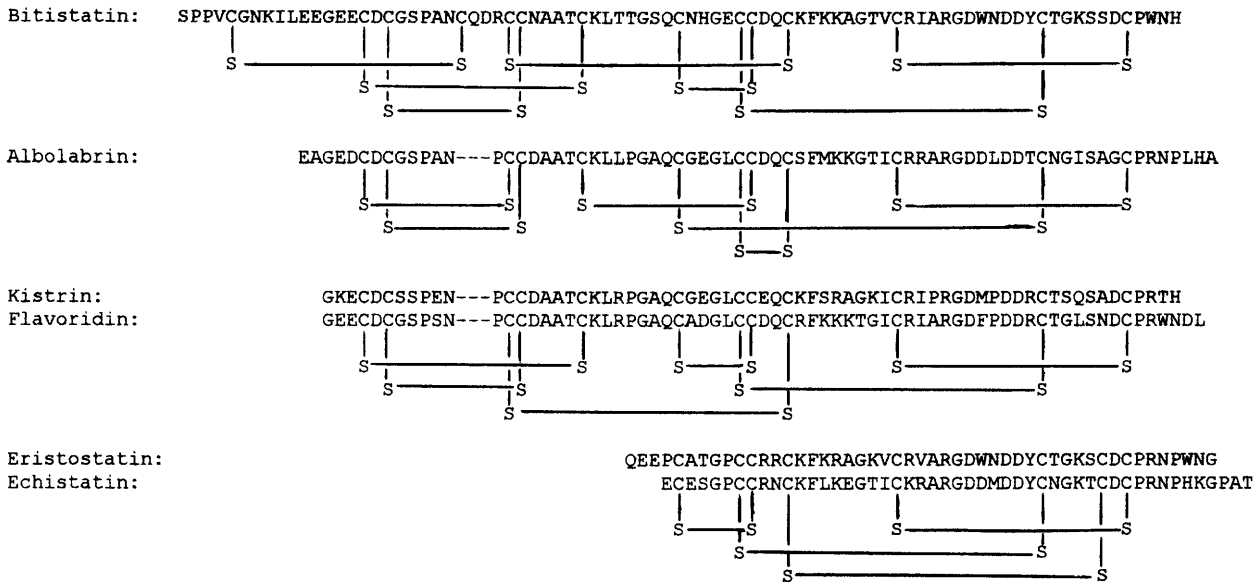

Figure 2. Pattern of intramolecular bridges in disintegrins (26).

gested that disintegrins were also potent inhibitors of cell adhesion. Thus, it has been found that disintegrins also interact with integrins other than αIIbβ3 and show different binding affinity and selectivity toward these integrins. The inhibitory effect of disintegrins on the adhesion of fibroblasts and murine melanoma cells to fibronectin, laminin, and vitronectin has been demonstrated (42, 44, 45). In a study with isolated receptors and ligands in solid phase assay, flavoridin and echistatin appeared to be the most potent promiscuous disintegrins, binding with similar avidity to αIIbβ3, αvβ3 and α5β1 integrin. Eristostatin, bitistatin, and barbourin showed a high degree of specificity to αIIbβ3; kistrin appeared to interact with equal potency with αvβ3 and αIIbβ3 but not with α5β1 (15, 46–48). There is evidence that integrins α3β1, α5β1, αvβ1, αvβ5, αvβ6 and αvβ8 recognize the RGD sequence in their ligands (49). Moreover, ligand binding to α2β1 and α4β1 can be inhibited by cyclic RGD peptides (50). However, there are scarce literature data about disintegrin interaction with integrins other than αIIbβ3, αvβ3 and α5β1. Recently Chandra Kumar *et al.* (51) provided evidence that the disintegrin echistatin does not interact with αvβ1 although it is a potent antagonist of αvβ3. Additionally and surprisingly, kistrin appeared to be a strong inhibitor of αLβ2 interaction with ICAM-1 (Bodary S, personal communication).

McLane *et al.* (52) have shown that disintegrin interaction with αIIbβ3 depends on the activation state of this receptor. They found that eristostatin bound with the same high affinity to resting platelets and to platelets activated by ADP or thrombin. On the other hand, bitistatin, echistatin, and albolabrin bound with a much higher affinity to platelets activated by ADP or thrombin than to resting platelets. The RGD-containing peptide, decorsin, bound with low affinity to resting and to ADP-activated platelets and with high affinity to thrombin-activated platelets (53). Disintegrins are very potent inducers of the conformational changes that depend on the exposure of ligand-induced binding sites (LIBS) in both αIIbβ3 and αvβ3 receptors as evidenced by the increased binding of anti-LIBS antibodies to both receptors (54, 55). Their activity is higher by at least four orders of magnitude compared to linear RGD peptides.

We have suggested that disintegrins, in conjunction with monoclonal antibodies, may represent a useful tool to map active epitopes of integrins. For example, there is evidence that eristostatin and echistatin bind to different sites on αIIbβ3 integrin and are displaced by different monoclonal antibodies. Echistatin binds to an epitope on αvβ3 that is recognized by monoclonal antibody 7E3 but not by mAb LM609 (55).

Short Disintegrins, Echistatin and Eristostatin

Echistatin and eristostatin, the shortest monomeric disintegrins, share significant sequence identity (62%) and have an identical disulfide bridge pattern among all eight conserved cysteine residues, yet the disintegrins show significant functional differences in interaction with various integrins. Eristostatin shows a high degree of specificity to αIIbβ3, whereas echistatin is a promiscuous disintegrin, recognizing αIIbβ3, αvβ3, and α5β1. We have developed a three-dimensional model of eristostatin structure using molecular dynamics simulation methods (56) and echistatin NMR coordinates. From the comparison of their 3-D structures, we hypothesized that the differences in biological activities may be due to structural differences in the amino acid sequences of their RGD loops (36). The presence of two large hydrophobic residues (V^{25} and W^{30}) around the RGD sequence in eristostatin makes the loop significantly wider than the RGD loop in echistatin, which has Arg and Asp residues in similar positions. A double mutant of echistatin (R22V/D27W), obtained by recombinant technology, had an RGD loop with the same shape and width as

eristostatin's. This mutant was the most potent inhibitor of αIIbβ3, and its anti-αvβ3 activity was partially decreased (36, 57). On the basis of these data, the authors hypothesized that the width and shape of the RGD loop are important structural features to affect fitting of the ligand to the binding pocket of αIIbβ3 and αvβ3. Using chemically synthesized echistatin mutants, Yamada (58) demonstrated that it is possible to increase the potency of a native disintegrin by introducing a conformational constraint in the RGD region.

The C-terminus of echistatin was proposed to have functional ability separate from that of the RGD loop. A peptide derived from residues 40–49 inhibited echistatin/αIIbβ3 binding, but it also activated this integrin's binding to immobilized vitronectin, fibronectin, and collagens I and IV (59). In contrast to RGD peptides, this C-terminal peptide did not inhibit binding of fibrinogen to αIIbβ3. In our laboratories, we found that truncation of the C-terminus of echistatin or of its mutants (Fig. 3) decreased 5–11 times their ability to inhibit ADP-induced platelet aggregation. The ability of truncated echistatin to compete with the binding of vitronectin to immobilized αvβ3 was diminished. Its ability to induce LIBS in the β3 subunit in either αIIbβ3 or αvβ3 was almost completely abolished after truncation (60). Interestingly, one echistatin mutation that replaced echistatin's C-terminal HKGPAT sequence with that of eristostatin's (WNG) did not significantly affect this protein's inhibitory potential with αvβ3 integrin, although it did decrease echistatin's inhibition of ADP-induced platelet aggregation by 1.6-fold (60). On the basis of these studies on the interaction of various analogs of echistatin, we proposed a hypothesis in which echistatin's RGD loop determines selective recognition of αIIbβ3, αvβ3 and α5β1 integrins, whereas the C-terminal domain supports its binding to resting integrins and is critical to the expression of the LIBS epitope and conformational changes of the receptor, leading to a further increase of the binding affinity and inhibitory effect of echistatin (54).

Medium-Sized Disintegrins

Medium disintegrins share reasonable sequence similarities with short disintegrins. However, the folding of the polypeptide backbone and the three-dimensional structures of kistrin and flavoridin are different from those of the short disintegrins. Furthermore, although seven cysteine residues are conserved between short and medium disintegrins, the disulfide bridge pattern differs significantly in these proteins. In short disintegrins, the RGD loop and the C-terminus of the protein are hypothesized to be involved in binding to the integrin receptors. In kistrin and flavoridin, the RGD loop is projected out about 14 Å from the rest of the globular structure. Our own computer modeling studies suggest that the substitution of amino acids that flank the RGD motif of kistrin has no significant effect on the shape of the loop itself. Accordingly, Dennis *et al.* (32) observed by alanine scanning mutagenesis that only the RGD motif in kistrin is important for the expression of antiplatelet aggregation activity.

Significant steps have been taken toward understanding the structure-function relationship of disintegrins through experiments using the 65–72 residue disintegrins, especially kistrin and elegantin (47, 61–64). Scarborough *et al.* (65) initially suggested that the placement of a hydrophobic residue (tryptophan) C-terminal to the RGD sequence would create a disintegrin conformation selective for binding to αIIbβ3. In contrast, disintegrins with the sequence RGDNP interacted more potently with αvβ3 or α5β1 than αIIbβ3. Most medium-sized disintegrins inhibit αIIbβ3 and αvβ3. Flavoridin strongly inhibits αIIbβ3, αvβ3, and α5β1, whereas barbourin (with the sequence KGDW) is a specific inhibitor of αIIbβ3. Studies on isoforms of elegantin (66) suggested that amino acids adjacent to RGD are important in determining selectivity of disintegrins. Rahman *et al.* (63) noted the dissimilarity of RGD loop sequences in kistrin (PRGDMP) and elegantin (ARGDNP), and the differences in integrin selectivity exhibited by each: kistrin preferentially inhibited fibrinogen/αIIbβ3 interactions compared with fibronectin/αIIbβ3, whereas elegantin showed just the opposite functional activity. They also noted that the disintegrin-like neurotoxin analog, dendroaspin (mambin) (67), resembled kistrin in both RGD loop sequence and the ability to bind to αIIbβ3 and not α5β1. Lu (61) carried this observation one step further, altering dendroaspin's binding selectivity by changing its kistrin-like sequence PRGDMP to elegantin's ARGDNP, creating a molecule that binds to the fibronectin site, but not the fibrinogen site, on αIIbβ3. Similarly, Tselepsis (64) replaced the entire RGD sequence in kistrin with LDV, a binding site in fibronectin connective segment I recognized by α4β1. The resulting protein acquired the ability to block α4β1 activity, but it did not block αvβ3 or α5β1.

In contrast to elegantin and recombinant dendroaspin (both ARGDNP) (Rahman S, personal communication), kistrin and dendroaspin (both PRGDMP) bound with high affinity to an αIIbβ3 mutant ($\beta 3_{S123A}$). Interestingly, an elegantin mutant with the sequence PRGDMP did not interact with this integrin mutant. Competition studies suggested that elegantin and kistrin bind to two distinct RGD-dependent binding sites within αIIbβ3 (63). This correlates with work reported by our laboratory that suggested

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Echistatin:                          ECESGPCCRNCKFLKEGTICKRARGDDMDDYCNGKTCDCPRNPHKGPAT
Echis R22V/D27W (truncated):                                V    W              RNPHKGPAT
Echis R22V/D27W/M28N (truncated):                           V    WN             RNPHKGPAT
Eristostatin:                    QEEPCATGPCCRRCKFKRAGKVCRVARGDWNDDYCTGKSCDCPRNPWNG
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Figure 3. Mutations in the RGD loop and C-terminus of echistatin.

echistatin (ARGDDMP) and eristostatin (ARGDWNP) also bind to two distinct sites within αIIbβ3 (55).

Bitistatin, A Long Disintegrin

Bitistatin, a long disintegrin, appears to be quite a selective antagonist of αIIbβ3 integrin, in some assays acting weakly with αvβ3. Bitistatin has the highest degree of homology with the disintegrin-like domain of some ADAMs proteins. RGD loops of bitistatin and eristostatin are almost identical; however, the former disintegrin is a much weaker inhibitor of platelet aggregation than eristostatin. Most recently, Calvete *et al.* (26) established the S-S bridge pattern in bitistatin (Fig. 2). Two cysteine residues at positions 5 and 24, found in bitistatin but not in other disintegrin molecules, are disulfide bridged. This linkage creates an additional large loop which, depending on whether the NMR structure of flavoridin or kistrin is used for modeling the structure of bitistatin, lay opposite or nearly parallel, respectively, to the biologically active RGD loop (26). In contrast to kistrin that blocks αvβ3 and inhibits adhesion of human umbilical vein endothelial cells (HUVEC) to vitronectin, bitistatin did not interfere with the adhesion of these cells to vitronectin and to other natural ligands. However, HUVEC adhered as well to immobilized bitistatin as to immobilized kistrin (47). Moreover, bitistatin stimulated proliferation of bovine aortic endothelial cells, whereas kistrin and echistatin had an inhibitory effect on this process (Sage EH, personal communication). The adhesive protein on the surface of endothelial cells to which bitistatin binds has not been determined. We propose that the N-terminal loop of bitistatin may be involved in its interaction with endothelial cells.

Dimeric Disintegrins

Recent studies by Markland's group (23) and studies in our laboratories identified a new class of viper venom disintegrins: dimeric disintegrins. The molecular weight of these disintegrins ranges from 13–15 kDa. After reduction and ethylpyridylation, they yield two subunits with Mr approximately 7 kDa each. These subunits may or may not be identical. The cysteines involved in the formation of intra- and intermolecular bridges of these disintegrins have not yet been identified.

Contortrostatin, isolated from the venom of *Agkistrodon contortrix contortrix* (22), appears to be a disulfide-linked dimer with two RGD sites. As determined by mass spectrometry, the molecular weight of nonreduced contortrostatin is 13,505, with the reduced and ethylpyridylated form being 8000 Da. Its complete amino acid sequence has not yet been reported; however, this protein has been characterized functionally. It inhibits melanoma cell metastases by blocking α5β1 integrin (22). Whereas monomeric disintegrins block platelet aggregation and inhibit phosphorylation of platelet proteins on tyrosine, contortrostatin triggers tyrosine phosphorylation, although it inhibits platelet aggregation (23).

Recently we isolated and characterized two novel heterodimeric disintegrins: EMF10 from the venom of *Eristocophis macmahoni* and EC3 from the venom of *Echis carinatus* (24, 25). Both EMF10 (Mr 14,576) and EC3 (Mr 14,761) bound to Jurkat cells (which express α4β1) and inhibited their adhesion to immobilized VCAM-1. Both disintegrins bound to K562 cells expressing α5β1 and inhibited their adhesion to immobilized fibronectin. After reduction and alkylation, EC3 and EMF10 each separated into two subunits (A and B). Each EC3 subunit contained 67 amino acids (10 cysteines) and showed a high degree of homology with other disintegrins, including alignment of conserved cysteines. However, the RGD sequence occurring in most disintegrins was substituted in EC3A with VGD, whereas in EC3B, it was replaced by MLD. EC3 blocked adhesion of Jurkat cells and CHO cells transfected with α4 to immobilized VCAM-1, with an IC_{50} of 10–30 n*M*. It also blocked adhesion of cells expressing α4β7 to immobilized MadCAM with the same potency. EC3B was inhibitory in this system (IC_{50} = 6 μ*M*) whereas EC3A was inactive, suggesting that the inhibitory activity of EC3 was mediated by the MLDGLN sequence. Both EMF10 subunits, EMF10A and EMF10B, showed a homology with other viper venom disintegrins (Fig. 4), and preliminary experiments suggested that activity to inhibit α5β1 integrin resides in both subunits. Amino acid sequencing of these subunits demonstrated that EMF10A and EMF10B represent precursors of eristocophin I and eristocophin II, respectively, previously described by Siddiqi *et al.* (68). EC3B and EMF10A showed 70% amino acid sequence identity. EMF10A and EMF10B expressed RGD and MGD sequences, respectively. Interestingly, Siddiqi *et al.* (68) identified two monomeric peptides rather than a dimeric molecule in *E. macmahoni* venom since they started puri-

Echistatin	C K R A	R G D	D M D D Y C
Trigramin	C R I A	R G D	D L D D Y C
Kistrin	C R I P	R G D	M P D D R C
Flavoridin	C R I A	R G D	F P D D R C
Elegantin	C R R A	R G D	N P D D R C
EMF10A	C K K G	R G D	N L N D Y C
EMF10B	C W P A	M G D	W N D D Y C
Eristostatin	C R V A	R G D	W N D D Y C
Bitistatin	C R I A	R G D	W N D D Y C
Barbourin	C R V A	K G D	W N D D R C
EC3B	C K R A	M L D	G L N D Y C
Atr E	C R V S	M V D	R N D D T C
Atr A	C R P A	R S E C D I A E S C	
HR1B	C R A A	E S E C D I P E S C	
JARA	C R A S	M S E C D P A E H C	
ADAM 1	C R P A	E D V C D L P E Y C	
ADAM 2	C R G S	S N S C D L P E F C	
ADAM 15	C R P T	R G D C D L P E F C	
TACE	C Q E A I N A T C K G V S Y C		

Figure 4. Amino acid sequences of disintegrin loops in viper venom disintegrins, hemorrhagins and ADAMs. Abbreviations; Atr E-atrolysin E; Atr A-atrolysin A; HRB1-trimerelysin; JARA-jararhagin; ADAM 1-mouse fertilin α; ADAM 2-mouse fertilin β; ADAM 12-meltrin α; ADAM 15-metargidin; TACE-tissue necrosis factor α converting enzyme.

fication procedures from venom treated with reducing agents. EMF10 was about 100 times less potent than EC3 in inhibiting adhesion of cells expressing $\alpha4\beta1$ to immobilized VCAM-1. EMF10 inhibited adhesion of K562 cells expressing $\alpha5$ to immobilized fibronectin with IC_{50} of 5 n*M*, and competed with a monoclonal anti-$\alpha5$ antibody (SAM-1). EC3 was about 40 times less active as an $\alpha5\beta1$ antagonist. Anti-$\alpha4$ mAb (HP2/4) blocked adhesion of Jurkat cells to immobilized EC3 and did not interfere with their adhesion to immobilized EMF10. Furthermore, RGDS, at a concentration of 1 m*M*, inhibited adhesion of Jurkat cells to immobilized EMF10 but not to EC3. In contrast to typical viper disintegrins, that are potent inhibitors of platelet aggregation (IC_{50} = 50–300 n*M*) but do not interact with Jurkat cells, EC3 and EMF10 inhibited platelet aggregation only at concentrations greater than 1.0 μM.

Disintegrins: Significance in Thrombosis and Vascular Biology

Whereas disintegrins exhibit variability in their *in vitro* selectivity/potency with isolated receptors and cells in tissue culture environments, they similarly show a wide range of responses when injected *in vivo*. Shebuski *et al.* (13) assessed the antithrombotic potential of bitistatin using a canine model of platelet-dependent coronary thrombus formation. Injection of bitistatin at 10–100 μg/kg resulted in a dose-dependent inhibition of both platelet aggregation *ex vivo* and platelet-dependent cyclical flow reductions. Research on disintegrins promoted development of synthetic platelet fibrinogen receptor antagonists that are now undergoing clinical trials to evaluate their effect to prevent platelet thromboembolism. For instance, Tcheng *et al.* (41) used the molecular structure of barbourin to design a cyclic peptide, Integrelin, as an antithrombotic agent in elective coronary intervention. Both echistatin (50 μg/ml) and flavoridin (80 μg/ml) inhibited *in vivo* retinal detachment induced by retinal pigment epithelial cells attaching to extracellular matrix, and consequent vitreous contraction (69). This inhibition appeared to be mediated by $\alpha v\beta3$. Triflavin (flavoridin) exerted a potent effect in inhibiting rat mesangial cell adhesion to fibronectin, collagen types I and III, and inhibited platelet-derived growth factor-promoted mesangial cell growth in serum-free medium (70). Yang *et al.* (69) showed the ability of triflavin and echistatin to inhibit retinal pigment cell adhesion to extracellular matrix. Jones *et al.*, (48) demonstrated that $\alpha v\beta3$ is needed in smooth muscle cell migration mediated by insulin-growth factor-1 stimulation, and that $\alpha v\beta3$ antagonists (like kistrin and echistatin) may modulate this process. Sheu *et al.* (71) demonstrated that triflavin (1 μm) significantly inhibited aggregating platelet-induced vasoconstriction in de-endothelialized rat aorta, whereas neither trigramin (10 μM) nor small RGD peptides (2 m*M*) showed any significant effect. Triflavin also inhibited serotonin release and formation of thromboxane A2 from aggregating platelets, whereas trigramin and RGD peptides showed no effect. Triflavin (2 μM) markedly reduced adhesion of platelets to the subendothelium in the same aorta. This effect may be due to triflavin's efficiently preventing activation of platelets after inhibition of serotonin release and thromboxane formation, and not related to any ability to inhibit adhesion of platelets to extracellular matrix. Thus, triflavin may be useful for treatment of thromboembolism and its associated angiospasm.

A number of disintegrins (kistrin, flavoridin, echistatin, and albolabrin) inhibit adhesion of human umbilical vein endothelial cells (HUVEC) to vitronectin and von Willebrand Factor (47). Endothelial cells also adhere to kistrin and echistatin, with this adhesion resulting in apoptosis (unpublished observations from our laboratory). Echistatin, kistrin, flavoridin, and the neurotoxin analog mambin (dendroaspin) at nanomolar concentrations inhibited binding of HUVEC to immobilized vitronectin. Echistatin (3 μM) partially inhibited HUVEC adhesion to fibronectin (47). Adhesion of HUVEC to vWF is mediated by two receptors: $\alpha v\beta3$, which is blocked by dendroaspin, albolabrin, and echistatin (72), and by GPIb, that is blocked by echicetin, a lectin-like protein from *Echin carinatus* (Tan *et al.*, manuscript submitted for publication).

Echistatin has been used to isolate rapidly and reproducibly an 88%–95% pure culture of mammalian perfusion osteoclasts (73). At low concentrations, it has also prevented bone destruction by interacting with $\alpha v\beta3$ expressed on osteoclasts (74).

Disintegrins and Cancer

Cancer cells represent a unique class of cells that use integrin-ligand interactions to evade destruction (75), promote the development of their own blood supply (76), and metastasize (77). As such, the disintegrins' feature as integrin antagonists has provided insights into carcinoma cell biological functions. For example, flavoridin inhibited hepatoma cell adhesion to fibronectin, collagen, laminin, and vitronectin; the integrins involved were identified as $\alpha5\beta1$, $\alpha3\beta1$ and $\alpha v\beta3$ (78). In contrast, triflavin inhibited adhesion of human cervical carcinoma (HeLa) cells to fibronectin, fibrinogen, and vitronectin, but had a limited effect with laminin and collagens I and IV. In addition, triflavin did not affect ^{3}H-thymidine uptake of the cancer cells during a 3-day incubation time (78). Antibodies to $\alpha5\beta1$ and to the $\beta1$ subunit, but not to the integrin $\alpha v\beta3$ or the $\alpha2$ subunit, effectively blocked adhesion of M24 human metastatic melanoma cells to immobilized dimeric disintegrin contortrostatin (22). Kistrin inhibited adhesion of Saos-2 human osteosarcoma cells to vitronectin, fibronectin, and collagen I, and it also inhibited platelet aggregation induced by the cancer cells (79). Beviglia *et al.* (45) demonstrated similar findings with B16F10 murine melanoma cells, with albolabrin (IC_{50} 130–165 n*M*), barbourin (IC_{50} 46–60 n*M*), echistatin (IC_{50} 74–75 n*M*), and eristostatin (IC_{50} 7–8 n*M*) preventing melanoma cell–induced platelet aggregation. Albolabrin was the most, and eristostatin the least, potent in inhibiting cell adhesion to immobilized fibronectin, laminin,

or vitronectin. The medium-length disintegrin, elegantin, inhibited B16-BL6 murine melanoma cell adhesion to immobilized fibronectin with greater potency than kistrin (66). Finally, in an attempt to identify the mechanism whereby disintegrins inhibit B16-BL6 melanoma cell adhesion, Staiano (80) showed that echistatin decreased p125FAK phosphorylation, while increasing disassembly of actin cytoskeleton and focal adhesions. These intracellular phenomena were observable well before the cells actually detached from a fibronectin matrix. The authors suggested that echistatin's action came from its inhibition of protein tyrosine kinase rather than by an activation of protein tryosine phosphorylation.

When Beviglia *et al.* (45) co-injected C57BL6 mice with B16F10 melanoma cells with or without four different disintegrins, all control mice developed lung metastases, but the mice injected with eristostatin (0.05 μM), albolabrin (1.0 μM), bitistatin (0.9 μM), and echistatin (3.7 μM/mouse) did not, at the 50% inhibitory concentrations indicated. There seemed to be a time factor involved also, since albolabrin, bitistatin, and echistatin could be injected 1 hr after the melanoma cells and still prevent development of metastases. Surprisingly, if eristostatin were injected 1 hr later than the melanoma cells, the mice were not protected. Since eristostatin, out of the four disintegrins tested, has the greatest potency for αIIbβ3 and the least inhibitory potency for cell adhesion to fibronectin, laminin, and vitronectin, these authors suggested that eristostatin interferes with metastasis at a step involving platelets, whereas albolabrin, barbourin, and echistatin block an adhesive step in the metastatic process. Morris *et al.* (53), however, provided visual evidence to dispute any involvement of platelets in eristostatin's protective effect against metastasis. They suggested that eristostatin prevents survival of melanoma cells after they invade the liver tissue. Danen *et al.* (81), using a human melanoma cell line (MV3) that expresses $\alpha 4\beta 1$ integrin, demonstrated the possible involvement of eristostatin interference with $\alpha 4\beta 1$-VCAM binding. In addition, the dimeric disintegrin contortrostatin, at 20 μg and 100 μg, inhibited lung colonization of human metastatic melanoma cells (M24met), when injected into SCID mice, by 51% and 73%, respectively, *via* a $\beta 1$ integrin-mediated process (22). Interestingly, using the disintegrin echistatin, Lang *et al.* (82) showed that $\alpha 3$, $\alpha 5$, and an RGD-dependent pathway are involved in prostate cancer metastasis to bone.

Disintegrins and Hemorrhagins

Snake venom metalloproteinases (SVMP) (hemorrhagins, reprolysins) are highly toxic, producing severe bleeding, inhibiting formation of a hemostatic plug, and hydrolyzing basement membranes and extracellular matrix components such as fibronectin, collagen type IV, nidogen, and laminin (2). Atrolysins (Ht toxins) from *Crotalus atrox,* trimerelysins (HR toxins) from *Trimeresurus flavoviridis,* jararacin from *Bothrops jararaca,* and catrocollastatin from *Crotalus atrox* have been studied most extensively. There is good evidence that the disintegrin domain, by attaching an SVMP molecule to a receptor expressed on the cell surface, potentiates proteolytic activity of SVMP. Recently Jia *et al.* (83) expressed the disintegrin-like/cysteine-rich domain of atrolysin A from *Crotalus atrox* in insect cells, and demonstrated that recombinant protein-inhibited collagen and ADP-induced platelet aggregation. The sequence CRASMSECDPAEHC occurring in jararhagin and catrocollastatin (which corresponds to the RGD loop of disintegrins) likely represents the active site of these hemorrhagins responsible for their inhibitory effect on collagen-induced platelet aggregation (84, 85). All cysteines in the disintegrin domain of hemorrhagin appear to be linked. Most probably, the cysteine occurring in the middle of the loop forms an S-S bridge with another cysteine in the cysteine-rich domain. Perhaps this makes the conformation of this protein resistant to proteolysis and may account for the fact that low molecular weight disintegrins with cysteine adjacent to an RGD sequence or to a corresponding sequence do not occur in viper venoms despite the fact that disintegrins and hemorrhagins appear to be derived from common or closely related precursors (21, 86, 87). To maintain its activity, the RSECD-cysteinyl residue in atrolysin A must be constrained by participation in a disulfide bond with another cysteinyl residue (83). Zhou *et al.* (84) demonstrated that the hemorrhagin catrocollastatin binds to collagen using its disintegrin domain. It is conceivable that this event may facilitate degradation of collagen and/or degradation of the $\alpha 2\beta 1$ collagen receptor by the metalloproteinase domain of the same molecule (88).

Disintegrins and ADAMs Proteins

In contrast to hemorrhagins, ADAMs are cell membrane–anchored proteins. They contain metalloproteinase, disintegrin-like, cysteine-rich, epidermal growth factor–like, transmembrane and cytoplasmic domain (4, 5) (Fig. 1). Several ADAMs proteins contain putative cytoplasmic signaling motifs and SH3 ligand domains (4, 89). Wolfsberg and White (4) list 13 different members of ADAMs that have been identified in the tissue of human, monkey, rabbit, rat, guinea pig, and bovine tissues and two ADAMs identified in *Xenopus* and in *Caenorhabditis elegans* tissue. Two ADAMs, fertilin and cyritestin, occur in mammalian testis and are hypothesized to participate in the egg-fusion process. Fertilin (previously called PH-30) is composed of α and β subunits (11). The precursor of fertilin α has a metalloprotease domain with a catalytic site consensus sequence, whereas the metalloprotease domain of the β subunit of fertilin does not have an active site (90). Almeida *et al.* (91) suggested that interaction of sperm fertilin with $\alpha 6\beta 1$ integrin expressed on mouse egg is an important step during egg-sperm fusion. Interestingly, anti-$\alpha 6$ antibody blocked the fertilization process in this animal. More recently, Yuan *et al.* (92) raised polyclonal antipeptide antibodies against the predicted integrin binding sequence of the mouse sperm fertilin β and cyritestin. Antibodies

against both proteins inhibited fertilization and bound to sperm in indirect fertilization assay thus providing evidence that the predicted binding sequence of an ADAMs protein is exposed and accessible on the cell surface. Primakoff and colleagues (Primakoff P, personal communication) recently created a fertilin $B^{-/-}$ knockout mouse that resulted in male infertility. Sperm from these mice are defective in binding to the egg plasma membrane. They also do not fuse well to eggs, but if fused, the resulting zygotes show normal activation. The fertilin $B^{-/-}$ sperm are also defective in migrating from uterus to oviduct, and in binding to the zona pellucida. Using echistatin to study human sperm adherence and penetration of hamster oolemma, Bronson *et al.* (93) found significantly decreased adherence of sperm pretreated with this disintegrin. However, echistatin did not inhibit oocyte penetration of sperm that had become adherent to oolemma despite the presence of echistatin. This suggested to these authors that sperm penetration consists of two distinct processes: one involving integrin recognition of fibronectin or vitronectin, and a second leading to actual gamete membrane fusion, independent of disintegrin-integrin interaction.

In contrast to mature fertilin, where the metalloproteinase domains have been removed during sperm maturation, most ADAMs represent membrane-anchored proteins with metalloproteinase and disintegrin domains. Metalloproteinase-disintegrins participate in cleaving at least one substrate: the proinflammatory membrane-anchored cytokine tumor necrosis factor (TNFα). TNFα is cleaved and released from the cell membrane by TACE (TNFα convertase), which represents an ADAMs family member with metalloproteinase and disintegrin domains (94, 95). Release of this cytokine is critical in pathogenesis of the inflammatory process.

It can be suggested that the disintegrin domain in ADAMs might be used to increase the efficiency of the proteinase by binding to integrin expressed on the cell surface near the metalloproteinase substrate. Alternatively, it is possible that the distintegrin domain might be used to target the metalloproteinase to another cell in trans *via* an integrin (5). Meltrin α (ADAM 12) is a protein occurring in skeletal muscle and bones of mice, and it participates in muscle development (96). Meltrin α occurs in two forms: "intact" molecule with metalloproteinase and disintegrin domains and a form with truncated metalloproteinase domain. Overexpression of this truncated form leads to an increase in muscle fusion whereas overexpression of the full-length protein leads to a decrease in observed fusion. The mechanism by which ADAMs cause cell fusion remains unexplained, but it is conceivable that interaction of the disintegrin domain with a counter ligand is a prerequisite step for fusion to occur.

It is well known that mechanisms of interaction of cells expressing integrins with extracellular matrix components are severely disturbed in cancer. Emi *et al.* (97) isolated a novel ADAMs gene that encodes a 524-amino acid metalloproteinase-like, disintegrin-like and cysteine-rich protein. This gene showed somatic rearrangements in two primary breast cancers. Interestingly, a number of ADAMs are also expressed in hematological malignancies (98). Most recently, Herren *et al.* (99) demonstrated expression of ADAM 15 in cultured vascular cells and observed its upregulation in atherosclerosis. It should be noted that ADAM 15 (metargidin), first reported by Kratzchmar *et al.* (100), contains an RGD sequence and inhibits $\alpha_v\beta_3$ (101).

Disintegrins and Proteins from Plants, Leeches, and Slime Molds

Kieliszewski *et al.* (102) postulated a potential evolutionary link between plant chitin–binding domains and reptile disintegrins. While studying the amino acid sequence of potato (*Solanum tuberosum*) lectin, a chimeric chitin-binding protein composed of a lectin domain fused to a hydroxyproline-rich glycoprotein domain, they noted that all plant chitin–binding domains examined bore a remarkable sequence similarity, particularly in the spacing of Cys residues, to the disintegrins. This suggested that an archetypal polypeptide module gave rise to both plant chitin–binding and disintegrin domains.

Blumberg and colleagues (103) recently reported the isolation of a secreted protein from all growing *Dictyostelium* slime mold cells. This protein possesses multiple repeats of domains that share homology with disintegrins and with leech-derived ornatins (38), and seems to play an important role in slug migration, tissue proportioning, and morphogenetic movements that shape the fruiting body. Experiments with null mutant cells suggested that these proteins may function as adhesion-inhibiting molecules preventing premature cell-to-cell associations.

Disintegrin Loops in Disintegrins, Hemorrhagins, and ADAMs

Inspection of the amino acid sequences in the loops of disintegrins, hemorrhagins, and ADAMs reveals a variability of basic, acidic, and hydrophobic amino acids with conserved cysteines (Fig. 4). A typical disintegrin loop contains 11 amino acids flanked by two cysteines. Most disintegrins express an RGD sequence, whereas barbourin, EC3A, EC3B, EMF10B, and atrolysin E express KGD, VGD, MLD, MGD, and MVD sequences, respectively. Tryptophan adjacent to the C-terminus of the RGD or KGD sequence in eristostatin, barbourin, and bitistatin makes these disintegrins more selective for αIIbβ3. Asparagine adjacent to RGD in elegantin or EMF10 may increase its selectivity for α5β1 (61). The MLD sequence appears to increase affinity to α4. Disintegrin loops in hemorrhagins and in ADAMs have a cysteine inserted adjacent to the carboxyl end of the sequence corresponding to RGD. There are obvious differences between fertilin (which may interact with α6β1 integrin), α meltrin (participating in muscle development), and metargidin (which may interact with integrins expressed on endothelial cells). It can be speculated that various ADAMs may bind to different adhesive receptors and

that differences in amino acid composition of the disintegrin loop may determine selectivity of these proteins.

Summary

It is obvious that the disintegrin story is not limited to viper venom, and that the properties of proteins possessing a disintegrin domain can provide much information not only about adhesive ligands but also about their integrin receptors. We have undoubtedly only begun to uncover the species and tissues in which evolution has utilized this important adhesive domain.

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