Peptide Ligands for Integrin $\alpha_v\beta_3$ Selected from Random Phage Display Libraries

Judith M. Healy,*,‡ Ohoshi Murayama,§ Toshinaga Maeda,§ Kazuhiro Yoshino,§ Kiyotoshi Sekiguchi,§ and Masakazu Kikuchi‡

Protein Engineering Research Institute, 6-2-3 Furuedai, Osaka 565, and Research Institute, Osaka Medical Center for Maternal and Child Health, 840 Murodo-cho, Izumi, Osaka 590-02, Japan

Received November 15, 1994; Revised Manuscript Received January 13, 1995[⊗]

ABSTRACT: The integrin $\alpha_v\beta_3$ binds promiscuously to cell-adhesive proteins: vitronectin, fibronectin, and several others containing the RGD motif. We have explored molecular recognition by $\alpha_v\beta_3$ through selection of ligands from large random libraries of peptides displayed on phage. Ligands bound by $\alpha_v\beta_3$ consisted primarily of RGD peptides; however, these peptides showed considerable heterogeneity with respect to the identities of amino acids flanking RGD. The tolerance of $\alpha_v\beta_3$ for RGD peptides of diverse composition is consistent with its role *in vivo* as a versatile receptor for RGD-containing extracellular matrix proteins. Peptide ligands for $\alpha_v\beta_3$ also included a novel binding sequence, identical to a tetrapeptide found in vitronectin, which is a candidate for a synergistic site in this adhesive protein that may act in concert with RGD to promote molecular recognition.

Adhesion receptors of the integrin family are responsible for a wide range of cell−extracellular matrix (ECM)[1] and cell−cell interactions [reviewed in Hynes (1992) and Luscinskas and Lawler (1994)]. Each integrin consists of noncovalently associated α and β subunits, which pair to create heterodimers ($\alpha\beta$) with distinctive adhesive capabilities. As receptors for ECM proteins, integrins provide anchorage and convey signals that regulate cell growth, differentiation, and migration. The integrin $\alpha_v\beta_3$ is expressed on endothelial cells, osteoclasts, melanoma, and other cell types (Cheresh, 1987; Cheresh & Spiro, 1987; Pytela et al., 1985) where it plays a role in physiological processes that include angiogenesis and tissue repair, as well as pathological conditions such as osteoporosis, tumor cell metastasis, and adenoviral infection (Davis, 1992; Felding-Habermann et al., 1992; Seftor et al., 1992; Ross et al., 1993; Brooks et al., 1994; Nemerow et al., 1994). Though originally isolated as a receptor for vitronectin, $\alpha_v\beta_3$ actually recognizes a broad range of ECM protein ligands: vitronectin, fibronectin, fibrinogen, von Willebrand factor, thrombospondin, and osteopontin, all of which contain the classical integrin-recognition motif Arg-Gly-Asp (RGD) (Lawler et al., 1988; Charo et al., 1990; Ross et al., 1994). The relaxed specificity of $\alpha_v\beta_3$ contrasts sharply with the selectivity of the $\alpha_5\beta_1$ integrin, which binds exclusively to fibronectin and is apparently capable of recognizing the RGD sequence only within the context of the fibronectin molecule (D'Souza et al., 1991). Thus, though the RGD motif has been firmly established as a key determinant in the recognition of ECM protein ligands by $\alpha_v\beta_3$, $\alpha_5\beta1$, and other integrins, the molecular basis for differences in receptor−ligand specificity remains poorly understood.

Models for molecular recognition by integrins include the proposal that RGD adopts different functional conformations in different adhesive proteins and that these conformations are recognized selectively by one or more receptors (Ruoslahti & Pierschbacher, 1987). Indeed, cyclic peptides in which the conformation of RGD is fixed often exhibit considerably higher affinity and selectivity for integrins than their flexible linear counterparts (Pierschbacher & Ruoslahti, 1987; Gurrath et al., 1992; Pfaff et al., 1994). Studies of the integrin-binding specificities of synthetic peptides and of snake venom disintegrins also indicate that amino acids adjacent to RGD can influence the strength and selectivity of receptor−ligand interactions, perhaps by creating an extended RGD locus (Pierschbacher & Ruoslahti, 1987; Scarborough et al., 1993). Other models for integrin specificity invoke contacts between receptor active sites and amino acid residues at synergistic loci elsewhere within ligand molecules. Site-directed mutagenesis has delineated such synergistic regions within the eighth and ninth type III domain repeats of fibronectin that are required, in combination with the RGD-containing tenth type III repeat, for full recognition by $\alpha_5\beta_1$ (Obara et al., 1988; Aota et al., 1991; Yamada, 1991). Recently, the availability of phage display technology has provided a powerful new tool with which to probe ligand binding by integrins and to search for alternative recognition motifs (O'Neil et al., 1992; Koivunen et al., 1993). Phage library screening has identified $\alpha_5\beta_1$-binding sequences that may correspond to additional sites of ligand−receptor contact in fibronectin, as well as a novel, high-affinity ligand for $\alpha_5\beta_1$ that can substitute for the RGD tripeptide (Koivunen et al., 1994).

Here we report the first use of random phage display libraries to explore the ligand binding properties of $\alpha_v\beta_3$. Our results indicate that $\alpha_v\beta_3$ is capable of recognizing a remarkably broad range of peptides containing the RGD motif. Furthermore, we describe a non-RGD motif recognized by $\alpha_v\beta_3$ which matches a tetrapeptide sequence that is present in the ECM protein ligand, vitronectin. We suggest that this tetrapeptide may represent an accessory site in vitronectin that contributes to binding by $\alpha_v\beta_3$.

* Author to whom correspondence should be addressed (fax, (81) 06-872-8210).

‡ Protein Engineering Research Institute.

§ Research Institute, Osaka Medical Center for Maternal and Child Health.

⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1995.

[1] Abbreviations: ECM, extracellular matrix; RGD, arginylglycylaspartic acid; TBS, Tris-buffered saline; BSA, bovine serum albumin; CHO, Chinese hamster ovary.

0006-2960/95/0434-3948$09.00/0

MATERIALS AND METHODS

Reagents. Amplified portions of 2×10^8 clone 6-mer and 2.5×10^8 clone 15-mer peptide libraries constructed in the fUSE5 phage vector (Smith & Scott, 1993) were gifts of Dr. G. Smith (University of Missouri). Peptides were synthesized by Sawadei Technologies and purified by reverse-phase high-performance liquid chromatography (HPLC). Synthetic peptides containing cysteine residues were cyclized by treatment with dimethyl sulfoxide (Tam et al., 1991) prior to HPLC purification. Human vitronectin and fibronectin were supplied by Wako Pure Chemical Industries, Ltd.

Purification of Integrins. Procedures for purification of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins from human placenta have been described previously (Pytela et al., 1987). Proteins were extracted from approximately 100 g of blended placental tissue in TBS(+) buffer (25 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1 mM $CaCl_2$, 1 mM phenylmethanesulfonyl fluoride) containing 100 mM octyl glucoside. Integrins were isolated by affinity chromatography using GRGDSPK-Sepharose ($\alpha_v\beta_3$) or the cell-binding domain of human fibronectin coupled to Sepharose ($\alpha_5\beta_1$) and eluted from columns using GRGDSP peptide (Iwaki Glass) at 250 μg/mL. Purity of integrin preparations was confirmed by silver staining of SDS−polyacrylamide gels and immunoblot analyses using antibodies directed against β3 and β1 integrin subunits (Takara Biomedicals).

Selection of Integrin Binding Phage. Biopanning experiments were carried out essentially as described (O'Neil et al., 1992; Koivunen et al., 1993). All steps were performed at 4 °C. Integrin concentrations were adjusted to 5 μg/mL with TBS(+), and 0.5 μg of $\alpha_v\beta_3$ or $\alpha_5\beta_1$ was coated overnight onto wells of 96-well microtiter dishes (Costar). Wells were washed twice with TBS(+) and blocked for 1 h with 35 mg/mL BSA in TBS(+). Aliquots of 6-mer or 15-mer phage libraries (1×10^{10} transducing units) in incubation buffer [1 mg/mL BSA in TBS(+)] were pretreated in BSA-coated wells for 1 h. Phage unbound to BSA were then transferred to blocked, integrin-coated wells that had been washed twice with incubation buffer. After 1 h, wells were washed 10 times with TBS(+) containing 0.5% Tween 20. Integrin-bound phage were eluted with 0.1 mL of either 1 mM GRGDSP peptide in incubation buffer or 0.1 M glycine, pH 2.0, containing 1 mg/mL BSA. Acid eluates were neutralized by addition of 6 μL of 2 M Tris base. Eluted phage were amplified in strain K91kan (Smith & Scott, 1993), and biopanning was repeated twice for a total of three times.

Templates for nucleotide sequencing were prepared as described (Smith & Scott, 1993). Cycle sequencing using the primer 5′ TGAATTTTCTGTATGAGG 3′ was performed with the PRIZM Ready Reaction Dyedeoxy Terminator kit and model 373A DNA sequencer (Applied Biosystems).

Phage Attachment and Solid-Phase Integrin Binding Assays. Binding of individual phage clones to integrins was assayed by modification of established methods (Koivunen et al., 1993, 1994). Assays were performed with an input of 1×10^8 transducing units per well in 96-well microtiter dishes coated with integrin or BSA as described above. Phage bound to integrin in the absence or presence of increasing concentrations of GRGDSP peptide competitor were quantitated by monitoring the absorbance of K91kan cultures at 600 nm. Wells were washed 10 times with TBS(+) containing 0.5% Tween 20 prior to the addition of bacterial cells. Percent phage attachment was defined as the percentage of input phage bound nonspecifically to BSA-coated wells (consistently below 10%), subtracted from the percentage of input phage bound to integrin-coated wells. Data points denote means of duplicate measurements. Absorbance values for duplicate wells differed from the mean by less than 20%.

Activities of synthetic peptides were assayed by competitive inhibition of the binding of biotin-labeled adhesive proteins to immobilized integrins according to published procedures (Charo et al., 1990, 1991). Vitronectin and fibronectin were biotinylated at a concentration of 1 mg/mL using the NHS-LC-Biotinylation kit (Pierce). Biotin-labeled proteins were washed with TBS and concentrated on 30-kD Centricon ultrafilters (Amicon). The extent of biotinylation of each protein was estimated using the HABA [2-(4′-hydroxyazobenzene)benzoic acid] test (Pierce) to be three to four moelcules of biotin per molecule of vitronectin or fibronectin. Biotinylated adhesive proteins each exhibited high-affinity, saturable binding to integrin-coated microtiter wells (not shown) as described by others (Charo et al., 1990, 1991). In competitive assays of peptide activity, biotinylated vitronectin or fibronectin was added to a final concentration of 10 nM, and bound proteins were quantitated using an anti-biotin antibody conjugated to alkaline phosphatase (Sigma). Absorbance measurements at 405 nm were made using an ELISA reader (InterMed, model NJ-2000). Nonspecific binding, measured in each experiment by determining binding of biotinylated adhesive proteins to wells coated with BSA, represented less than 1% of maximum. All data points denote means of duplicate determinations. The absorbance values of duplicate wells consistently fell within 10−20% of the mean.

RESULTS

Selection of Peptides Binding to $\alpha_v\beta_3$ Integrin from a Random 6-mer Phage Library. To investigate the ligand recognition properties of $\alpha_v\beta_3$, we selected peptides binding to the receptor from a phage display library of six degenerate amino acids. Phage binding to integrin-coated microtiter wells were isolated by repeated rounds of selection and elution with low pH buffer or a commercially available RGD peptide. Three rounds of biopanning using highly purified $\alpha_v\beta_3$ produced a significant enrichment in the proportion of $\alpha_v\beta_3$-binding phage (from 10^{-3}% after round 1 to 10^{-1}% after round 3) over the level of background binding to BSA (10^{-4}%).

The majority of $\alpha_v\beta_3$-selected phage (70 of 76) from the random 6-mer library contained the RGD integrin recognition motif (Table 1), though $\alpha_v\beta_3$-binding sequences were quite heterogeneous with respect to the identities of residues flanking RGD. The amino acids most frequently observed to precede the RGD motif were serine and threonine, while a glycine or serine often followed RGD. However, amino acids adjacent to the N-terminus of the RGD tripeptide also frequently included residues with hydrophobic or charged side chains. Amino acids C-terminal to RGD were similarly diverse. In several independent isolates, RGD was flanked by cysteine residues, generating potentially cyclic disulfides

Table 1: Deduced Amino Acid Sequences of Peptides Binding to $\alpha_v\beta_3$ Integrin from a 6-mer Phage Display Library[a]

RGD peptide elution		low pH elution		low pH/RGD peptide elution	
CRGDCA(2)	FMTRGD	SCRGDC(2)	MRGDRG	TKRGDH	ARWRGD
CRGDCT	SRGDSF	WMRGDV	SRGDGI	RRGDHI	DSARGD
SCRGDC(2)	DTRGDW(2)	GSRGDF	RGDSYP	RGDGSS	RGDSLQ
NCRGDC	TVVRGD	NSRGDF	VLARGD	LQRGDW	TWRRGD(2)
SRGDNT	RGDAWL	FLTRGD	INSRGD	ATRGDT	SRLRGD
FSRGDR	RGDSLT	AFRGDS	RGDGYV	FRGDFS	RGDRSL
LNRGDG	TMRGDV	VLFRGD	RENRGD	VLRGDN	RGDGWL
WLGRGD	VRGDGV	KSMRGD	SLPRGD(2)	TRRGDT	PTQRGD
DRGDTY	TRGDPA	GRGDGS	GFRGDG	QIARGD	RGDYMD
	RGDSFN	PVRRGD	TIRGDQ	FPVRGD	NGLRGD
NGRIPD				RGDFFS	TRGDSL
TNGRGP	RSRNGR	TWNHLS		NGLRGD	RGDFAF
NGRSRF	NGRNTV			QYLRGD	

[a] Selection of phage binding to $\alpha_v\beta_3$ was carried out as described in Materials and Methods. Biopanning was repeated three times, and bound phage were eluted with either commercially available GRGDSP peptide or low pH. In one experiment, phage were eluted with low pH in the first round of screening, followed by elution with GRGDSP peptide in the second and third rounds. Amino acid sequences of hexapeptides displayed on the phage surface as GPIII fusions were deduced by nucleotide sequencing of phage DNA. The number of sequences encoding the same peptide is indicated in parentheses. RGD and NGR motifs are highlighted in bold.

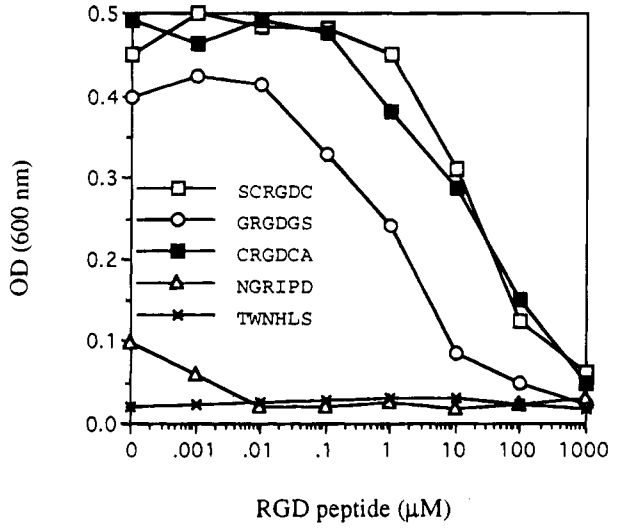

FIGURE 1: Binding of phage displaying six amino acid peptides to $\alpha_v\beta_3$ integrin. Phage attachment to microtiter wells coated with $\alpha_v\beta_3$ was assayed in the absence or presence of increasing concentrations of the synthetic GRGDSP peptide. The input of each phage was 1×10^8 transducing units per well. Bound phage were quantitated after extensive washing by monitoring the absorbance at 600 nm of bacterial cultures. The data represent means from duplicate wells.

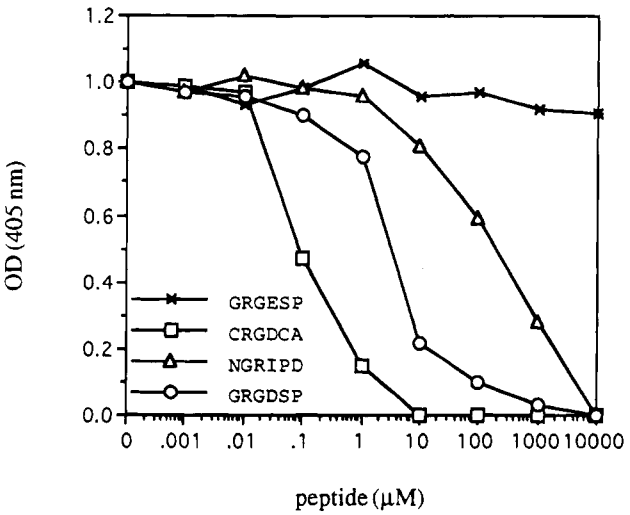

FIGURE 2: Competitive inhibition of fibronectin binding to $\alpha_v\beta_3$ integrin by synthetic peptides. Binding of biotinylated fibronectin to $\alpha_v\beta_3$ was assayed in the presence of the indicated competing peptides as described in Materials and Methods. The data represent means from duplicate wells.

in which the RGD peptide is conformationally constrained. CRGDC-containing isolates were especially prominent in the RGD peptide-eluted phage pool, which included four distinct potentially cyclic sequences: CRGDCA, CRGDCT, SCRGDC, and NCRGDC.

Notably, six $\alpha_v\beta_3$-selected phage encoded peptides that did not contain RGD. Of these, five different isolates carried a sequence, NGR, also known from phage library screening as a motif recognized by the fibronectin receptor, $\alpha_5\beta_1$ (Koivunen et al., 1993, 1994). One phage, TWNHLS, recovered from the acid-eluted pool, did not bear any resemblance to a known intergin binding sequence.

Binding Affinities of Selected 6-mer Sequences for $\alpha_v\beta_3$. To characterize the affinities of sequences identified through phage library screening, we examined binding to $\alpha_v\beta_3$ of both phage-encoded and synthetic peptides. Figure 1 shows inhibition of attachment of phage to $\alpha_v\beta_3$ by increasing concentrations of competitor RGD peptide. As anticipated, two phage displaying potentially cyclic RGD motifs, SCRGDC and CRGDCA, exhibited the strongest binding to $\alpha_v\beta_3$. Attachment of CRGDC-containing phage remained high even at competitor concentrations sufficient to abolish attachment of phage encoding a linear phage peptide. A representative of the group of phage containing the NGR motif, NGRIPD, showed weak but significant binding to $\alpha_v\beta_3$ that could be overcome at low concentrations of RGD peptide competitor. In contrast, phage containing the sequence TWNHLS failed to bind $\alpha_v\beta_3$ significantly over background even in the absence of inhibitory RGD peptide.

We synthesized peptides corresponding to several phage-displayed sequences and confirmed the results of phage attachment experiments in solid-phase binding assays using immobilized $\alpha_v\beta_3$. As shown in Figure 2, the cyclic peptide CRGDCA (IC_{50} 2×10^{-7} M) was a considerably more potent inhibitor of fibronectin binding to $\alpha_v\beta_3$ than the linear fibronectin-derived peptide GRGDSP (IC_{50} 2×10^{-6} M). The activity of linear CRGDCA peptide, not cyclized by oxidation, was largely indistinguishable from that of GRGDSP (not shown), suggesting that the rigidity contributed by

Table 2: Deduced Amino Acid Sequences of Peptides Binding to $\alpha_v\beta_3$ Integrin from a 15-mer Phage Display Library[a]

RGD peptide elution		low pH elution	
S**RGD**GGFIKVLHGSW(3)	**RGD**AIVTFPGSFMLY(2)	V**RGD**SLLFGVQAVLH	HAAFEP**RGD**VRHTLL(2)
RGDMSLLGEFTSPYG(8)	A**RGD**VFYEGSRGAWY	GE**RGD**GSFFAFRSPF(2)	S**RGD**GGFIKVLHGSW(2)
P**RGD**AFPHMQSSASV	R**RGD**IGPRFESAIVD(2)	AG**RGD**SLGNYRNFNS	**RGD**MSLLGEFTSPYG(2)
PEVIA**RGD**VVFFLRP	**RGD**AYAFGNAGVDLI	RGV**RGD**SFFLVMDAH	SL**RGD**HRVRWVLTPH
DALSC**RGD**CVWPTRG		P**RGD**AFPHMQSSASV	DNSHWFRRIS**RGD**AG
		G**RGD**RTDGSSGHVWG(2)	MWVFS**RGD**SSLFCCG
ACGSAGTCSPHLRRP		**RGD**AIVTFPGSFMLY(4)	RAL**RGD**RGWIVFWDP

[a] Selection and sequencing of phage bound to integrin $\alpha_v\beta_3$ were performed as described in Materials and Methods. The number of sequences encoding the same peptide is indicated in parentheses. The RGD motif is highlighted in bold.

disulfide bonding is important for high-affinity binding to $\alpha_v\beta_3$. Binding of a peptide containing the NGR motif to $\alpha_v\beta_3$ (2×10^{-4} M) was significantly greater than that of a biologically inert peptide, GRGESP. The NGRIPD peptide also competitively inhibited binding of vitronectin to $\alpha_v\beta_3$ (IC_{50} 3×10^{-3} M) in solid-phase assays (not shown).

Selection of Peptides Binding to $\alpha_v\beta_3$ Integrin from a Random 15-mer Phage Library. To further examine classes of peptides recognized by $\alpha_v\beta_3$, we screened a library of 15 degenerate amino acids constructed in the same phage vector as the 6-mer library for phage capable of binding to $\alpha_v\beta_3$. The longer insert displayed by 15-mer phage provided an opportunity to gain further insight into sequences utilized during ligand recognition. The yield of $\alpha_v\beta_3$-binding phage increased from 10^{-4}% to 10^{-2}% following three rounds of biopanning of the 15-mer library, as described above, using the same preparation of integrin used in biopanning of the 6-mer library. 15-mer sequences containing the RGD motif were highly enriched by repeated selection with $\alpha_v\beta_3$. Almost all (42 of 43) of the $\alpha_v\beta_3$-binding phage displayed RGD peptides (Table 2). In most cases, the RGD sequence was positioned at or near the N-terminal region of the 15 amino acid insert. Only one isolate carried RGD in proximity to the C-terminus of the displayed region, suggesting that the conformational presentation of RGD in the phage peptide may be an important factor in determining its biological activity. As noted for $\alpha_v\beta_3$-selected hexapeptides (Table 1), strong preferences for a particular amino acid at positions flanking the RGD motif were not generally observed among 15-mer isolates. Serine and alanine were favored slightly at positions preceding and following RGD, respectively, though charged and hydrophobic residues were also tolerated. One potentially cyclic peptide, DALSCRGDCVWPTRG, with homology to CRGDC-containing sequences identified from the 6-mer library, was recovered among phage eluted with RGD peptide. Another cysteine-rich peptide identified in the same eluate, ACGSAGTCSPHLRRP, was the only sequence bound to $\alpha_v\beta_3$ that did not include the RGD motif.

Binding Affinities of Selected 15-mer Sequences for $\alpha_v\beta_3$. The affinities of two RGD-containing 15-mer sequences, one potentially cyclic and the other linear, were compared to that of the novel $\alpha_v\beta_3$-binding sequence, ACGSAGTCSPHLRRP, in phage attachment assays. Phage in which RGD is flanked by cysteines bound much more avidly to $\alpha_v\beta_3$ than phage displaying the linear RGD peptide (Figure 3). On the other hand, attachment of ACGSAGTCSPHLRRP-containing phage to $\alpha_v\beta_3$ was much weaker than that of either RGD-containing phage tested. ACGSAGTCSPHLRRP phage showed a low but significant level of binding to $\alpha_v\beta_3$ that was easily displaced by competing RGD peptide and was reminiscent of the activity of phage containing the NGR motif (Figure 1). Examination of the efficiencies of synthetic peptides in inhibiting binding of vitronectin to immobilized $\alpha_v\beta_3$ verified the high affinity of the cyclic DALSCRGDCVWPTRG peptide compared to a linear sequence derived from vitronectin (Figure 4). Furthermore, the ACGSAGTCSPHLRRP peptide also showed activity in competitive assays (IC_{50} 2.5×10^{-3} M), confirming that this novel, non-RGD peptide is capable of weak binding to $\alpha_v\beta_3$ (Figure 4). To determine whether activity of this sequence could be localized to the interval flanked by cysteine residues, we tested a shorter derivative, ACGSAGTCS, in integrin binding assays. The IC_{50} value of ACGSAGTCS was similar to that of the longer sequence (Figure 4), suggesting that the $\alpha_v\beta_3$-binding activities of these peptides reside in the conformationally contrained segment delimited by cysteines.

Selection of Peptides Binding to $\alpha_5\beta_1$ Integrin from a Random 15-mer Phage Library. To directly compare ligand recognition by $\alpha_v\beta_3$ with that of a more selective integrin, we screened the 15-mer phage library with purified fibronectin receptor, $\alpha_5\beta_1$. Phage bound by $\alpha_5\beta_1$ also consisted primarily of sequences encoding the RGD motif (Table 3). $\alpha_5\beta_1$-selected phage included just one non-RGD sequence, SEELLVESSAIRSE, recovered among phage eluted from integrin with low pH buffer. However, this isolate failed to bind $\alpha_5\beta_1$ over background in subsequent phage attachment assays (not shown). Though most $\alpha_5\beta_1$-binding phage (43 of 44) contained RGD, $\alpha_5\beta_1$ apparently preferred a smaller subset of the RGD-containing peptides represented in the random 15-mer library than did $\alpha_v\beta_3$. Only three phage (RGDMSLLGEFTSPYG, RRGDAIVPREFSAIVD, and DALSCRGDCVWPTRG) were selected by both integrins. The collection of $\alpha_5\beta_1$-binding phage showed less diversity (9 compared to 19 different sequences) and more pronounced preferences for particular types of amino acid residues flanking RGD than $\alpha_v\beta_3$-selected phage. In about half of the RGD-containing sequences bound to $\alpha_5\beta_1$, an arginine preceded the RGD tripeptide, and in nearly every case, the amino acid following RGD was hydrophobic.

Differential Binding of RGD Peptides to $\alpha_v\beta_3$ or $\alpha_5\beta_1$ Integrins. We surveyed the binding affinities of selected sequences for $\alpha_v\beta_3$ or $\alpha_5\beta_1$ using phage attachment assays. Three phage showed considerable selectivity for $\alpha_v\beta_3$, attaching severalfold more efficiently to this receptor than to immobilized $\alpha_5\beta_1$ (Figure 5). Notably, attachment to $\alpha_v\beta_3$ of the phage peptide GERGDGSFFAFRSPF rivaled that of phage displaying the potentially cyclic peptide DALSCRGDCVWPTRG. Some phage peptides, including the constrained RGD sequence, showed approximately equivalent levels of binding to each integrin, while phage bearing the sequence ISRRGDLSGLSFSRL exhibited a degree of pref-

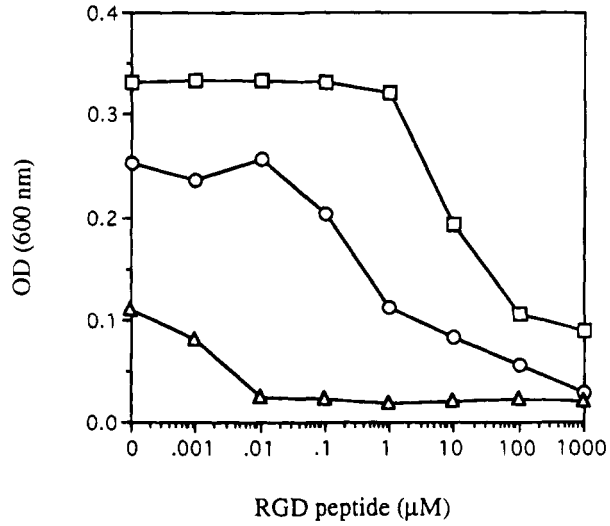

FIGURE 3: Binding of phage displaying 15 amino acid peptides to $\alpha_v\beta_3$ integrin. Phage attachment to immobilized $\alpha_v\beta_3$ was quantitated in the absence or presence of competing RGD peptide as described in the legend of Figure 1. Symbols: (□) DALSCRGDCVWPTRG phage; (○) HAAFEPRGDVRHTLL phage; (△) ACGSAGTCSPHLRRP phage. The data represent means from duplicate wells.

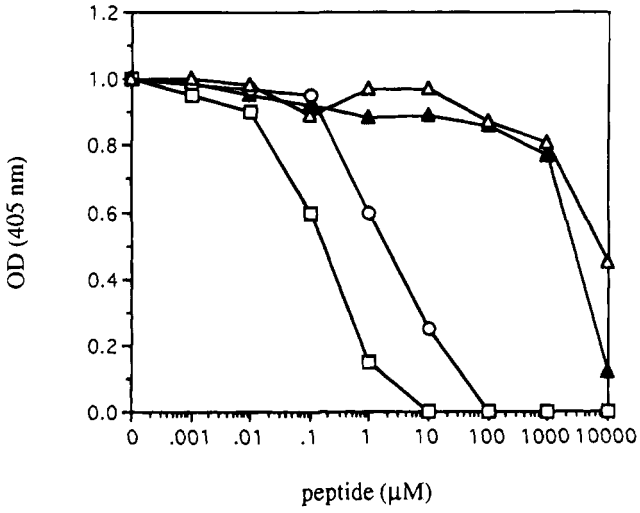

FIGURE 4: Inhibition of vitronectin binding to $\alpha_v\beta_3$ integrin by synthetic peptides containing RGD and SAGT motifs. Binding of biotinylated vitronectin to $\alpha_v\beta_3$ was assayed in the presence of the following synthetic peptides: (□) DALSCRGDCVWPTRG; (○) VTRGDVF; (▲) ACGSAGTCSPHLRRP; (△) ACGSAGTCS. The data represent means from duplicate wells.

erence for $\alpha_5\beta_1$. Phage containing the novel $\alpha_v\beta_3$-binding sequence ACGSAGTCSPHLRRP did not bind detectably to the fibronectin receptor.

Integrin binding assays using synthetic peptides essentially confirmed the results of phage attachment studies (Table 4). Peptides were tested for the ability to competitively inhibit binding of the highest affinity ligand for each integrin: vitronectin ($\alpha_v\beta_3$) or fibronectin ($\alpha_5\beta_1$). As anticipated, the cyclic peptide DALSCRGDCVWPTRG bound with highest efficiency to $\alpha_v\beta_3$. However, the conformationally constrained sequence showed almost no integrin specificity, inhibiting adhesive protein binding to either $\alpha_v\beta_3$ or $\alpha_5\beta_1$ with equivalent IC_{50} values. Among the linear synthetic peptides examined, GERGDGSFFAFRSPF was the most potent inhibitor of vitronectin binding to $\alpha_v\beta_3$. Furthermore, this RGD peptide exhibited a striking preference for $\alpha_v\beta_3$ over $\alpha_5\beta_1$ in competitive binding assays (Table 4).

DISCUSSION

Integrin $\alpha_v\beta_3$ Selects Diverse RGD Peptides from Random Phage Display Libraries. Our results confirm the key role of the RGD motif in ligand recognition by the integrin $\alpha_v\beta_3$. Sequences containing RGD were selected predominantly by $\alpha_v\beta_3$ from vast phage libraries of degenerate peptides, and in several instances, RGD was displayed on phage in apparently cyclic configurations which bound to $\alpha_v\beta_3$ with improved affinity. Notably, though the majority of peptides binding to $\alpha_v\beta_3$ from both 6-mer and 15-mer phage libraries contained the RGD motif, little obvious enrichment for particular amino acid residues was noted at positions either preceding or following the RGD tripeptide. The apparent tolerance of $\alpha_v\beta_3$ for heterogeneous RGD-containing sequences validates the view of $\alpha_v\beta_3$ as a multifunctional receptor designed to recognize the RGD motif in a variety of different structural contexts (Charo et al., 1990). Indeed, the relaxed specificity exhibited by $\alpha_v\beta_3$ for RGD-containing peptides resembled its promiscuity *in vivo* for ECM protein ligands.

Screening of $\alpha_v\beta_3$- and $\alpha_5\beta_1$-binding peptides from the same 15-mer phage library in parallel experiments facilitated direct comparison of the types of RGD-containing peptides preferred by each integrin. We were also able to compare hexapeptides selected by $\alpha_v\beta_3$ and $\alpha_5\beta_1$, since an earlier study (Koivunen et al., 1993) described screening of the same 6-mer library used here with the fibronectin receptor. Those authors reported significant enrichment for arginine or lysine at the position just before, and leucine at the position just after, RGD in hexapeptides bound by $\alpha_5\beta_1$. Among $\alpha_5\beta_1$-binding 15-mer sequences, we also detected a preference for arginine at the position preceding RGD, while the position following RGD was almost invariably occupied by a hydrophobic residue. Thus, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ bound distinguishably to the set of RGD peptides encoded by the same phage display libraries, in a way which suggested that these integrins differ in the extent to which ligand binding by each relies on a particular preferred presentation of the RGD locus. Certain types of RGD peptides recognized readily by $\alpha_v\beta_3$ were apparently unsuitable for recognition by $\alpha_5\beta_1$. The comparatively narrow range of RGD-containing peptides preferred by $\alpha_5\beta_1$ is consistent with the idea that $\alpha_5\beta_1$, unlike $\alpha_v\beta_3$, is specialized to recognize the RGD motif in relatively few structural settings.

Selective Binding to $\alpha_v\beta_3$ of an RGD Peptide with Homology to Vitronectin. Several RGD peptides that we identified through screening of the 15-mer library were recognized more efficiently by $\alpha_v\beta_3$ than by $\alpha_5\beta_1$ in integrin binding assays. Presumably, such peptides are unable to adopt a conformation preferred by $\alpha_5\beta_1$ or contain amino acids flanking RGD that impair binding primarily to the fibronectin receptor. Though not entirely selective, one $\alpha_v\beta_3$-binding sequence, GERGDGSFFAFRSPF, showed unexpectedly high specificity for $\alpha_v\beta_3$. Interestingly, this peptide shares more extensive sequence homology with the primary natural ligand of $\alpha_v\beta_3$, vitronectin, than other RGD peptides that we isolated. The phage-encoded sequence SFFAFR is quite similar to the sequence SLFAFR found at positions 152−157 of vitronectin. We do not know whether these residues of vitronectin contact $\alpha_v\beta_3$ directly or contribute to

Table 3: Deduced Amino Acid Sequences of Peptides Binding to $\alpha_5\beta_1$ Integrin from a 15-mer Phage Display Library[a]

RGD peptide elution	low pH elution	
RRGDIGPREFSAIVD(4)	RRGDIGPREFSAIVD(5)	RVFRGDLGYRTPYIG
PRDHIARRGDLAFQA	ISRRGDLSGLSFSRL(4)	RGDVWTLWSVGDTRS
SPARGDLFRFMGAVH(2)	RGVKMRRGDFSTIMD	DALSCRGDCVWPTRG(3)
RGDMSLLGEFTSPYG(10)	RGDMSLLGEFTSPYG(4)	
DALSCRGDCVWPTRG(6)	SPARGDLFRFMGAVH	SEELLVESSAIRSRE

[a] Selection and sequencing of phage bound to integrin $\alpha_5\beta_1$ were performed as described in Materials and Methods. The number of sequences encoding the same peptide is indicated in parentheses. The RGD motif is highlighted in bold.

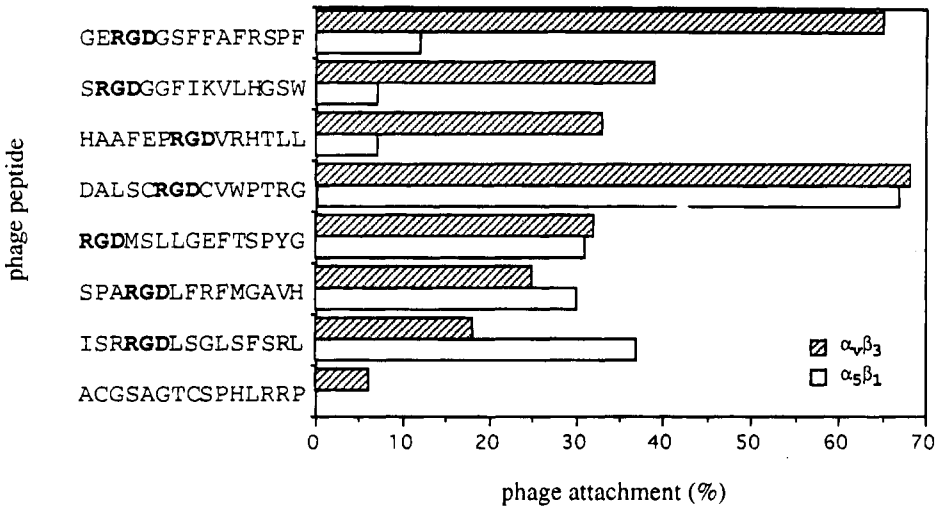

FIGURE 5: Specificities of selected phage for $\alpha_v\beta_3$ or $\alpha_5\beta_1$ integrins. Phage binding to $\alpha_v\beta_3$ or $\alpha_5\beta_1$ integrins was assayed as described in Materials and Methods. Percent phage attachment represents the percent of the input phage titer adhering specifically to integrin-coated microtiter wells. The data represent means of duplicate determinations from one of two similar experiments.

Table 4: Binding of Synthetic Peptides Containing RGD to $\alpha_v\beta_3$ or $\alpha_5\beta_1$ Integrins[a]

peptide	IC_{50} (M) inhibition of vitronectin binding to $\alpha_v\beta_3$	IC_{50} (M) inhibition of fibronectin binding to $\alpha_5\beta_1$
DALSCRGDCVWPTRG	1.5×10^{-7}	1.0×10^{-7}
GERGDGSFFAFRSPF	4.0×10^{-7}	1.5×10^{-5}
HAAFEPRGDVRHTLL	3.0×10^{-6}	4.0×10^{-5}
SRGDGGFIKVLHGSW	3.0×10^{-6}	1.0×10^{-5}
ISRRGDLSGLSFSRL	8.0×10^{-6}	4.0×10^{-6}

[a] IC_{50} values for the inhibition of binding of adhesive proteins to $\alpha_v\beta_3$ or $\alpha_5\beta_1$ integrins by synthetic peptides were measured using a solid-phase assay as described in Materials and Methods.

binding indirectly by exerting a favorable influence on the conformation of the RGD motif. Nevertheless, it is attractive to speculate that the presence of an additional vitronectin-derived sequence is a factor in the avidity with which the GERGDGSFFAFRSPF peptide is bound by $\alpha_v\beta_3$, perhaps through stabilization of the interaction between RGD and the integrin active site. These observations suggest that an efficacious approach for the design of RGD peptides binding with improved affinity to cognate integrins might be to construct "mini-ligands" that recapitulate certain defined intervals of amino acids present in ECM proteins. Introduction of conformational constraints, by cyclization (Pierschbacher & Ruoslahti, 1987; Pfaff et al., 1994) or insertion into a rigid protein scaffold (Maeda et al., 1989; Barbas et al., 1993; Yamada et al., 1993, 1994), could further enhance selective recognition of such tailored peptides. Selectivity is a prerequisite for the development of therapeutic agents, *e.g.*,for use in treatment of osteoporosis, that must target the function of $\alpha_v\beta_3$ with great precision.

Non-RGD Peptides Recognized by $\alpha_v\beta_3$. We identified two novel $\alpha_v\beta_3$-binding motifs by phage library screening, both of which match sequences found in ECM protein ligands of $\alpha_v\beta_3$: (1) the RGD-related sequence NGR (fibronectin) and (2) SAGT (vitronectin). Motifs present in natural ligands of $\alpha_v\beta_3$ were the only non-RGD sequences displayed on phage which bound to $\alpha_v\beta_3$ significantly above background levels.

Peptides containing NGR comprised the major class of non-RGD sequences binding to $\alpha_v\beta_3$. The NGR tripeptide occurs in human fibronectin near the carboxy terminus of the ninth type III domain repeat (positions 1401−1403). This motif has also been identified by phage library screening as a peptide ligand for the fibronectin receptor, $\alpha_5\beta_1$ (Koivunen et al., 1993, 1994). An NGR-containing peptide was shown to compete with RGD for binding to $\alpha_5\beta_1$ and to block adhesion of CHO cells mediated by either $\alpha_5\beta_1$ or $\alpha_v\beta_3$ (Koivunen et al., 1993). In our studies, five different sequences containing NGR were selected by $\alpha_v\beta_3$, and binding of NGR-containing phage to $\alpha_v\beta_3$ was inhibited by RGD peptide. Moreover, in solid-phase assays a synthetic NGR peptide competitively inhibited binding of either fibronectin or vitronectin (which lacks an NGR sequence) to $\alpha_v\beta_3$. At present, we cannot distinguish whether the NGR motif represents a synergistic site of fibronectin that is recognized by both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins in conjunction with RGD or whether NGR merely represents a low-affinity analogue of RGD. In support of the former view, the RGD and NGR sequences of fibronectin are each likely to reside on exposed conformationally mobile loops that should be

accessible to the active sites of integrins (Main et al., 1992). However, a recent study using a homology scanning approach to define the minimal region of the ninth type III repeat of fibronectin sufficient to enhance the cell-adhesive activity of the RGD-containing tenth repeat emphasized the importance of an alternative sequence in recognition by $\alpha_5\beta_1$ (Aota et al., 1994). Two-domain chimeras of fibronectin which assayed various portions of the ninth domain for augmentation of cell adhesion identified the motif PHSRN (positions 1376−1380) as a key synergistic site. Furthermore, a peptide derived from the same region of fibronectin, DRVPHSRNSIT, was also shown to bind to the platelet integrin $\alpha_{IIb}\beta_3$ (Bowditch et al., 1994). Interestingly, however, this 11 amino acid peptide was not utilized by $\alpha_v\beta_3$ (Bowditch et al., 1994). Thus, in contrast to $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ integrins, the Asp^{1373}−Thr^{1383} interval of fibronectin seems unlikely to function as a synergistic site that is recognized by $\alpha_v\beta_3$.

The sequence ACGSAGTCSPHLRRP was the only non-RGD sequence selected by $\alpha_v\beta_3$ from the 15-mer phage library. The SAGT tetrapeptide was of particular interest since it is found in human vitronectin at positions 307−310, just N-terminal to the positively charged heparin-binding domain (Suzuki et al., 1984; Preissner, 1991). Identification of this motif may have been facilitated by the presence of flanking cysteine residues, which potentially form a cyclic disulfide on the phage surface in which the conformation of the intervening amino acid sequence is fixed. SAGT-containing peptides competed with vitronectin for binding to $\alpha_v\beta_3$, though the interaction of the SAGT motif with $\alpha_v\beta_3$ was at least 1000-fold weaker than that of RGD. Inhibition of SAGT-phage binding to $\alpha_v\beta_3$ by a synthetic RGD peptide further suggested that the SAGT and RGD motifs could bind to similar or overlapping sites on $\alpha_v\beta_3$. SAGT-containing phage, on the other hand, did not bind detectably to $\alpha_5\beta_1$. These data are consistent with a role for SAGT as an accessory site in vitronectin that acts in concert with the RGD tripeptide at positions 45−47 to promote recognition by the $\alpha_v\beta_3$ integrin. Examination of the cell-adhesive activities of CNBr-cleavage fragments of vitronectin (Suzuki et al., 1984) and limited studies of site-directed or deletion mutants (Cherny et al., 1993; Zhao & Sane, 1993) have not provided evidence for the existence of sites in vitronectin, other than RGD, which mediate significant levels of cell attachment. However, the initial binding of $\alpha_v\beta_3$ to vitronectin has been shown to result in a subsequent stabilized interaction that is resistant to inhibition by RGD peptides (Orlando & Cheresh, 1991). Such stability could be achieved by multiple contacts between $\alpha_v\beta_3$ and synergistic regions of vitronectin. Little information is currently available concerning the three-dimensional structure of vitronectin, though several reports indicate a folded tertiary structure, as well as conformational transition(s) (Preissner, 1991; Morris et al., 1994). The folded state of the vitronectin molecule proposed to result from electrostatic interactions between the heparin-binding region and negatively charged residues near the N-terminus could conceivably place the SAGT motif in proximity to the RGD region.

Contacts between integrin active sites and multiple regions of ECM ligands, including the essential RGD locus, appear to be a general feature of ligand recognition by integrins (Aota et al., 1994; Bowditch et al., 1994; Koivunen et al., 1994). Binding to synergistic loci is expected to confer added strength or specificity to integrin−ligand associations beyond that determined by the composition or conformation of the RGD motif itself. Definition of the roles of the SAGT and NGR sequences identified here in ligand binding and cell adhesion mediated by the $\alpha_v\beta_3$ integrin will require further investigation. Nevertheless, this study underscores the utility of phage display approaches in discerning subtle differences in the binding preferences of cellular receptors and in providing clues to mechanisms of molecular recognition.

ACKNOWLEDGMENT

We thank Dr. G. Smith for his gift of 6- and 15-mer peptide libraries and Dr. T. Tanaka for much helpful advice concerning the preparation and cyclization of synthetic peptides. We also thank Dr. M. Ikehara for his support and Dr. T. Yamada for continued interest in this work.

REFERENCES

Aota, S., Nagai, T., & Yamada, K. M. (1991) *J. Biol. Chem. 266*, 15938−15943.

Barbas, C. F., III, Languino, L., & Smith, J. W. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10003−10007.

Bowditch, R. D., Hariharan, M., Tominna, E. F., Smith, J. W., Yamada, K. M., Getzoff, E. D., & Ginsberg, M. H. (1994) *J. Biol. Chem. 269*, 10856−10863.

Brooks, P., Clark, R., & Cheresh, D. A. (1994) *Science 264*, 569−571.

Charo, I. F., Nannizzi, L., Smith, J. W., & Cheresh, D. A. (1990) *J. Cell Biol. 111*, 2795−2800.

Charo, I. F., Nannizzi, L., Phillips, D., Hsu, M. A., & Scarborough, R. M. (1991) *J. Biol. Chem. 266*, 1415−1421.

Cheresh, D. A., & Spiro, R. C. (1987) *J. Biol. Chem. 262*, 17703−17711.

Cheresh, D. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6471−6475.

Cherny, R. C., Honan, M. A., & Thiagarajan, P. (1993) *J. Biol. Chem. 268*, 9725−9729.

Davis, G. (1992) *Biochem. Biophys. Res. Commun. 182*, 1025−1031.

D'Souza, S. E., Ginsberg, M. H., & Plow, E. F. (1991) *Trends Biol. Sci. 16*, 46−250.

Felding-Habermann, B., Ruggeri, Z., & Cheresh, D. A. (1992) *J. Biol. Chem. 267*, 5070−5077.

Gurrath, M., Muller, G., Kessler, H., Aumailley, M., & Timpl, R. (1992) *Eur. J. Biochem. 210*, 911−921.

Hynes, R. O. (1992) *Cell 69*, 11−25.

Koivunen, E., Gay, D. A., & Ruoslahti, E. (1993) *J. Biol. Chem. 268*, 20205−20210.

Koivunen, E., Wang, B., & Ruoslahti, E. (1994) *J. Cell Biol. 124*, 373−380.

Lawler, J., Weinstein, R., & Hynes, R. O. (1988) *J. Cell Biol. 107*, 2351−2361.

Luscinskas, F. W., & Lawler, J. (1994) *FASEB J. 8*, 929−938.

Maeda, T., Oyama, R., Ichihara-Tanaka, K., Kimizuka, F., Kato, I., Titani, K., & Sekiguchi, K. (1989) *J. Biol. Chem. 264*, 15165−15168.

Main, A. L., Harvey, T. S., Baron, M., Boyd, J., & Campbell, I. D. (1992) *Cell 71*, 671−678.

Morris, C. A., Underwood, P. A., Bean, P. A., Sheehan, M., & Charlesworth, J. A. (1994) *J. Biol. Chem. 269*, 23845−23852.

Nemerow, G. R., Cheresh, D. A., & Wickham, T. J. (1994) *Trends Cell Biol. 4*, 52−55.

Obara, M., Kang, M., & Yamada, K. M. (1988) *Cell 53*, 649−657.

O'Neil, K. T., Hoess, R. H., Jackson, S., Ramachandran, N. S., Mousa, S., & DeGrado, W. F. (1992) *Proteins: Struct., Funct., Genet. 14*, 509−515.

Orlando, R. A., & Cheresh, D. A. (1991) *J. Biol. Chem. 266*, 19543−19550.

Pfaff, M., Tangemann, K., Muller, B., Gurrath, M., Muller, G., Kessler, H., Timpl, R., & Engel, J. (1994) *J. Biol. Chem. 269*, 20233−20238.

Pierschbacher, M. D., & Ruoslahti, E. (1987) *J. Biol. Chem. 262*, 17924−17928.

Preissner, K. T. (1991) *Annu. Rev. Cell Biol. 7*, 275−310.

Pytela, R., Pierschbacher, M. D., & Ruoslahti, E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5766−5770.

Pytela, R., Pierschbacher, M. D., Argraves, S., Suzuki, S., & Ruoslahti, E. (1987) *Methods Enzymol. 144*, 475−492.

Ross, F., Chappel, J., Alvarez, J., Sander, D., Butler, W., Farach-Carson, M., Mintz, K., Robey, T., Teitelbaum, S., & Cheresh, D. A. (1993) *J. Biol. Chem. 268*, 9901−9907.

Ruoslahti, E., & Pierschbacher, M. D. (1987) *Science 238*, 491−497.

Scarborough, R. M., Rose, J. W., Naughton, M., Phillips, D., Nannizzi, L., Arfsten, A., Campbell, A. M., & Charo, I. F. (1993) *J. Biol. Chem. 268*, 1058−1065.

Seftor, R., Seftor, E., Gehlsen, K., Stetler-Stevenson, W., Brown, P., Ruoslahti, E., & Hendrix, M. J. C. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1557−1561.

Smith, G. P., & Scott, J. K. (1993) *Methods Enzymol. 217*, 228−257.

Suzuki, S., Pierschbacher, M. D., Hayman, E. G., Nguyen, K., Ohgren, Y., & Ruoslahti, E. (1984) *J. Biol. Chem. 259*, 15307−15314.

Tam, J., Cui-Rong, W., Liu, W., & Zhang, Z. W. (1991) *J. Am. Chem. Soc. 113*, 6657−6662.

Yamada, K. M. (1991) *J. Biol. Chem. 266*, 12809−12812.

Yamada, T., Uyeda, A., Otsu, M., Matsushima, M., Sekiguchi, K., & Kikuchi, M. (1993) *J. Biol. Chem. 268*, 10588−10592.

Yamada, T., Uyeda, U., Kidera, K., & Kikuchi, M. (1994) *Biochemistry 33*, 11678−11683.

Zhao, Y., & Sane, D. C. (1993) *Biochem. Biophys. Res. Commun. 192*, 575−582.

BI9426569