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Arg–Gly–Asp constrained within cyclic pentapeptides

Strong and selective inhibitors of cell adhesion to vitronectin and laminin fragment P1

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Cyclic Arg–Gly–Asp–Phe–Val peptides with either D-Phe or D-Val residues were 20- to more than 100-fold better inhibitors of cell adhesion to vitronectin and/or laminin fragment P1 when compared to a linear variant or Gly–Arg–Gly–Asp–Ser. No or only little increase in inhibitory capacity was observed for fibronectin adhesion and for the binding of platelet receptor αIIbβ3 to fibrinogen. NMR studies of the two most active cyclic peptides showed for both an all-*trans* conformation with a βII′ and γ turn. Subtle conformational differences, however, exist between both peptides and may contribute to selectivity of inhibition.

Conformation; Fibrinogen receptor; Integrin; NMR; Synthetic peptide

1. INTRODUCTION

The identification of the Arg–Gly–Asp (RGD) sequence as a cell adhesion site in fibronectin [1,2] was a major breakthrough in the molecular characterization of cell–matrix interactions. It was subsequently shown that several more proteins including vitronectin, von Willebrand factor, fibrinogen, thrombospondin and laminin are RGD-dependent adhesion proteins which bind to a variety of either specific or promiscuous integrin receptors [3–6]. Each residue in the tripeptide sequence appears to be crucial since conservative replacements in most cases abolish activity of synthetic peptides [2,7]. Contributions by residues adjacent to RGD are also known [8]. Yet these data do not readily explain the selectivity of RGD recognition by distinct integrins nor the possibility that RGD in a number of proteins may be non-functional. The variation of cell adhesion-inhibiting activity by restricting the conformational space of active peptide sequences, by using them in cyclic form, could lead to components with improved activity and receptor selectivity [9]. This seems feasible as shown for a cyclic synthetic RGD-peptide being a better inhibitor of vitronectin than fibronectin adhesion [8] and for several snake venom RGD-containing peptides named disintegrins [10]. In both cases conformational constraints imposed by disulfide bridges [11,12] were important for improving biological activity. In the present approach we used RGD pentapeptides in which a single D-amino acid induces defined conformational motifs and facilitates cyclization not requiring disulfide bonds. Two variants showed 20- to 100-fold higher inhibiting activity than linear GRGDS for cell adhesion to some but not all protein substrates tested. Based on 2D NMR spectroscopy and molecular dynamics (MD) simulations in solution, we also propose a distinct relationship between activity and conformation.

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2. EXPERIMENTAL

Laminin fragments P1 [13] and E8 [14] were prepared from the laminin–nidogen complex obtained from the mouse Engelbreth-Holm-Swarm tumor. Human plasma fibronectin (Behringwerke, AG) and vitronectin [15] were obtained by chromatography on heparin-Sepharose. Fibrinogen from human plasma was a kind gift of Dr H. Hörmann, Martinsried. Integrin αIIbβ3 (GPIIb/IIIa) was purified from human platelets [16] and biotinylated with NHS-LC-biotin following the manufacturer's (Pierce) instruction. Cyclic peptides were synthesized by the solid phase method with Fmoc protected amino acids using dicyclohexylcarbodiimide/1-hydroxybenzotriazole as coupling reagents. After release from the resin by mild acid treatment, cyclization was achieved in high dilution with diphenylphosphoryl azide at pH 8.5, followed by removal of side chain protecting groups under strong acidic conditions. The peptides were then purified by reversed phase HPLC and characterized by fast atomic bombardment mass spectroscopy. Linear GRGDS was supplied by Dr W. König, Hoechst, AG.

Conformation of cyclic peptides was studied by NMR and MD simulations [17,18]. All NMR spectra were acquired on Bruker AMX 500 and AMX 600 spectrometers and processed on a Bruker X32 computer. The ^{1}H assignment and the extraction of conformationally relevant parameters (coupling constants, temperature coefficients, interproton distances) were achieved by a set of 500 MHz and 600 MHz

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1D and 2D NMR spectra (TOCSY, E.COSY, NOESY). For structure refinement restrained MD simulations were performed in vacuo and in solution over 150 ps. All calculations were performed on Silicon Graphics 4D/25GT, 4D/70GTB and 4D/240SX computers using the software package INSIGHT (BIOSYM) for graphical display and model building and the programs from the Groningen molecular simulation system (GROMOS) for all energy minimization and molecular dynamics simulations [19]. The NOEs were included in the potential energy function with a harmonic potential. Parameters for MD simulations in DMSO are based on unpublished work [31].

Optimal substrate coating of microtiter wells and analysis of adherent cells by a colorimetric assay have been described [20]. The human cell lines usesd were melanoma A375, mammary epithelia HBL-100 and fibrosarcoma HT 1080 [21,22]. In the inhibition assay, a cell suspension ($0.5–2 \times 10^5$ cells/ml) in Dulbecco's modified Eagle's medium was mixed with the peptide solution and immediately placed into the coated wells. Biotinylated integrin $\alpha IIb\beta 3$ was used in binding assays with immobilized ligands [23] using streptavidin–peroxidase conjugate and 5-amino-2-hydroxybenzoic acid for detection. In inhibition a fixed concentration of biotinyl-$\alpha IIb\beta 3$ (100 nM) was preincubated with increasing amounts of peptides (1 h, 25°C), prior to adding the mixture to the fibrinogen coat for another hour.

3. RESULTS AND DISCUSSION

Previous studies have shown that levels of inhibition of cell adhesion by a linear GRGDS peptide or other linear variants differ with the substrate used, being high (IC_{50} ~ 10–50 μM) for vitronectin and laminin fragment P1 but low (IC_{50} ~ 100–1000 μM) for fibronectin [7,13]. We have compared these substrates using cyclic RGDFV peptides and linear GRGDS as inhibitors. Introduction of D-Phe into the cyclic structure (cRGDFV) increased inhibition of A375 cell adhesion to laminim P1 by 20-fold and to vitronectin by 100-fold compared to GRGDS (Fig. 1). The use of D-Val in the cyclic sequence was more selective as it showed a distinct increase in inhibitory activity only for laminin P1 substrate. These observations were confirmed with 2 other cell lines and extended to more variants of the cyclic structure (Table I). Introduction of a single D-Arg or D-Asp into the cyclic peptide produced a distinct drop in inhibiting activity below the level of linear GRGDS. The same was observed for replacing Gly in the cyclic peptides by either D-Ala or L-Ala, in the latter case the D-Phe being maintained.

The increase in activity for some cyclic peptides is apparently due to cyclization rather than introducing a D-amino acid as shown for linear RGDFV which in most cases was of distinctly less inhibitory activity compared to GRGDS (Table I). Variations in this activity have been previously recognized in some, but not all, linear RGD sequences being adjacent to a hydrophobic amino acid residue [8,24].

The adhesion of 2 cell lines (A375, HBL-100) to vitronectin very likely includes in part the classical vitronectin receptor $\alpha v\beta 3$ present on these cells to a variable extent [21]. Since we could only partially inhibit adhesion by either a $\beta 3$-specific monoclonal antibody C17 [25] or an antiserum against fibronectin receptor with a strong titer for the $\beta 1$ subunit, the more recently described vitronectin receptors [6] $\alpha v\beta 1$ and possibly $\alpha v\beta 5$ may also participate. Similar antibody inhibition patterns were also observed for adhesion to laminin P1 [21] while an inhibiting monoclonal antibody to the fibronectin receptor $\alpha 5$ subunit was inactive. This indicates that high inhibiting activity of cyclic pentapeptides may involve several but not all RGD-dependent integrins.

The 3 cell lines (Table I) also adhere strongly to fibronectin and 2 of them, HT1080 and HBL-100, to collagen type IV and laminin fragment E8. These interactions were used to compare GRGDS with the most active cRGDFV peptide. Both peptides were of similar inhibiting activity (IC_{50} ~ 270–500 μM) for fibronectin adhesion demonstrating that the high activity of the cyclic D-Phe peptide (IC_{50} = 0.1–4 μM) with vitronectin and laminin P1 substrates is an effect which is specific for the receptor(s) recognizing these substrates. Cell ad-

Table I

Inhibitory capacity (IC_{50}) of cyclo (Arg-Gly-Asp-Phe-Val) derivates containing D-amino acids or Gly-to-Ala substitutions for cell adhesion on vitronectin (VN) or laminin fragment P1 and for $\alpha IIb\beta 3$ integrin binding to fibrinogen (Fb). Comparison was with linear GRGDS and RGDFV peptides

Inhibitor	IC_{50} (μM)						
	A375		HBL-100		HT1080		$\alpha IIb\beta 3$
	P1	VN	P1	VN	P1	VN	Fb
cRGDFV	1.0	0.2	0.1	0.1	0.2	4	12
cRGDFV	1.9	20	0.9	30	1.0	>120	2
cRGDFV	114	>120	25	>120	18	>120	150
cRADFV	>120	46	28	41	18	>120	>750
cRADFV	>120	>120	>120	>120	>120	>120	80
cRGDFV	>120	>120	20	>120	49	>120	nt
GRGDS	18	15	5	14	4	80	6
RGDFV	29	82	42	>170	92	>150	nt

nt = not tested.

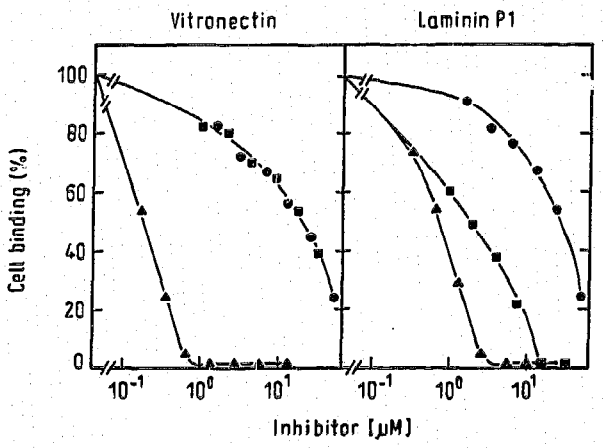

Fig. 1. Inhibition of A375 cell adhesion to vitronectin and laminin fragment P1 substrates by linear GRGDS peptide (●) or the cyclic pentapeptide RGDFV possessing either D-Phe (▲) or D-Val (■). Cell adhesion in the absence of inhibitors was set at 100%.

hesion to collagen IV and laminin E8 substrates was insensitive to inhibition by both peptides, with IC_{50} values distinctly above 500 μM. This is in accordance with previous data [21,22,26] showing that these adhesions are mediated by integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$ or $\alpha 6\beta 1$ which belong to the RGD-insensitive cell receptors [6]. It also indicates that the high inhibiting activity of cRGDFV peptide for vitronectin and laminin P1 adhesion is not due to cytotoxic or other effects unrelated to integrin recognition.

Purified platelet integrin $\alpha IIb\beta 3$ was used to analyze RGD-dependent binding to fibrinogen. In the biotinylated form it bound strongly to immobilized fibrinogen but not to vitronectin and laminin P1. The binding to fibrinogen could be most efficiently inhibited by soluble fibrinogen with IC_{50} = 0.17 μM. About 10-fold higher concentrations were required for the same inhibition with the most active cyclic peptide cRGDFV (Fig. 2). Other peptides tested were of either moderately

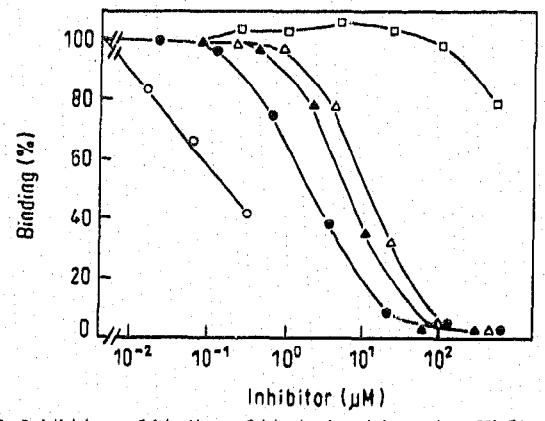

Fig. 2. Inhibition of binding of biotinylated integrin $\alpha IIb\beta 3$ to immobilized fibrinogen. Inhibitors used were fibrinogen (○), linear GRGDS (▲) and the cyclic peptides cRGDFV (●), cRGDFV (△) and cRADFV (□). Non-inhibited binding is set at 100%. Underlined residues correspond to D-isomers.

(GRGDS, cRGDFV) or distinctly lower inhibitory capacity (Table I).

The conformations of the 2 most active inhibitors cRGDFV and cRGDFV, were established by 2D NMR spectroscopy and MD simulations performed in a DMSO solvent box [17,18,27]. Both peptides were similar in adopting an all-*trans* conformation with a βII′ turn and γ turn structure in which the D-amino acid occupies the i+1 position of the βII′ turn (Fig. 3). The major difference is the position of the RGD sequence relative to the βII′ turn and γ turn, and the formation of a different amide–carbonyl hydrogen bond stabilizing the βII′ turn. These bonds are between Arg–amide and Asp–carbonyl in the D-Phe peptide and between Gly–amide and Phe–carbonyl in the D-Val peptide. Interestingly, the distance between Phe-carbonyl and Gly-amide in the D-Phe peptide is relatively short (3.1 Å) strongly suggesting that the peptide may change to the conformation of the D-Val variant. Model building studies in fact support such a transition from one to another βII′γ conformation. One can simulate by MD with dihedral restraining a conformational transition of cRGDFV via an intermediate $\beta I\gamma_i$ turn arrangement, shifted by one residue compared to the initial conformation. A conversion of the γ_i into a γ turn results in a backbone conformation identical to that determined for cRGDFV. A comparable turn rearrangement is not so easy to perform for cRGDFV, because it lacks the structural requirements for the interconversion into the conformation of cRGDFV. For example, the large distance (7 Å) between Asp–carbonyl and Arg–amide in cRGDFV prevents a similar conformational change. Hence, the proposed transition between 2 conformations could easily explain why the D-Phe peptide efficiently competes against cell adhesion to both, vitronectin and fragment P1, while the less flexible D-Val peptide may be a more selective inhibitor for laminin P1 adhesion.

The strong reduction in inhibiting activity after Gly to Ala substitution in the cyclic pentapeptide is most likely explained by steric hindrance of binding by a single methyl group such as found before for linear RAD sequences [2,7]. This is particularly obvious for cRADFV which should have a conformation almost identical to that of active cRGDFV while loss of activity in cRADFV may also be due to a shift in the positions of the βII′ and γ turns. Such shifts could also explain the low activity of the peptides cRGDFV and cRGDFV which by analogy to the conformations shown in Fig. 3 would place the essential RGD sequence into a different context within the βII′γ turns. The low activity of the cyclic D-Arg variant was of particular interest, since replacement of L-Arg by D-Arg in the linear GRGDSP peptide did not cause any substantial change in inhibiting activity for vitronectin and fibronectin adhesion substrates [8].

In order to further characterize the conformational

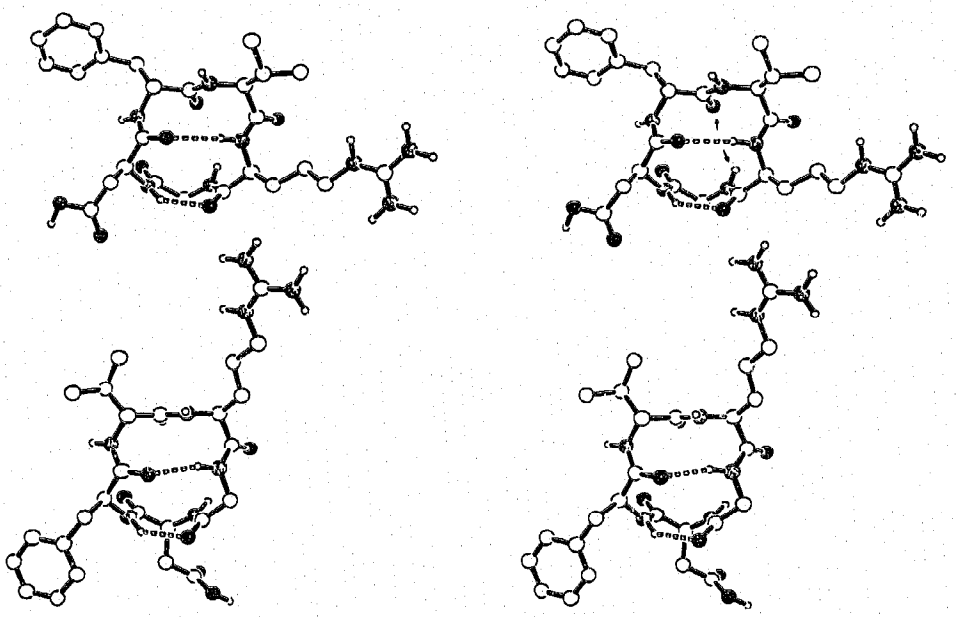

Fig. 3. Stereoplots of the conformations of the most active peptides cRGDEV (top) and cRGDFV (bottom) based on 2D NMR and MD simulation. Large circles show carbon (open), oxygen (filled) and nitrogen (stippled) atoms. Polar hydrogen atoms are indicated by small circles. The βII' turn is shown at the top of each peptide. Essential hydrogen bonds stabilizing the βII' turn and the γ turn are indicated by dashed lines. Arrows in the upper peptide indicate amide and carbonyl groups involved in a possible transition to the conformation of the lower peptide. Both conformations are consistent with experimental NOE data and the temperature coefficients of the NH resonances of protons involved in hydrogen bonds being in the range of $\Delta\delta/\Delta T$ = −0.75 to 3.78 ppb/K.

constraints which impose high biological activity upon the cyclic RGDEV peptide, several cyclic hexapeptides cRGDFVA containing a single D-amino acid were synthesized. Peptide cRGDEVA, which most closely resembles one of the active cyclic pentapeptides, was of 3–5-fold lower inhibiting activity for laminin P1 adhesion when compared to linear GRGDS. Conformational analysis of this hexapeptide showed, as expected, an all-*trans* conformation with D-Phe in i+1 position of a βII' turn, but a βII turn with Arg in i+1 and Gly in i+2 positions instead of a γ turn. The distance between β-carbons of Arg and Asp which was 7.8 Å in the hexapeptide and only 6.6 Å in the pentapeptide was a further substantial difference. We assume that these conformational differences are mainly responsible for the large differences in biological activity between both cyclic peptides. We have previously also utilized a synthetic peptide for inhibition [13] comprising the authentic laminin A chain sequence [28] CQAGTFALRGDNPQGCSP-amide. This peptide, when used in either linear or cyclic disulfide-bonded form, was no better an inhibitor than GRGDS for P1 substrates. This emphasizes that the steric restrictions imposed in small rings are crucial for the specific conformation required for efficient binding to RGD-sensitive integrins. In addition, the precise position of RGD within βII' and γ turns as well as the chirality of the spacer residues (Phe, Val) may contribute to the activity and selectivity of binding.

Other studies have shown that the disulfide-bonded peptide GPenGRGDSPCA (where Pen is penicillamine) inhibits cell adhesion to vitronectin about 10-fold better than the linear variant [8]. NMR analysis of this peptide [11] demonstrated 2 consecutive βI turns and a γ turn. This particular conformation may explain the lower relative inhibiting activity observed for this structure when compared to cRGDEV (Table I). A comparable 100-fold increase in inhibiting activity for vitronectin adhesion was recently reported for the cyclic heptapeptide cGRGDSPA when compared with the linear form, while smaller cyclic RGD peptides were far less active [29]. Here, it remains interesting to analyze to what extent the conformation of the cyclic heptapeptide comprises that of cRGDEV.

The high inhibiting activity of cyclic RGDEV and RGDFV peptides for certain but not all adhesion reactions was not predictable. However, the synthetic approach adopted was successful and also permitted the correlation of activity with distinct conformational features. The selectivity of inhibition will be useful in analyzing the receptors involved and to study their biological functions. In addition, such cyclic peptides are likely to be metabolically more stable than linear sequences, which may be an advantage for in vivo studies of developmental processes or tumor metastasis [30].

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