A Peptide Isolated from Phage Display Libraries Is a Structural and Functional Mimic of an RGD-binding Site on Integrins

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Abstract. Many integrins recognize short RGD-containing amino acid sequences and such peptide sequences can be identified from phage libraries by panning with an integrin. Here, in a reverse strategy, we have used such libraries to isolate minimal receptor sequences that bind to fibronectin and RGD-containing fibronectin fragments in affinity panning. A predominant cyclic motif, *CWDD$^{G/}$$_L$WLC*, was obtained (the asterisks denote a potential disulfide bond). Studies using the purified phage and the corresponding synthetic cyclic peptides showed that *CWDDGWLC*–expressing phage binds specifically to fibronectin and to fibronectin fragments containing the RGD sequence. The binding did not require divalent cations and was inhibited by both RGD and *CWDDGWLC*–containing synthetic peptides. Conversely, RGD-expressing phage attached specifically to immobilized *CWDDGWLC*–peptide and the binding could be blocked by the respective synthetic peptides in solution. Moreover, fibronectin bound to a *CWDDGWLC*–peptide affinity column, and could be eluted with an RGD-containing peptide. The *CWDDGWLC*–peptide inhibited RGD-dependent cell attachment to fibronectin and vitronectin, but not to collagen. A region of the β subunit of RGD-binding integrins that has been previously demonstrated to be involved in ligand binding includes a polypeptide stretch, KDDLW (in β3) similar to WDD$^{G/}$$_L$WL. Synthetic peptides corresponding to this region in β3 were found to bind RGD-displaying phage and conversion of its two aspartic residues into alanines greatly reduced the RGD binding. Polyclonal antibodies raised against the *CWDDGWLC*–peptide recognized β1 and β3 in immunoblots. These data indicate that the *CWDDGWLC*–peptide is a functional mimic of ligand binding sites of RGD-directed integrins, and that the structurally similar site in the integrin β subunit is a binding site for RGD.

INTEGRINS are heterodimeric glycoproteins formed by the association of an α and a β subunit; they mediate cell–matrix and cell–cell interactions that are important in biological events such as cell differentiation, malignant transformation, immune recognition, and blood coagulation (13, 16, 19, 38). Some of the integrin effects in these phenomena are the consequence of physical adhesion, others are mediated by signal transduction through integrin cytoplasmic domains (17, 20, 30, 46).

An important integrin binding site is the tripeptide RGD, present in a variety of integrin ligands. In fibronectin, it is located in the tenth type III repeat (III$_{10}$)[1] (34, 35), the structure of which has been elucidated by NMR (26) and crystallography (11). Studies with fibronectin fragments and phage display libraries have suggested that

other sites in this region of fibronectin are also needed for full integrin-binding activity (4, 23, 31).

Contact regions for the RGD sequence have been identified in the integrin subunits (14). Affinity cross-linking of RGD peptides and site-directed mutagenesis localize a ligand-binding site in the αIIbβ3 integrin at amino acid residues 109-172 of the β subunit (2, 3, 6, 8, 33). Studies with the αvβ3 integrin place an RGD-binding site at amino acids 61-203 (41), and a similar cross-linking region, amino acids 120-140 in the β1 subunit, has also been found to be involved in ligand binding (43). In each case, the RGD-binding site is near or at a site that binds divalent cations. The α subunit also contains one or more ligand-binding sites; as with the β subunits, these sites localize to the divalent cation binding sequences (5, 7, 12, 25, 27).

Our laboratory has used random peptide libraries displayed on phage (40) to study the structural requirements in the RGD-type peptide ligands for their binding to integrins. Searches of such libraries have confirmed the central role of the RGD sequence in ligand binding by several integrins and have also revealed auxiliary binding sites in the ligands (15, 21–24, 29, 42). Here we have used the reverse

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1. *Abbreviation used in this paper:* III$_{10}$, tenth type III repeat.

strategy in an attempt to elucidate the sequences in integrins that are important for ligand binding. We report here the identification and characterization of an RGD-binding sequence, *CWDDGWLC*, from a random phage display library. This peptide is similar to key residues in the integrin ligand binding site and thus appears to be a structural mimic of an RGD contact point in integrins.

Materials and Methods

Materials

Human plasma fibronectin was from the Finnish Red Cross (Helsinki, Finland). A 110-kD fragment of fibronectin was prepared as described previously (34). Recombinant fibronectin fragments containing type III repeats 8 and 9, 9 and 10, 10 and 11, 10 alone, and 8 through 11 were produced as described (10), using GST (Pharmacia, Uppsala, Sweden) (for the 8 through 11 fragment) and His-Tag (Qiagen, Chatsworth, CA) (for the other four fragments) fusion protein systems. A fragment encompassing the alternatively spliced cell attachment domain of fibronectin (amino acids 1860-2140) was also produced using the His-Tag system. Vitronectin was purified from human plasma as described (47). Collagen was from Collaborative Research (Bedford, MA), sheep red blood cells were from Sigma. Purified $\alpha IIb\beta3$ was from Enzyme Research Laboratories Inc. (South Bend, IN). Anti-$\beta1$ monoclonal antibody TS2/16 was a gift from Dr. Martin Hemler (Dana Farber Cancer Institute, Harvard Medical School, Boston, MA). Peptides were synthesized on a synthesizer (Model 430A; Applied Biosystems, Foster City, CA) by standard Merrifield solid phase synthesis protocols and t-butoxycarbonyl chemistry. Cyclic peptides were prepared by oxidizing with 0.01 M $K_3Fe(CN)_6$ at pH 8.4 overnight and purified by reverse-phase HPLC. The peptide structures were confirmed by mass spectroscopy. Phage display libraries were made as described (22-24) using the fuse 5 vector (40).

Panning of Phage

Aliquots of the libraries were screened with fibronectin fragments coated on microtiter wells. Panning was performed on each fragment individually. In the first and second panning the coating concentration of protein was 5 μg/well. To increase the stringency of the panning, the wells were coated with decreasing concentrations of protein (1 and 0.1 μg/well). Phage were selected for further amplification from the well with the lowest protein concentration that showed phage binding over background. In the fourth panning, the concentration was 10 ng/well. To recover the bound phage, the wells were eluted with 1 mM solution of GRGDSP or *CELRGDGWC* peptides, 2 mM EDTA, or were directly incubated with 50 μl of bacteria. Phage were sequenced from randomly selected clones as described (22).

Phage Attachment Assay

Binding of individual cloned phage to insolubilized fibronectin and fibronectin fragments was studied in microtiter assays (22, 23). The coating concentration for the proteins was 10 μg/ml. Coating with peptides was carried out at 10-100 μg/ml overnight with or without 1 mM divalent cations. Phage binding was determined by growing K91kan bacteria in the presence of the selection marker tetracyclin. The absorbance at 600 nm was read after 16-24 h of incubation at room temperature (22). Alternatively, the phage binding was also quantified by using sheep anti-M13 polyclonal antibodies (1 μg/ml; Pharmacia). Readings at 450 nm were analyzed after incubation with alkaline phosphatase conjugated anti-sheep IgG (1:10,000; Sigma).

Affinity Chromatography

Peptides were coupled to Sepharose-CH (Pharmacia) according to the manufacturer's instructions. Fibronectin and fibronectin fragments at 2 mg/ml in TBS were applied onto the *CWDDGWLC*-Sepharose or to a control peptide column. After extensive washing with TBS, bound material was eluted with GRGDSP and GRGESP peptides (1 mM) or glycine (0.1 M) containing NaCl (0.1 M, pH = 3.0). Samples were concentrated when necessary and analyzed in SDS-PAGE pre-cast gradient mini gels. Proteins were visualized by Coomassie blue staining.

Cell Attachment Assays

An osteosarcoma cell line, MG-63, which attaches to fibronectin, vitronectin, and collagens through its complement of several integrins (37), was used in cell attachment assays to examine peptide inhibition of integrin function. Microtiter wells were coated with fibronectin, vitronectin or type IV collagen at concentrations that resulted in 60% of maximal attachment (~5 μg/ml). Free binding sites on plastic were blocked with BSA. Approximately 1×10^5 cells per well were allowed to attach for 30 min in the presence or absence of competing peptides and the bound cells were quantitated by staining with crystal violet (28).

Immunization and Immunoblot Analysis

RBF-Dnj mice (Jackson Laboratories, Bar Harbor, ME) were immunized with the *CWDDGWLC* peptide coupled to sheep red blood cells (Sigma) according to the manufacturer's instructions.

Purified $\alpha IIb\beta3$ (2 μg/lane) and MG-63 cell extracts (20 μl from a vol/vol detergent/cell pellet solution) were separated on 4-12% gradient SDS-PAGE and transferred to Immobilon-P membranes. After blocking of non-specific sites, filters were probed with anti-*CWDDGWLC* (1:200) and anti-$\beta3$ cytoplasmic domain polyclonal serum (1:1,000). Normal mouse and rabbit sera were used as negative controls. Reactivity of antibodies was detected with anti-mouse or rabbit IgG, and chemiluminescence (ECL; Amersham).

Results

Isolation of Phage Capable of Binding to Fibronectin Fragments

To identify peptide motifs that interact with the RGD-containing 10th type III domain of fibronectin (III_{10}), recombinant fibronectin fragments were used to select clones from a mixture of peptide libraries by successive rounds of affinity panning and elution with RGD-containing peptide. Decreasing protein coating concentration in the second and third rounds, and the use of excess of phage to introduce binding competition, allowed for selection of specific, high-affinity phage 50- to 150-fold enrichment was achieved on III_{10}-bearing fragments in the third round of panning (Fig. 1). The fragment containing the 10th and 11th type III repeats of fibronectin was the most efficient binder of specific phage. Enrichment was also seen on the recombinant fibronectin fragment bearing the III_{10} domain alone, but not on the fragment from the alternatively spliced fibronectin domain containing the CS-1 binding site for $\alpha4\beta1$ integrin (18) (results not shown). Binding of phage to fragment $III_{8,9}$ was also low, indicating that the III_{10} domain was important for the enrichment of RGD-eluted phage. GST and BSA were used as controls for non-specific attachment and showed negligible phage binding. Enrichment of specific phage was also seen with the RGD-coating fragments when the phage were eluted with EDTA or collected by direct infection of bacteria added to the washed wells (see below).

Phage Selected by RGD-containing Fibronectin Fragments Display the *CWDD$^G/_L$WLC* Peptide Motif

Sequences of the insert in the phage eluted with RGD peptides, EDTA, or recovered by direct incubation of bacteria showed that $\sim80-85\%$ of the clones displayed the motif WDDGWL (Table I). Some of the other motifs found were similar to the WDDGWL sequence; the glycine residue was frequently replaced by leucine. Further-
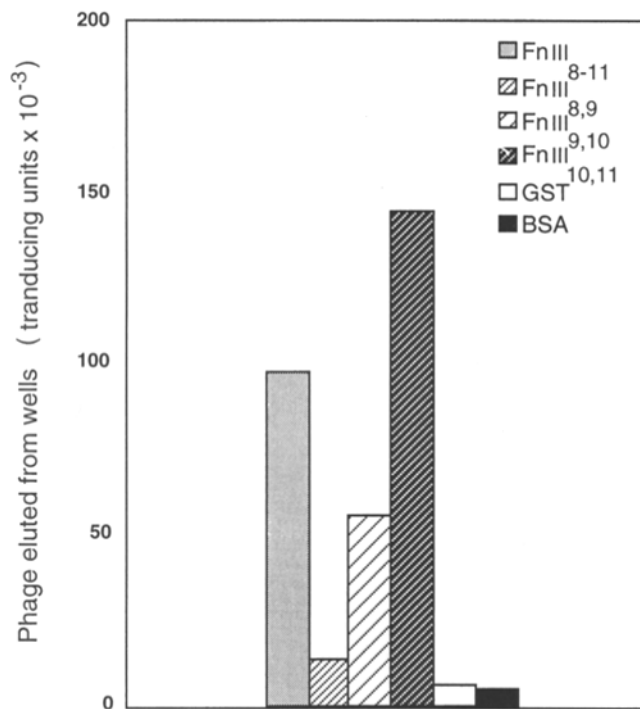
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Figure 1. Panning of phage peptide libraries on fibronectin fragments. Fibronectin fragments were immobilized onto microtiter wells at 1 µg/well. Phage libraries displaying CX_5C, CX_6C, CX_7C, and CX_9 peptides were plated and bound phage were eluted with a cyclic RGD peptide. Results illustrate the number of phage (transducing units $\times 10^{-3}$) eluted from each well coated with individual fragments in the third round of panning.

more, the WDDGWL sequence was not encoded by a single clone, since there was variation at the nucleotide level among the phage.

Binding of WDDGWL Phage to the III_{10} Domain Is Blocked with Synthetic Peptides

The specificity of the WDDGWL-phage binding to fibronectin and fibronectin fragments was tested in a microtiter assay. The binding was dependent on the presence of the III_{10} domain (Fig. 2 *a*). Binding to $III_{8,9}$, to control proteins (BSA and GST) and also to the fragment encompassing the alternatively spliced cell attachment domain of fibronectin (not shown) was minimal. An unrelated phage

Table I. Selection of Peptides Binding to Fibronectin Type $III_{10(RGD)}$-containing Fragments from Phage Display Peptide Libraries

RGD elution	EDTA elution	Direct infection of wells
WDDGWL (131)	WDDGWL (21)	WDDGWL (45)
WDDLWWL (10)	LVWLLVQFY (2)	SWDDGWL (6)
WDDGLM (7)	TFGGGIGRV (1)	PDDLWWL (3)
WDDGWM (4)	TLRFQRS (1)	DGWLGF (2)
		QRIVLGFT (1)
		DYWLGF (1)
		FVLWLV (1)
		GNRLR (1)

Peptide sequences displayed by phage isolated by different elution strategies. The number of phage displaying the same amino acid motif is indicated in parenthesis.

displaying the peptide RDPRAQDL showed no binding to fibronectin or any of its fragments.

Two cyclic peptides, *CWDDGWLC* and A*CRGDGWMC*G, were synthesized and tested for their ability to inhibit phage binding to the $III_{10,11}$ fragment. Both peptides, but not an irrelevant cyclic one, inhibited WDDGWL–phage binding in a dose-dependent manner (Fig. 2 *b*). EDTA did not inhibit the binding of WDDGWL–phage (not shown), although we had detected WDDGWL–phage in the EDTA eluates after affinity panning. The recovery of the WDDGWL–phage with EDTA (Table I) may have represented non-specific release of the bound phage rather than specific elution, because less phage was eluted than with the RGD peptide.

Phage Attachment to Immobilized Peptides

The binding of WDDGWL and RGD-displaying phage to RGD and WDDGWL-containing peptides was analysed in phage attachment assays using *CWDDGWLC*, A*CRGDGWMC*G, and an irrelevant cyclic peptide as substrates. We were able to show that WDDGWL–phage can bind to the RGD-containing peptide. Conversely, RGD–phage can bind to the *CWDDGWLC*-containing peptide (Fig. 3 *a*). The RGD phage also showed slight, but consistent, binding to the peptide displaying the same RGD motif, RGDGW. Addition of either the *CWDDGWLC* or A*CRGDGWMC*G peptide in solution blocked the WDDGWL–phage binding (Fig. 3 *b*), whereas an unrelated peptide had no effect (not shown). The binding of RGD–phage was also inhibited by both peptides, and was unaffected by the control peptide (Fig. 3 *c*) or by EDTA (not shown).

Fibronectin Binds to *CWDDGWLC* Sepharose

Affinity chromatography showed that fibronectin bound to *CWDDGWLC*-Sepharose, and was eluted with the GRGDSP peptide but not with the GRGESP peptide (Fig. 4 *a*). Fibronectin was not retained in a control unrelated peptide column (not shown). Fibronectin fragments lacking the III_{10} domain and unrelated proteins (BSA and type IV collagen) were not retained in the *CWDDGWLC*-Sepharose column. The difference between the binding of the $III_{9,10}$ (RGD+) and $III_{8,9}$ (RGD–) fragments to the *CWDDGWLC* column is shown in Fig. 4 *b*.

CWDDGWLC Inhibits RGD-dependent Cell Adhesion

The *CWDDGWLC* peptide inhibited cell adhesion when either fibronectin or vitronectin were used as substrates, but not on collagen (Fig. 5). An unrelated cyclic peptide had no effect on any of the substrates. *CWDDGWLC* was slightly less effective than the standard RGD peptide, GRGDSP (not shown).

CWDD$^G/_L$WLC Resembles a Peptide from β3 Integrin Subunit

A DDLW sequence from the ligand binding region of the β subunits shows similarity to our RGD binding peptide motif. A synthetic peptide containing the DDLW sequence of the β3 subunit (amino acids 109-133; DYP-
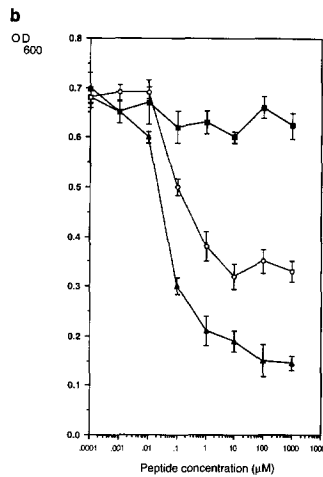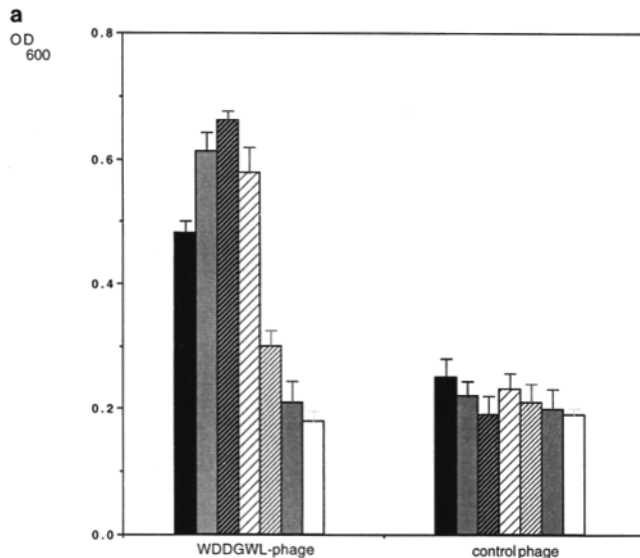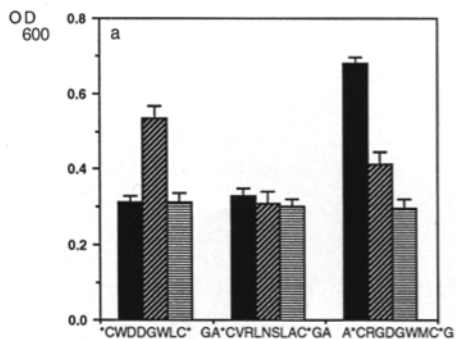
a

b

Figure 2. Phage attachment assay. (*a*) Purified WDDGWL phage was incubated in microtiter wells coated with various fibronectin fragments immobilized onto microtiter wells and bound phage were quantified by the absorbance indicative of relative bacterial growth. These results are representative of three separate experiments. Points represent the mean of triplicate determinations with standard error less than 10% of the mean. ■, Fn; ▨, FnIII$_{8-11}$; ▨, FnIII$_{10,11}$; ▱, FnIII$_{9,10}$; ▨, FnIII$_{8,9}$; ■, BSA; □, no phage. (*b*) Inhibition of the binding of *CWDDGWLC *–expressing phage to the III$_{8-11}$ fibronectin fragment by synthetic peptides. WDDGWL–phage binding to the III$_{8-11}$ fragment was analyzed as described in *a*, in the presence of the competing peptides indicated. The bound phage was quantified as described above. —■—, GA*CVRLNSLAC*GA; —▲—, A*CRGDGWMC*G; —○—, *CWDDGWLC*.

Figure 3. Binding of phage to immobilized peptides in the presence or absence of soluble peptides. RGD- and WDDGWL-containing peptides were coated on microtiter wells at 20 μg/ml and used to bind phage expressing the same motifs. Soluble peptides were added at 1 mM concentration. The data represent mean from triplicate wells with standard error less than 10% of the mean. (*a*) ■, WDDGWL phage; ▨, ELRGDGW phage; ▤, RD-PRAQDL phage. (*b* and *c*) ■, *CWDDGWLC*; ▨, *CRGDG-WMC*; ▨, no inhibition.

VDIYYLMDLSYSMK DDLWSIQN) binds to an RGD peptide (9). As shown in Fig. 6, RGD–phage bound to the β3(109-133) peptide. The binding required the presence of divalent cations, either while the peptide was coated onto plastic or during the phage binding (Fig. 6 *a*). The binding of the RGD–phage binding was blocked by the addition of soluble *CWDDGWLC* or GRGDSP peptides (Fig. 6 *b*). The two peptides were equally effective; in titration experiments 50% inhibition was achieved at about 1 μM concentration of the peptides (not shown). However, EDTA did not block phage binding when peptides were allowed to coat in the presence of cations.
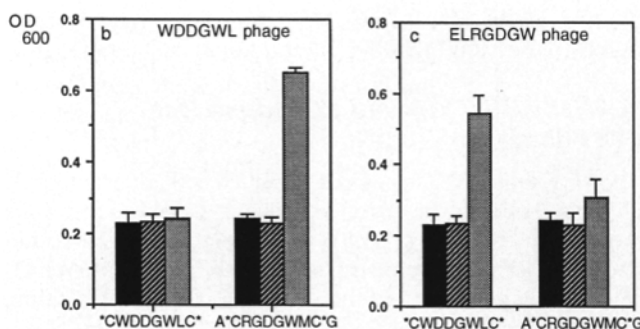
To establish whether the DDLW sequence in the β3 (109-133) peptide was important for the RGD–phage binding, we synthesized a variant β3 peptide in which both Asp 126 and Asp 127 were replaced by Ala (DYPVDI-YYLMDLSYSMKAALWSIQN). RGD-phage binding to this AA variant was much weaker than to the DD peptide (Fig. 6), indicating an important role for the two aspartate residues in the interaction. The residual binding to the AA peptide had the same cation requirements as the binding to the wild type peptide.

Antibodies against *CWDDGWLC* Peptide Recognize β3 and β1 in Immunoblots

Further evidence for the structural similarity between *CWDDGWLC* and β subunits was obtained with antibodies raised against *CWDDGWLC* peptide. When purified αIIbβ3 was probed in immunoblots with anti-*CWD-DGWLC*, a band was detected that had the expected molecular size of β3 and aligned with the band detected by anti-β3 cytoplasmic domain antiserum (Fig. 7 *a*). Reactivity of the anti-*CWDDGWLC* serum could be abrogated by preincubation of the serum with either the *WDDG-WLC* or the β3 (109-133) peptide (Fig. 7 *a*, lanes *C* and *D*, respectively). The anti-*CWDDGWLC* serum also reacted with bands that co-migrated with β1 and β3 subunits in MG-63 total cell extracts (Fig. 7 *b*, lane *E*). Anti-β1 monoclonal antibody TS2/16 against β1 was used as a pos-
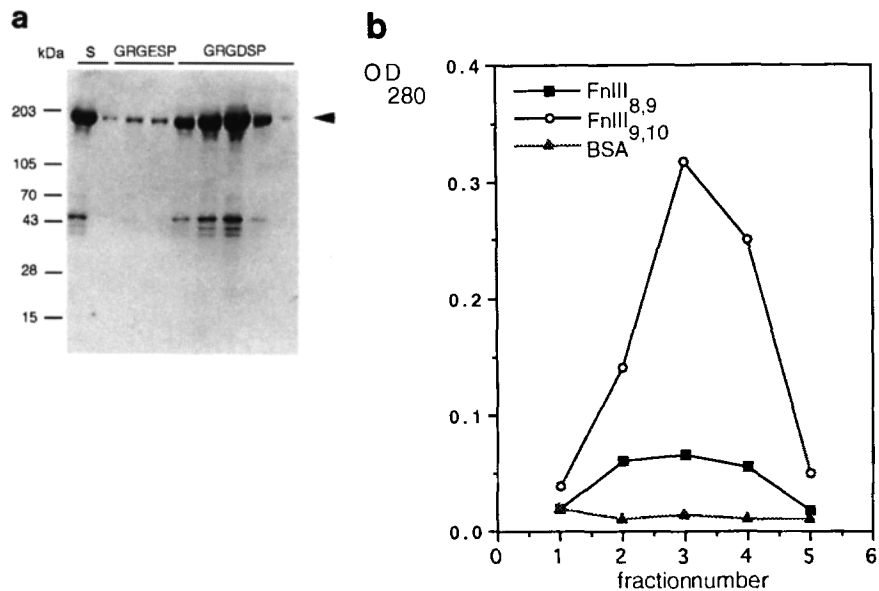
Figure 4. Binding of fibronectin and fibronectin fragments to *CWDDG-WLC*-Sepharose. (*a*) Two mg of purified fibronectin were applied into a *CWD-DGWLC*-Sepharose column (lane *S* represents a sample of the starting material). Bound material was eluted by applying GRGDSP or GRGESP peptides at 1 mM. The eluted fractions were analyzed in SDS-PAGE and protein was visualized by Coomassie blue staining. (*b*) Fibronectin fragments $III_{9,10}$ and $III_{8,9}$ and BSA as a control were fractionated on a *CWDDG-WLC*-Sepharose column. Bound material was eluted with glycine/NaCl at pH 3.0 and the fractions were analyzed for protein by measuring OD 280.

itive control (Fig. 7 *b*, lane *E*). The reactivity of anti-WDDGWL serum was abrogated by pre-incubation of the antiserum with either *WDDGWLC* or β3 (109-133) (Fig. 7 *b*, lanes *C* and *D*, respectively); these peptides had no effect on the reactivity of the positive control antibodies in *a* or *b*.

Discussion

We have used phage peptide libraries to identify a cyclic peptide motif, represented by the sequence WDDGWL, that binds to the RGD-containing domain of fibronectin. This motif seems to be a structural mimic of an RGD-binding site in integrin β subunits.

The WDDGWL sequence and its variations (flanked by cysteine residues engineered into the libraries) was by far the most frequently isolated sequence on the RGD-containing fibronectin fragments. The sequences that were not related to the WDDGWL motif were hydrophobic and/or seen only once. The binding of these phage is likely to have been non-specific; they were lost in the subsequent high affinity screening steps and were not seen at all when specific elution with an RGD peptide was used.

The binding of the WDDGWL phage to the RGD-containing fragments was specific because only background binding was seen when fragments lacking the RGD-containing III_{10} domain, or control proteins, were used. Moreover, panning performed using the function-blocking anti-αIIbβ3 monoclonal antibodies, PAC-1, OPG2, and LJ-CP3, which each contain an RYD sequence as an RGD mimic (1, 44, 45), also yields the WDDGWL motif as the predominant motif (Pasqualini, R., and E. Ruoslahti, unpublished data). Finally, the specificity of the WDDGWL-phage binding to the fibronectin fragments was also supported by specific inhibition of the interaction both by peptides representing the motif itself and by RGD-peptides. This latter result and the ability of a cyclic peptide containing the WDDGWL motif to bind phage that display the RGD sequence also show that the binding site for the WDDGWL encompasses the RGD sequence itself.

The DDL sequence, which according to our data is an RGD-contact region, is conserved in several β subunits. Variability in the amino acid residues adjacent to DDL (see Table II) may explain the differences in the ability of integrins containing different β subunits to bind to RGD-containing ligands. Because several contact areas may be necessary for ligand binding, regions in the α chain are also likely to affect integrin specificity. In the β subunit binding site, the tryptophan residue, only present in β3, may be important because it was nearly invariant in the phage that bound avidly to the RGD ligands.

Phage–peptide binding studies revealed a preliminary result that deserves comment. As shown in Fig. 3 *a*, there was some binding by phage displaying the sequence RG-DGW to an immobilized peptide containing this same motif. As the RGDGW sequence has some similarity with WDDGWL, it may be that the RGDGW motif mimics the integrin while also being self-complementary. It has been proposed that receptor-ligand pairs arise in evolution from sequences that occur frequently in proteins, because the probability for a newly emergent receptor (or ligand) would find a useful ligand (or receptor) is greatest when the reciprocal binding sequence is common (32); Our results suggest an alternative scenario for the emergence of the integrin-RGD recognition system, i.e., self complementarity.

The fact that the *CWDDGWLC* peptide binds to both fibronectin and vitronectin (and apparently also to antibodies that mimic RGD-containing ligands) suggests that such peptide might be useful in the isolation of unknown integrin ligands. Conversely, antibodies made against the peptide may be used to identify new integrin β subunits.

The WDDGWL motif places the RGD binding site to the same region of the β subunit that was identified in earlier studies (Table II) (2, 3, 6, 8). This motif also pinpoints the binding site further. The smallest RGD-binding peptide from integrin sequences so far has been the 23–amino acid peptide of D'Souza et al. (9) which contains both a divalent cation binding site and the WDDGWL similarity
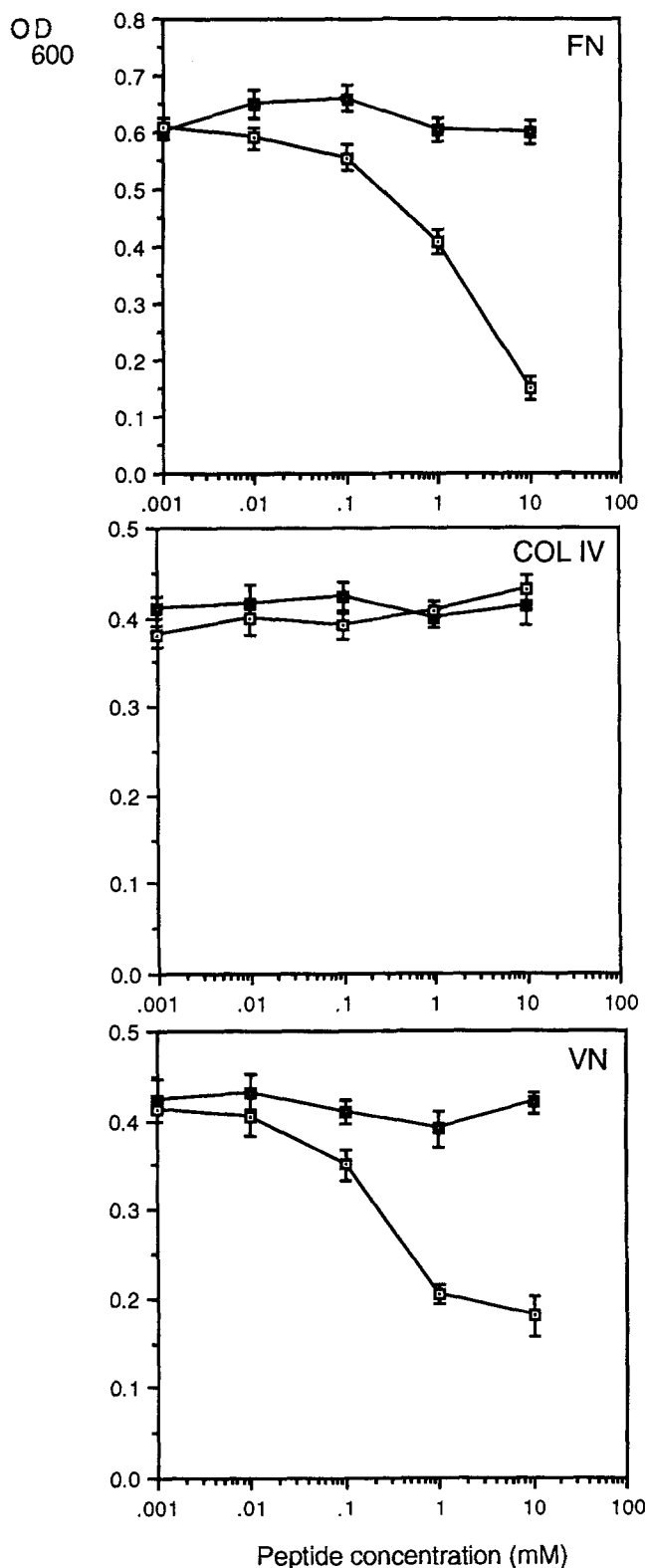
OD
600

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0

FN

.001   .01   .1   1   10   100

0.5
0.4
0.3
0.2
0.1
0.0

COL IV

.001   .01   .1   1   10   100

0.5
0.4
0.3
0.2
0.1
0.0

VN

.001   .01   .1   1   10   100

Peptide concentration (mM)

OD
405

0.6
0.5
0.4
0.3
0.2
0.1
0.0

a

DD   AA   DD   AA

coating condition   no cations   + cations

0.6
0.5
0.4
0.3
0.2
0.1
0.0

b

DD   AA   DD   AA

no cations   + cations

Figure 5. Inhibition of cell attachment to fibronectin and vitronectin by *CWDDGWLC* peptide. Fibronectin (*Fn*), type IV collagen (*Col*), or vitronectin (*Vn*) were coated onto 96-well plates. The MG-63 osteosarcoma cells were allowed to attach in the presence of increasing concentrations of the indicated peptides. Attached cells were fixed and stained with crystal violet. The data represent means of OD_{600} values from triplicate wells. —□—, *CVRLNSLAC*; —◙—, *CWDDGWLC*.
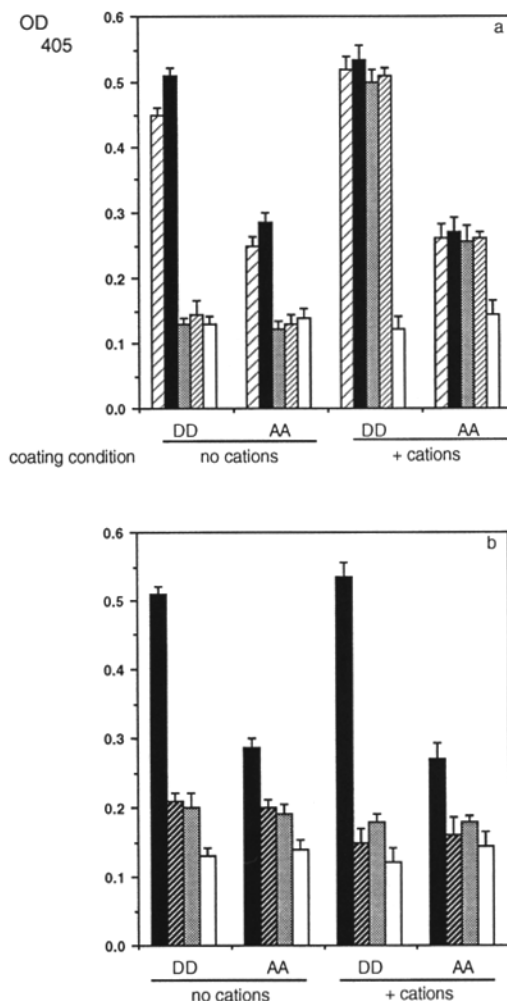
Figure 6. Binding of RGD-phage to peptides from the β3 integrin subunit. (*a*) Synthetic peptide (DYPVDIYYLMDLSYS-MKDDLWSIQN), "DD" and (DYPVDIYYLMDLSYSMKAA-LWSIQN), "AA" were coated at 100 μg/ml onto microtiter wells in the presence or absence of 1 mM divalent cations. EL-RGDGW–phage was incubated in the presence or absence of EDTA (10 mM) or calcium or magnesium (1 mM). Antibodies against M13 phage were used to quantify the amount of bound phage. Phage displaying an unrelated peptide sequence, RD-PRAQDL was tested as a control phage. (*a*) ▨, Calcium; ■, magnesium; ▧, no cations; ▨, EDTA; □, ctr phage. (*b*) The effect of soluble GRGDSP or *CWDDGWLC* peptides on the binding of phage to the same integrin peptides as in *a* was analyzed. The data in *a* and *b* represent the means from triplicate wells with standard error less than 10% of the mean. ■, Magnesium; ▨, *CWDDGWLC*; ▨, GRGDSP; □, ctr phage.

region. Mutagenesis of residues in the cation binding motif eliminates divalent cation binding or reduces ligand binding by the whole integrin. However, one such mutation of Asp 119 to tyrosine (see Table II), while rendering the whole integrin inactive, fails to affect RGD binding by the binding region peptide (9). Our results with the β3 subunit peptide are similar to those of D'Souza et. al. (9), but we found that the presence of divalent cations is required for RGD binding either when the peptide was coated onto plastic, or during the binding assay. We were using a cyclic RGD peptide displayed on phage, whereas D'Souza et al.
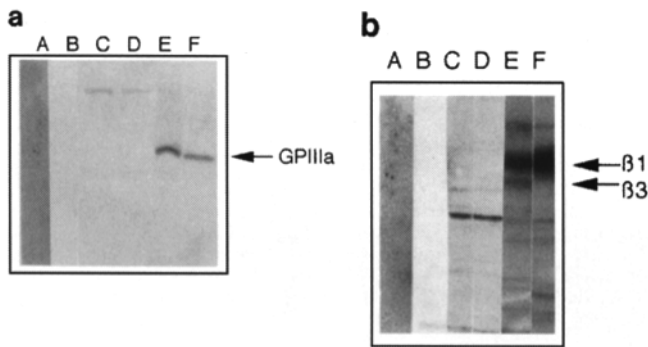
a

A B C D E F

← GPIIIa

b

A B C D E F

← β1
← β3

Figure 7. Immunoblot analysis of anti-*CWDDGWLC* antiserum. (*a*) Purified αIIbβ3 was separated on SDS-PAGE and transferred to Immobilon-P membranes. The filters were probed with normal anti-*CWDDGWLC* serum (1:500) and anti-β3 cytoplasmic domain sera (1:2000) (lanes *E* and *F*, respectively). (*b*) MG-63 osteosarcoma cell extracts were processed in the same way and probed with anti-*CWDDGWLC* and with TS2/16 (10 μg/ml), an anti-β1 monoclonal antibody (lanes *E* and *F*, respectively). Normal rabbit and mouse sera were used as controls both in *a* and *b* (lanes *A* and *B*). In lanes *C* and *D*, filters were incubated with anti-*CWDDGWLC* serum in the presence of 100 μM of the *CWDDGWLC* or the β3 (119-133) peptides in solution.

used a synthetic RGD peptide, and the divalent cation requirements for the binding of different ligands to the same binding structure on the integrin may vary (25). As the WDDGW motif lacks the divalent cation binding sequence, which is at the NH_2 terminus of the peptide (9), we suggest the following model for the RGD binding: The actual RGD binding site is predominantly located in the WDDGW similarity region, and in the intact integrin the activity of this site depends on the binding of a cation to the adjoining divalent cation binding site; the cyclic WDDGWL peptide may assume the RGD-binding conformation without divalent cation binding because the disulfide bond stabilizes the RGD-binding conformation. The binding of an RGD ligand may in turn affect the divalent cation binding site, as it causes extrusion of the cation from the integrin (9).

One earlier result would not appear to agree with our hypothesis that the DDLW sequence in the integrin could be important in ligand binding; Bajt et al. (3) found that mutating the aspartic acid residues in this sequence to alanines did not appreciably affect the binding of the αIIbβ3 integrin to fibrinogen. A possible explanation of this result is that αIIbβ3 is known to recognize the γ chain KQGDV sequence in fibrinogen, and unlike RGD peptides which bind to the β subunit, this sequence binds mostly to the α

subunit as measured by affinity labeling (36, 39). The binding of the mutant αIIbβ3 integrin to the RGD-mimic OPG2 antibody was lost and, as the WDDGWL similarity region appears to be the RGD binding site, it may be that changes in this region within intact integrins would affect only the binding of RGD ligands.

Table II. Comparison of the RGD-binding Sequence from Peptide Library with Integrin Sequences

		C W	D D D^q_L W	L C	
β3:	DLYYLM**D**LSYSMK	**D D L W**	S IQNLGTKLAT		(113-153)
β1:	DLYYLMDLSYSMK	D D L	E NVKSLGTDLMN		
β5:	DLYYLMDLSLSMK	D D L	D NIRSLGTKLAEE		
β6:	DLYYLMDLSAAMD	D D L	N TIKELGSGLSKE		

The amino acid sequences of integrin β subunits are shown using the *single-letter code*. Residues D119, D126, and D127 within the β3 subunit are underlined.

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