Differential Structural Requirements for Fibrinogen Binding to Platelets and to Endothelial Cells

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Abstract. The cytoadhesins represent a group of RGD receptors that belongs to the integrin superfamily of adhesion molecules. Members of this cytoadhesin family include the platelet GPIIb-IIIa and the vitronectin receptors. These glycoproteins share the same β-subunit, which is associated with different α subunits to form an α/β heterodimer. In the present study, we have analyzed the fine recognition specificity of the cytoadhesins from platelets and endothelial cells for the adhesive protein, fibrinogen. Two sets of synthetic peptides, RGDX peptides and peptides corresponding to the COOH terminus of the fibrinogen γ chain, were compared for their structure-function relationships in the two cellular systems. The results indicate that: (*a*) both RGDX and γ-chain peptides inhibit the binding of fibrinogen to platelets and endothelial cells; (*b*) a marked influence of the residue at the COOH- and NH_2-terminal positions of each peptide set can be demonstrated on the two cell types; and (*c*) RGDX and γ peptides have differential effects on platelets and endothelial cells with respect to fine structural requirements. These results clearly indicate that while the platelet and endothelial cytoadhesins may interact with similar peptidic sequences, they express a different fine structural recognition.

MEMBERS of a broadly distributed superfamily of adhesion receptors, which have been termed the integrins (23), mediate a wide variety of cell-cell and cell-substrata interactions. This superfamily is comprised of noncovalent heterodimers, and the α and β subunits of each heterodimer contain membrane-spanning domains. Three distinct groups of adhesion receptors, with specific structural and functional features have already been identified within the integrin superfamily: (*a*) the Leu-CAM family which is found on leukocytes and includes LFA-1, MAC-1, and p150,95 (48); (*b*) the VLA family, of which there are at least five distinct members that are found on a variety of cell types, including the fibronectin receptor from fibroblasts (21, 50); and (*c*) the cytoadhesin family (16, 39, 40). Members of the cytoadhesin family include GPIIb-IIIa from platelets and megakaryocytes and the vitronectin receptors, which are found on endothelial (15), melanoma (5), and osteosarcoma (41) cells. Members of each family have a similar β subunit, which associates with different α subunits to create functionally distinct adhesion receptors. Thus, the platelet and the endothelial cell cytoadhesin share a similar β subunit, GPIIIa, but distinct α subunits (15). The three β subunits, one from each family, are highly related to one another, exhibiting 40–50% identity at the amino acid sequence level (5, 11, 26).

Certain members of the cytoadhesin family exhibit recognition specificity that permits them to interact with more than a single adhesive protein. For instance, GPIIb-IIIa from platelets can interact with at least three adhesive proteins—fibrinogen (1, 31), von Willebrand factor (12, 45), and fibronectin (13, 33)—and the cytoadhesins from endothelial cells have been shown to mediate the attachment of these cells to vitronectin, fibrinogen, and von Willebrand factor (2–4, 6, 7). Considerable progress has been made in defining the fine recognition specificities involved in the binding of the various adhesive proteins to GPIIb-IIIa. Specifically, an Arg-Gly-Asp (RGD) recognition is involved in these interactions since RGD-containing peptides inhibit the binding of all three adhesive ligands to GPIIb-IIIa (14, 19, 38, 43). The RGD recognition specificity is not restricted to the cytoadhesin family but is also involved in the recognition of extracellular ligands by certain members of the other two families within the integrin superfamily (42, 49). In addition, a second recognition specificity has been implicated in the binding of adhesive proteins to GPIIb-IIIa; these interactions can be inhibited by a second peptide set that corresponds in sequence to the extreme COOH terminus of the γ chain of fibrinogen. This sequence of γ 400–411 is HHLGGAKQAGDV (20, 37). Despite these indications of a relaxed recognition specificity of GPIIb-IIIa, the RGD and γ-chain peptides inhibit the binding of the recognized adhesive proteins to platelets with a fine structural specificity. For example, conservative amino acid substitutions at the Arg, Gly, or Asp position markedly diminish the potency of the peptides as inhibitors of ligand binding (46). Similarly, acetylation of the

Lys residue within the γ-chain peptide renders it inactive in inhibiting ligand binding to platelets (27, 28). In contrast to this detailed information on the recognition specificity of GPIIb-IIIa, only limited data are available on the recognition specificity of the endothelial cell cytoadhesin. RGDS has been shown to interfere with the attachment of these cells to fibrinogen and other adhesive proteins, and a monoclonal antibody that inhibits fibrinogen binding to platelets also inhibits endothelial cell attachment to fibrinogen (2, 4).

With the ability to quantitatively analyze the binding of fibrinogen to endothelial cells and the evidence that this interaction is mediated by the endothelial cell cytoadhesin, it is now possible to dissect the fine recognition specificity of the cytoadhesin from these cells for fibrinogen and compare it to that of platelets. Such an analysis has been undertaken in this study.

Materials and Methods

Proteins and Synthetic Peptides

Human fibrinogen was purified by differential ether fractionation and was radiolabeled with carrier-free $Na^{125}I$ (Amersham International, Buckinghamshire, England) to a specific activity of 1 μCi/μg by a modified chloramine T (Eastman Kodak Co., Rochester, NY) procedure, as previously described (31). The purity of the protein, including its freedom from contaminating plasminogen, thrombin, factor VIII, von Willebrand factor, and fibronectin has already been detailed (31). Contaminating fibronectin was reduced to a trace amount by affinity chromatography on gelatin Sepharose and never exceeded 5–10 μg fibronectin/mg fibrinogen (29).

Human fibronectin was purified from human plasma by affinity chromatography on gelatin Sepharose, as previously described (10). The purity and the integrity of the protein was verified by SDS-PAGE.

Synthetic peptides were prepared by the solid phase method, described by Merrifield (32), using a synthesizer (Peptomat, Sherbrooke, Canada) with Merrifield resins and t-Boc-amino acids (Fluka AG, Buchs, Switzerland). The first amino acid was attached to the resin by the Merrifield procedure (32) or the Gisin method (17). The general synthesis protocol used was the following: 40% TCA and 0.10% acetyl-D-L-tryptophan in dichloromethane for deprotection, 5% *N*-ethyldiisopropylamine in dichloromethane for neutralization, and two- or threefold excess of t-Boc-amino acids with direct *N-N'*-dicyclohexylcarbodiimide/1-hydroxy-benzatriazol coupling, for 2 h. The completeness of coupling and deprotection was estimated according to the test by Kaiser et al. (25), and each coupling was followed by an acetylation with acetic anhydride. Protecting groups and the peptide–resin link were cleaved with liquid HF-anisole (Interchim) (reference 10; 1 vol/vol) for 1 h at 0°C. After evaporation of HF the resin was washed with anhydrous diethylether. Crude peptides were extracted with 10–45% acetic acid according to their hydrophobicity and freeze-dried twice. Peptides were first purified by gel filtration on Sephadex G10. The amino acid composition was verified by amino acid analysis using an alpha analyzer (LKB Instruments Inc., Stockholm, Sweden) and was consistent with the sequence of the peptides. The homogeneity of the peptides was verified by analytical reverse-phase HPLC, using a C18-ODS 2 column with a 0–80% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Peptides were generally 95% homogenous and, if not, they were purified by preparative HPLC.

Cells

Platelets were isolated from fresh human blood drawn into acid-citrate-dextrose by differential centrifugation followed by gel filtration in a modified Tyrode's buffer, pH 7.2, containing 2% BSA (31). To avoid the formation of microaggregates, 0.1 μM PGE_1 was maintained throughout the procedure and was removed from the platelet suspension during the gel filtration on Sepharose CL 2B.

Human endothelial cells were obtained from human umbilical veins according to the method of Jaffé et al. (24). Optimal plating efficiency and growth rate of endothelial cells were obtained when cells were seeded on fibronectin matrices (30). Thus, petri dishes were coated as follows: 2 ml of a 0.25-mg/ml fibronectin solution in Hanks' buffered saline (HBS) containing 2 M urea were deposited on petri dishes (Falcon Labware, Oxnard, CA) under laminar flow hoods for 20 min at 37°C. The fibronectin solution was removed from the dishes and the cells were plated at a 10^5-cells/ml density and cultured in medium 199 containing 30% pooled serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine. Endothelial cells were passaged by incubating the cells with either HBS/0.05% EDTA or HBS/0.01% EDTA, 0.1% trypsin (Boehringer Mannheim GmbH, Mannheim, FRG). Control experiments indicated that both detachment procedures gave the same results. For example, the binding of ^{125}I-fibrinogen and endothelial cell adhesion was identical when detachment was performed in the presence of EDTA alone or EDTA plus trypsin.

For binding experiments, cells obtained from the second or third passage were washed three times with PBS, pH 7.3, containing trypsin inhibitor (0.1 mg/ml) and resuspended in the binding buffer.

Binding Assays

Binding of fibrinogen to platelets and its inhibition by various peptides were performed as previously described (31). Briefly, platelets at a final concentration of 2×10^8 cells/ml were incubated with ^{125}I-fibrinogen, at a final concentration of 0.1 μM, and varying concentrations of peptides. The binding reaction was initiated by the addition of 5 μM ADP in the presence of 0.5 mM $CaCl_2$, and the interaction was quantified after 30 min at room temperature by separating bound from free ligands by centrifugation of 50-μl aliquots of the reaction mixture through 20% sucrose. Interaction of fibrinogen with endothelial cells was performed with cells in suspension. Briefly, 2×10^6 cells/ml were resuspended in PBS, 2% BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and ^{125}I-fibrinogen was added at a final concentration of 0.04 μM. After 3 h at 37°C, ^{125}I-fibrinogen bound to cells in the presence or absence of the selected peptides was measured. Nonspecific binding was evaluated by measuring ^{125}I-fibrinogen binding in the presence of a large excess of unlabeled fibrinogen (20 μM). The inhibitory capacity of the peptides was expressed as the IC_{50} value, the concentration which produced 50% inhibition of specific binding.

In parallel experiments, the viability of the cells was controlled by (*a*) dye exclusion procedure with fluorescein (44) for viable cells and ethidium bromide for dead cells; and (*b*) the ability of the cells to attach and spread under various conditions. In all experiments it was observed that after 3 h of incubation, the viability of the cells ranged from 80 to 90%, and ~85 ± 10% of the cells readhered and respread.

In control experiments, it was verified that the viability of the cells in the presence of the peptides was unaffected as shown by the dye exclusion procedure. Other experiments were performed to determine if the different peptides were hydrolyzed by endothelial cells or by platelets. For these control experiments, the peptides were incubated for 3 h with either platelets or endothelial cells, the cells were centrifuged, the supernatant was treated with 15% TCA, and the soluble peptides were analyzed by HPLC. These controls indicated that none of the peptides tested were hydrolyzed by the cells during the time of the assay.

Functional Assays

Platelet aggregation was performed using washed platelets. Platelets were isolated from human blood drawn into acid-citrate-dextrose by differential centrifugation followed by gel filtration in a modified Tyrode's buffer or medium 199, both containing 2% BSA. Aggregation was measured in a dual-channel aggregometer (Model 800B; Payton Scientific Inc., Scarborough, Ontario, Canada) at 37°C at a stirring rate of 1,000 rpm. The reaction mixture consisted of 400 μl buffer, 10 μl solution of synthetic peptides, 10 μl $CaCl_2$ (1 mM final concentration), 10 μl fibrinogen (0.5 μM final concentration), and 10 μl ADP (5 μM final concentration).

For adhesion assays, endothelial cells were labeled for 48 h with tritiated thymidine (0.2 μCi/ml) (Amersham International). Confluent cells were detached as described above and resuspended in medium 199 containing 2% BSA. Petri dishes were coated with purified fibrinogen at 0.25 mg/ml. The fibrinogen concentration on the plastic was determined using the radiolabeled protein as a tracer and was 8 $\mu g/cm^2$. 1 ml of radiolabeled cells was seeded in petri dishes, and attachment was measured in the presence of various concentrations of peptides after 120 min at 22°C. Each peptide was tested in triplicate with several different preparations of endothelial cells.

The spreading was estimated on a four-point scale ranging from rounded cells (0) to extensive spreading (++++). The scale was based on double immunofluorescent localization of actin (see Results).

Double Immunofluorescent Localization of Actin

Human antiactin was a gift of Dr. Micouin, from the Blood Center of Gre-

Table I. Comparative Effect of RGD Peptides on the Binding of Fibrinogen to ADP-stimulated Platelets and to Endothelial Cells

Peptides		IC_{50} Platelets	IC_{50} Endothelial cells
		μM	μM
RGD		>1,000	>1,000
RGDS	(Fn, vWF, Fg)	55 ± 20	200 ± 60
RGDQ	(C_3bi)	45 ± 10	>1,000
RGDC	(Pro-vWF)	35 ± 10	320 ± 20
RGDV	(Vn)	18 ± 7	350 ± 20
RGDF	(Fg)	9 ± 3	150 ± 50
NIMEILRGDF	(Fg)	160 ± 70	40 ± 5
ILRGDFSSAN	(Fg)	>500	50 ± 5

Platelets were stimulated with 5 μM ADP. The results are the mean value of three separate experiments. Each peptide corresponds to a naturally occurring sequence within the structure of adhesive proteins: (*Fn*, fibronectin; *Fg*, fibrinogen; *vWF*, von Willebrand Factor; *Vn*, vitronectin; and *C_3bi*, activated complement factor C_3b. The single letter amino acids are as follows: *R*, arginine; *G*, glycine; *D*, aspartic acid; *S*, serine; *Q*, glutamine; *C*, cysteine; *V*, valine; *F*, phenylalanine; *L*, leucine; *A*, alanine; *I*, isoleucine; *N*, asparagine; *M*, methionine.

noble. The cells were detached, resuspended in serum-free medium 199 containing 2% albumin, and seeded on fibrinogen-coated coverslips in the presence of various peptide concentrations as described for adhesion assays. Immunofluorescent localization of actin in cells was carried out according to a modified method of Granger and Lazaridés (18). Briefly, cells were washed three times with PBS at 37°C and then immersed in 3.7% formaldehyde in PBS at 37°C for 10 min. Cell permeation to antibodies was performed by dipping the coverslips for 5 min in a solution containing 0.5% Triton X-100 in PBS. The antiactin solution was added to coverslips during 30 min at 37°C. The cells were then washed for 15 min with the Triton-PBS buffer. The fluorescein conjugated Fab_2' fragments of goat anti-human IgG (The Jackson Laboratory, Bar Harbor, ME) were deposited during 30 min at 37°C. Finally, the cells were washed for 15 min and mounted in 50% glycerol-PBS. Observations were carried out with a photomicroscope (Reichert Jung, Vienna, Austria) equipped for epifluorescence. Fluorescence images were recorded on AGFA film, 400 ASA, and developed with T. MAX (Eastman Kodak Co.).

Results

The Effects of RGD Peptides

The involvement of an RGD recognition specificity in ligand-receptor interactions has been demonstrated by the capacity of RGDX (where X can be one or several permissive amino acid substitutions) peptides to interfere with binding of the ligand to the receptor. To define more precisely the RGD recognition specificity involved in mediating the binding of fibrinogen to endothelial cells, a series of RGDX peptides was synthesized and their capacity to inhibit the interaction of fibrinogen with these cells was examined. The effect of these peptides on fibrinogen binding to platelets was analyzed in parallel experiments to permit comparison of the recognition specificities of the two cells. With both cell types, the RGD tripeptide was ineffective since it did not produce detectable inhibition of fibrinogen binding at a 1-mM concentration (Table I). Thus, the three-amino acid sequence is not sufficient to inhibit fibrinogen binding to either cell type. In contrast, several RGDX tetrapeptides were identified that inhibited fibrinogen binding to both cell types, indicating that an RGD recognition specificity is involved in the interaction of this ligand with both cells. As shown in Table I, RGDS, RGDC, RGDV, and RGDF were all found to inhibit fibrinogen binding to both endothelial cells and platelets. Each of these four tetrapeptides was more potent in inhibiting the interaction of the ligand with platelets than with endothelial cells. As the relative potency of the peptides in inhibiting the interaction of fibrinogen with the two cell types differed considerably (RGDS was 4-fold more active with platelets, whereas RGDV was 20-fold more active), the recognition specificity of platelets and endothelial cells for fibrinogen cannot be identical. The most telling example of this disparity was observed with the RGDQ tetrapeptide. This peptide inhibited fibrinogen binding to platelets with an IC_{50} of 45 ± 10 μM, whereas no inhibition of fibrinogen binding to endothelial cells was observed at 1 mM.

As noted in Table I, extension of the RGDF tetrapeptide in either the NH_2- or COOH-terminal direction resulted in peptides that were less potent than the tetrapeptide itself in inhibiting fibrinogen binding to platelets. In contrast, such extensions resulted in peptides that were more potent than the parent tetrapeptide in inhibiting fibrinogen binding to endothelial cells. The two decapeptides shown in Table I correspond to the natural amino acid sequences surrounding the RGDF sequence at A α 95–98 of human fibrinogen (8). These extended peptide derivatives were more potent in inhibiting fibrinogen binding to endothelial cells than to platelets. To emphasize the differential effects of the extended peptides on fibrinogen binding to the two cell types, the full-dose titration inhibition curves of the two decapeptides and the parent RGDF tetrapeptide are presented in Fig. 1. The similarity in the effects of the three peptides on fibrinogen binding to endothelial cells is apparent and contrasts with their markedly different effects on platelets.

In examining the inhibitory potencies of the RGDX peptides on fibrinogen binding to platelets, a clear influence of the residue in the X position is observed. In the peptides compared in Table I, the more hydrophobic was the residue at the fourth position, the more potent was the peptide in inhibiting fibrinogen binding to platelets. This point is illustrated in Fig. 2 in which the hydrophobicity of the residue at the X position has been assigned a value, according to the scale of Hopp and Woods (22), and plotted against its IC_{50} value. A relationship between inhibitory potency and hydrophobicity is observed. Such a relationship is not observed for fibrinogen binding to endothelial cells (Table I). For example, RGDV has a more hydrophobic residue at the X position than RGDS, but RGDS is more potent in inhibiting fibrinogen binding to endothelial cells.

The Effects of Fibrinogen γ-Chain Peptides

Peptides derived from or corresponding in sequence to the extreme COOH-terminal region of the γ chain of fibrinogen have been shown to inhibit the interaction of fibrinogen and other adhesive proteins with GPIIb-IIIa (27, 37). A panel of peptides corresponding to the sequence of this region was synthesized and used, first, to determine if they interfered with fibrinogen binding to endothelial cells and, second, to compare the structure-activity relationship of these γ-chain peptides on platelets and endothelial cells. As shown in Table II and consistent with previously published data (27, 37), the dodecapeptide, which is designated H12 and corresponds to

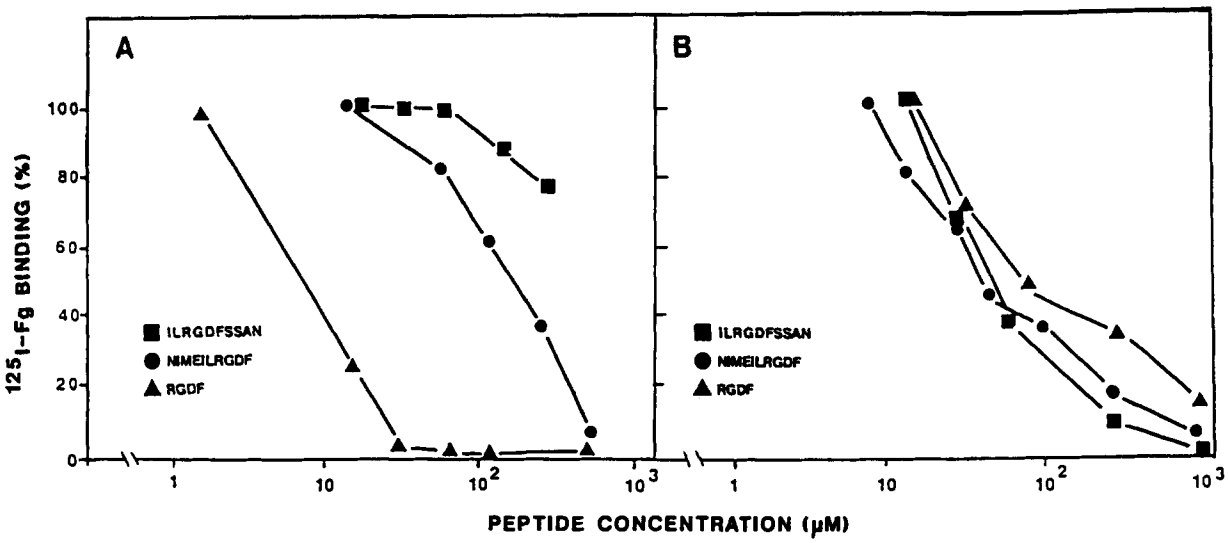

Figure 1. Effect of synthetic peptides corresponding in sequence to the fibrinogen A α chain on the binding of fibrinogen to ADP-stimulated platelets (*A*) and to endothelial cells (*B*). Binding assays were performed as described in Materials and Methods. Endothelial cells (2×10^6 cells/ml) in PBS, 2% BSA, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ were incubated for 3 h at 37°C in the presence of 0.04 μM ^{125}I-fibrinogen. Platelets (2×10^8 cells/ml) in Tyrode's buffer, 2% BSA containing 1 mM $CaCl_2$ were incubated 30 min at 22°C in the presence of 0.1 μM ^{125}I-fibrinogen and 5 μM ADP. Binding was measured in the presence of various concentrations of RGDF (▲), corresponding to the A α 95–98 sequence, or in the presence of two different decapeptides, corresponding to the natural sequence surrounding RGDF in the A α chain: ILRGDFSSAN (■) and NIMEILRGDF (●).

His 400 to Val 411 of the γ chain of fibrinogen, inhibited the binding of fibrinogen to ADP-stimulated platelets with an IC_{50} of 240 ± 20 μM. This level of inhibitory potency was maintained as the size of the peptide was reduced from the dodecapeptide down to the hexapeptide K6. Moreover, as shown in Fig. 3 *A*, the inhibition produced by these peptides exceeded 85%. Further reduction in the size of the peptides to generate the pentapeptide Q5 resulted in a sharp decline in inhibitory activity. No inhibition of fibrinogen binding to platelets was detected with Q5, A4, or G3 at 1 mM.

As shown in Table II, certain γ-chain peptides, including H12 as well as some of the smaller derivatives, also inhibited fibrinogen binding to endothelial cells. Thus, the endothelial cell cytoadhesin shares a γ peptide recognition specificity with platelets, but this specificity was not identical for the two cell types as most notably demonstrated with the K6 peptide. This hexapeptide inhibited fibrinogen binding to platelets with a potency similar to those of the longer γ-chain peptides, but it did not inhibit fibrinogen binding to endothelial cells at the highest concentration tested. This difference is

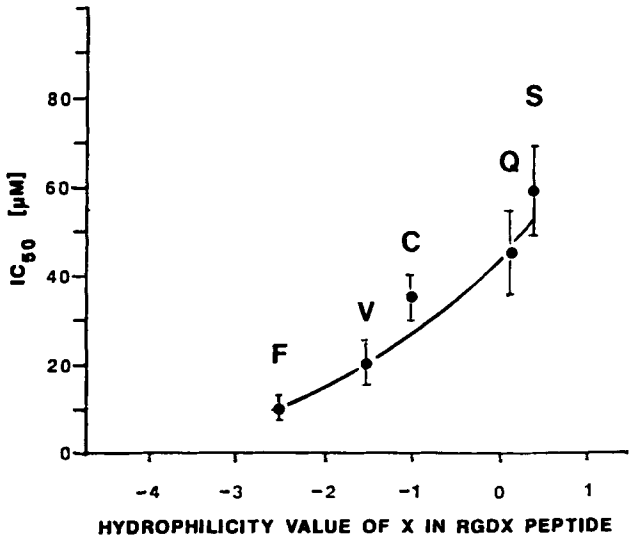

Figure 2. The effect of the hydrophilicity of the fourth amino acid residue at position X on the inhibitory potency of RGDX tetrapeptides on the platelet–fibrinogen interaction. The IC_{50} values, the means from three separate experiments, are plotted against the hydrophilicity of the X residue according to the scale of Hopp and Woods (22). The amino acid residues are (*F*) phenylalanine, (*V*) valine, (*C*) cysteine, (*Q*) glutamine, and (*S*) serine.

Table II. Inhibition of Fibrinogen Binding to ADP-stimulated Platelets and to Endothelial Cells by Synthetic Peptides Corresponding to the COOH-Terminus of the Fibrinogen γ Chain

	Peptides	IC_{50} Platelets	IC_{50} Endothelial cells
		μM	μM
H12	HHLGGAKQAGDV	240 ± 20	140 ± 20
H11	HLGGAKQAGDV	240 ± 20	360 ± 10
L10	LGGAKQAGDV	170 ± 50	130 ± 80
G9	GGAKQAGDV	180 ± 30	330 ± 20
K6	KQAGDV	210 ± 20	>1,000
Q5	QAGDV	>1,000	>1,000
A4	AGDV	>1,000	
G3	GDV	>1,000	

Binding of ^{125}I-fibrinogen to platelets and to endothelial cells was measured as described in Fig. 1. The IC_{50} values represent the mean value of four separate experiments and were derived from the dose-dependent inhibition curves.

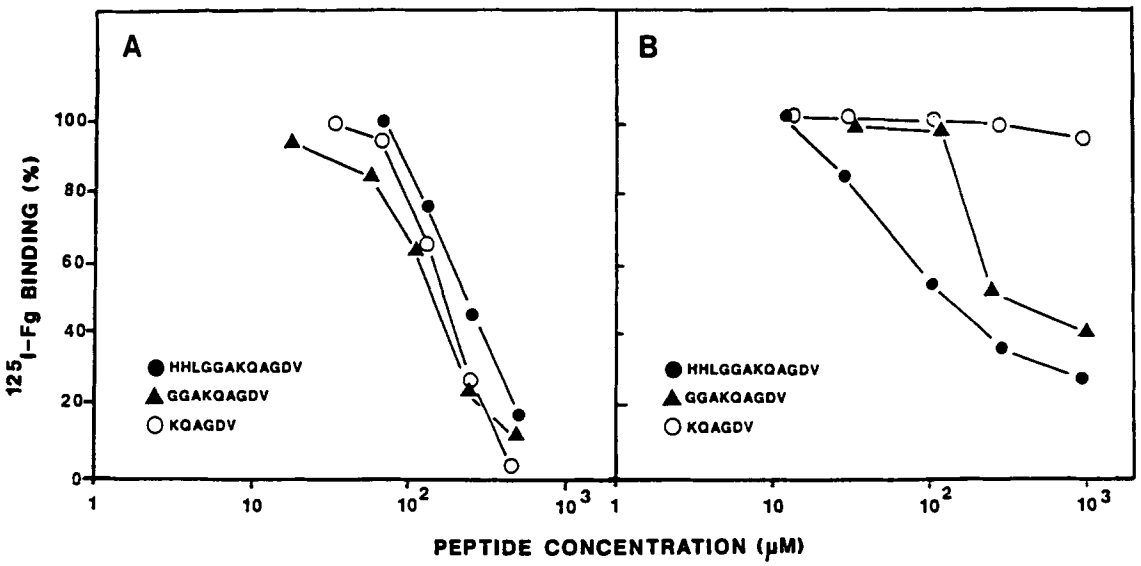

Figure 3. Effects of three selected synthetic peptides, corresponding to the extreme COOH-terminal sequence of the fibrinogen γ chain, on fibrinogen binding to platelets (*A*) and to endothelial cells (*B*). The conditions for measuring fibrinogen binding are those described in Fig. 1. The selective peptides are H12 (●), HHLGGAKQAGDV; G9 (▲), GGAKQAGDV; and K6 (○), KQAGDV. *H*, histidine; *L*, leucine; *G*, glycine; *A*, alanine; *K*, lysine; *Q*, glutamine; *D*, aspartic acid; and *V*, valine.

apparent from the data shown in Fig. 3 *B*. At 1 mM, K6 failed to inhibit endothelial cell binding of fibrinogen, whereas it completely inhibited binding of the ligand to platelets. Also noted in Fig. 3 *B* is a difference in the extent of inhibition attained at high concentrations of the inhibitory peptides. While the inhibition of fibrinogen binding to platelets with peptides such as H12 and G9 was complete, these same peptides only partially inhibited the binding of the ligand to endothelial cells. Thus, this partial inhibition pattern and the marked difference in the inhibitory potency of K6, distinguish the γ-chain recognition specificity of endothelial cells from platelets.

The Fine Recognition Specificity of Platelets and Endothelial Cells for Fibrinogen

The above observations indicate that fibrinogen binding to endothelial cells and platelets is inhibited by both RGDX and γ-chain peptides. In parallel experiments, it was established that the two sets of peptides do not have a synergistic effect on these reactions. Thus RGDX and γ peptides produced an additive inhibition when mixed at different concentrations (data not shown). However, the precise structural requirements for the inhibitory activity of these peptides differ for the two cell types. In subsequent experiments, this difference was further explored with RGDF and KQAGDV serving as the prototypic structures of the two peptide sets. Single amino acid substitution profoundly diminished the inhibitory potency of RGDF peptides on fibrinogen binding to platelets. As shown in Table III, substitution of a Gly (RGGF) for the Asp or a His (HGDF) or a Gln (QGDF) for the Arg resulted in a shift in the IC_{50} of RGDF from 9 μM to >1 mM. This precise structure-function relationship is consistent with previous studies demonstrating that conservative amino acid substitutions at either the Arg, Gly, or Asp positions also resulted in peptides that poorly inhibited fibrinogen binding to platelets (38). Thus, conservation of the RGD structure is essential for peptides that are potent inhibitors of fibrinogen binding to platelets. In contrast, the three substituted peptides, which were inactive on fibrinogen binding to platelets, interfered with fibrinogen binding to endothelial cells and were of a potency similar to the parent RGDF peptide (see Table III). The two peptides in which His or Gln were substituted for the Arg residue were as effective as RGDF, and the peptide in which the Asp was replaced by a Gly was only slightly less inhibitory. These data establish a clear difference in the capacity of endothelial cells and platelets to distinguish among these related peptide structures.

A similar conclusion was derived from analysis performed with variants of the γ-chain peptide K6. Substitution of an

Table III. Structure-Function Relationship of RGD and γ Peptides in the Inhibition of ^{125}I-Fibrinogen Binding to Platelets and to Endothelial Cells

Peptides	IC_{50}	
	Platelets	Endothelial cells
	μM	*μM*
RGD analogues		
RGDF	9 ± 3	150 ± 50
RGGF	>1,000	340 ± 40
HGDF	>1,000	80 ± 40
QGDF	>1,000	150 ± 30
γ analogues		
KQAGDV	210 ± 20	>1,000
RQAGDV	>1,000	600 ± 100
HQAGDV	>1,000	180 ± 20

Binding of ^{125}I-fibrinogen to platelets or to endothelial cells was assayed as described in the text or in Fig. 1. The IC_{50} values were obtained from the dose-dependent curves of inhibition and are the mean value of three separate experiments.

Table IV. The Effects of Different Peptides on the Adhesive Reactions of Platelets and Endothelial Cells

Peptides	Inhibition		
	Platelet aggregation	Endothelial cell attachment	Endothelial cell spreading
	%	%	
None	0	0	++++
RGDF	100	84 ± 6	0
HGDF	0	38 ± 8	0
KQAGDV	84 ± 7	7 ± 5	++
HQAGDV	0	0	++

Endothelial cell attachment and spreading were estimated in medium 199, 2% BSA in the presence of a 2-mM concentration of each peptide. Aggregation was measured with washed platelets suspended in the medium 199 in the presence of 2 mM peptides and 20 μM ADP. The scale used to estimate endothelial cell spreading was based on double immunofluorescent localization of action. Maximal spreading was noted (++++) and corresponded to Fig. 4 *A*; incomplete spreading was noted (++) and corresponded to Fig. 4 *B*; minimal spreading was noted (0) and corresponded to Fig. 4 *C*.

Arg (RQAGDV) or a His (HQAGDV) for the Lys residue at the NH_2 terminus yielded peptides that no longer inhibited fibrinogen binding to platelets; i.e., these replacements decreased inhibitory potency at least fivefold. In contrast, these variant peptides inhibited fibrinogen binding to endothelial cells, even though the parent K6 peptide was inactive. HQAGDV was at least fivefold more potent in inhibiting fibrinogen binding to endothelial cells than to platelets and was at least fivefold more potent than K6 on endothelial cells.

Effects of the Peptides on Platelet and Endothelial Cell Function

To place the above described inhibition experiments in a functional perspective, the effects of selective peptides on fibrinogen-dependent platelet aggregation and endothelial cell adhesion and spreading were examined. To compare the relative potency of each peptide under the same conditions, both cells were suspended in medium 199. The results obtained with platelets in medium 199 (Table IV) were identical to those obtained when the cells were washed and suspended in the routine tyrode–2% albumin buffer. Adhesion and spreading of endothelial cells were performed with fibronectin-depleted fibrinogen. Spreading was estimated with a double immunofluorescent staining procedure using antiactin antibodies. A typical pattern in the absence of peptides was shown in Fig. 4 *A*: extensive spreading led to stress fiber organization. As noted in Table IV and Fig. 1, RGDF inhibited fibrinogen binding on platelets and platelet aggregation. This peptide on endothelial cells also inhibited fibrinogen binding, adhesion, and spreading. The inhibition curves were dose dependent (Fig. 5). HGDF, which inhibited fibrinogen binding to endothelial cells (Fig. 5) but not to platelets (Table III), had no effect on platelet aggregation (Table IV). As shown in Fig. 5, HGDF and RGDF produced the same dose-dependent inhibition curves for the binding of fibrinogen to endothelial cells, but the adhesive properties of these two peptides were quite different. While RGDF inhibited endothelial cell adhesion with a similar dose-dependent curve, HGDF had a weaker effect (Fig. 5). HGDF, however, produced a complete inhibition of endothelial cell spreading as shown in Fig. 4 *C*, where the rounded cells without stress fiber organization were shown. Thus RGDF was a potent inhibitor of endothelial cell attachment and spreading, whereas HGDF was only a potent inhibitor of spreading. This difference suggests that the mechanisms for ligand binding in the attachment and spreading reactions are probably different.

Two different hexamers from the γ-chain series were also tested. KQAGDV, at 2-mM concentration, produced an almost complete inhibition of the aggregation of washed platelets but had no effect on endothelial cell adhesive reactions (Table IV). The modified peptide, HQAGDV, which was inactive on fibrinogen binding to platelets, did not inhibit platelet aggregation. This peptide, which inhibited fibrinogen binding on endothelial cells, although to a lesser extent than RGDF, did not modify endothelial cell attachment but produced a slight inhibition of endothelial cell spreading on fibrinogen. Partial stress fiber organization was formed in the cells shown in Fig. 4 *B*.

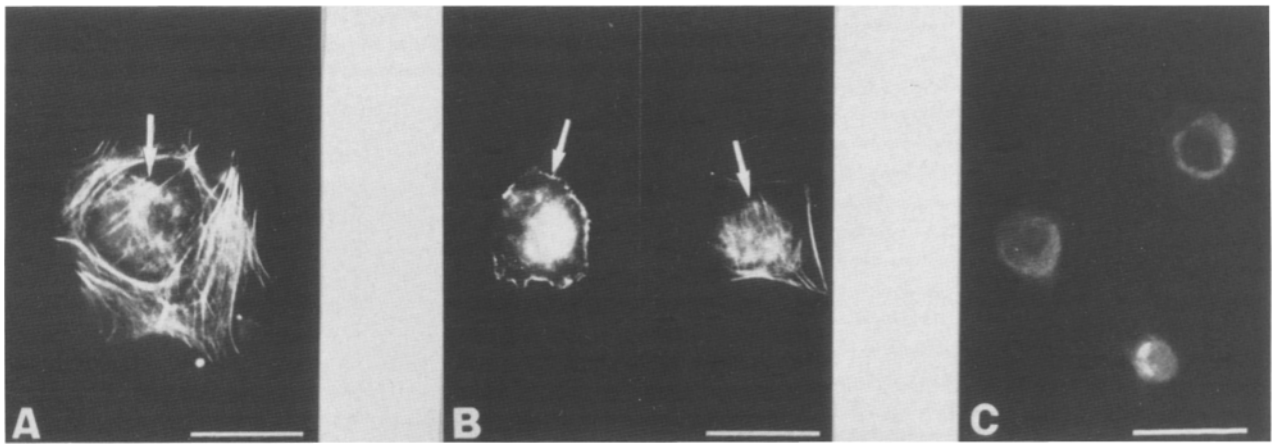

Figure 4. Actin microfilament bundle organization in endothelial cells cultured for 2 h on fibronectin-depleted fibrinogen substrates in the presence or in the absence of inhibitory peptides. Cells were fixed and stained immunofluorescently with human antiactin antibodies. *A* represents extensive cell spreading and stress fiber formation (*arrow*) in the absence of peptides (++++). *B* represents partial spreading and minimal actin bundles (*arrows*) in the presence of 2 mM of the peptides KQAGDV or HQAGDV (++). *C* shows no spreading and no microfilament bundles in the presence of 2 mM of the peptide HGDF (0). Bars, 10 μM.

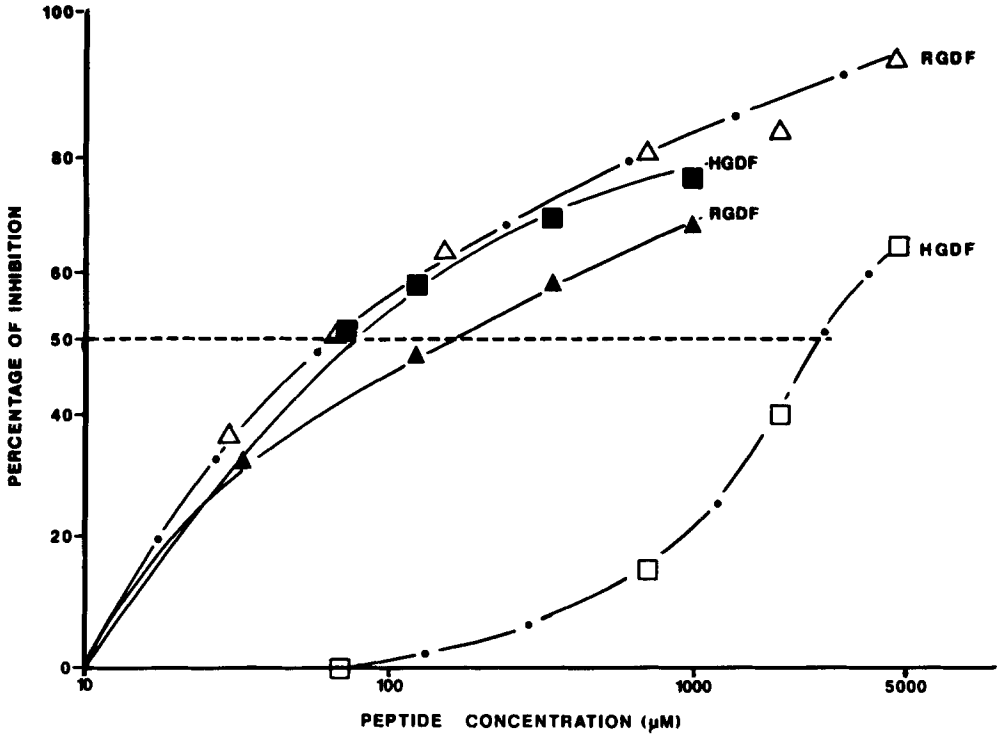

Figure 5. Comparison of the inhibitory capacity of the peptides RGDF and HGDF to inhibit, respectively, fibrinogen binding to endothelial cells and adhesion to fibrinogen-coated dishes. The binding and the adhesion assays were performed as described in Materials and Methods. RGDF in the adhesion (△) and binding (▲) assays had a similar inhibitory effect, whereas HGDF has an appreciable effect in the binding assay (■), but a weak effect in the adhesion assay (□).

Discussion

The platelet fibrinogen receptor is the membrane glycoprotein GPIIb-IIIa, a calcium-dependent heterodimer that belongs to the RGD adhesion receptor superfamily. In addition to fibrinogen, this receptor interacts with two other RGD-containing proteins, fibronectin (13, 36) and von Willebrand factor (12, 45), suggesting that GPIIb-IIIa has a broad recognition specificity. Two sets of synthetic peptides have been shown to inhibit the binding of these adhesive proteins to GPIIb-IIIa and their related adhesive reactions: peptides containing the sequence RGD, common to all the interactive ligands, and peptides corresponding to the COOH terminus of the fibrinogen γ chain with the structure HHLGGAKQAGDV which is only present within the fibrinogen molecule. Both sets of peptides interact with GPIIb-IIIa at specific and related sites (9, 28, 47). The molecular entity for the fibrinogen binding sites on endothelial cells has not yet been clearly identified. However, recent findings have indicated that the endothelial cell binding site for fibrinogen also has an RGD specificity and that certain GPIIb-IIIa antibodies can inhibit this interaction (29). To compare the specificity of the endothelial binding sites with that of GPIIb-IIIa, we have analyzed the structure–function relationship of both sets of peptides in the two cellular systems.

Consistent with previously published observations concerning other RGD adhesion receptors (33, 35), the tripeptide RGD itself was inefficient in inhibiting the binding of fibrinogen to platelets and to endothelial cells. A fourth amino acid residue at position X in the sequence RGDX was necessary for the synthetic peptides to express inhibitory activity on both cells. With platelets, the more hydrophobic this residue was, the more potent the RGDX peptide was. For instance, RGDF was six times more potent than RGDS. This structure–function relationship was not observed on endothelial cells as RGDF and RGDS were equally potent. These results are consistent with the recent findings that the amino acid residues at position X may considerably influence the stereochemistry and potency of RGDX peptides in inhibiting RGD receptors on other cells (34). A differential effect was observed when the arginine residue in RGDF was replaced by a more acidic residue (HGDF) or a neutral residue (QGDF). These peptides had contrasting effects on fibrinogen binding to the two cell types.

The shortest γ-chain peptide that was active on platelets was the hexapeptide, KQAGDV, which corresponds to Lys 406 to Val 411 of the γ chain of fibrinogen. This peptide inhibited fibrinogen binding to platelets with an IC_{50} similar to that of the dodecapeptide, HHLGGAKQAGDV. This result is somewhat different from those obtained by Kloczewiack et al. (27), who have shown that the dodecapeptide was more potent than the heptapeptide AKQAGDV, and our own previous study (37), in which the nonapeptide was found to be less active than the dodecapeptide. At the present time, we have no explanation for these discrepancies; however, all peptides tested have now been synthesized on multiple occasions and have consistently given the results shown in the present study. Again, it is noteworthy that the amino acid residue present at the COOH terminus of the hexapeptide K6 is a Val, which has hydrophobic properties, and that this peptide was inactive on endothelial cells. However, when the lysine residue was replaced by the more acidic histidine residue, the activity was diminished for platelets and enhanced for endothelial cells.

From these results, the following conclusions can be drawn: (*a*) both RGDX and γ-chain peptides inhibit the binding of fibrinogen to platelets and to endothelial cells; (*b*) a clear influence of the residue at the COOH position and the

NH_2 position of each peptide set can be demonstrated on the two cell types; (*c*) although RGDX and γ peptides have a differential effect on platelets and on endothelial cells, their structural requirements for inhibiting fibrinogen binding to one cell type appear to be consistent; for instance, HGDF and HQAGDV were both inactive on platelets but were potent inhibitors on endothelial cells; and (*d*) inhibition of fibrinogen binding correlated with an inhibition of the adhesive reactions of each cell type. This relationship between inhibition of binding and function is, however, more complex for endothelial cells. In previous studies, we have shown that the plasmin degradation product fragment D of high molecular weight, which contains the COOH-terminal domain of the γ chain, did not significantly inhibit the binding of fibrinogen to endothelial cells. The γ-chain sequence may have an affinity lower than the RGDF sequence that is present in the fibrinogen A α chain for endothelial cells. It is noteworthy that none of the γ-chain peptides completely inhibited binding of fibrinogen to endothelial cells. Accordingly, the fibrinogen recognition site on endothelial cells may interact predominantly with the RGDF sequence in the A α chain. The observation that RGDF-containing decapeptides with the adjacent sequence from the A α chain were more potent than RGDF itself supports this hypothesis.

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