Isolation of a Highly Specific Ligand for the $\alpha_5\beta_1$ Integrin from a Phage Display Library

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Abstract. Our previous studies showed that the $\alpha_5\beta_1$ integrin selects cysteine pair-containing RGD peptides from a phage display library based on a random hexapeptide. We have therefore searched for more selective peptides for this integrin using a larger phage display library, where heptapeptides are flanked by cysteine residues, thus making the inserts potentially cyclic. Most of the phage sequences that bound to $\alpha_5\beta_1$ (69 of 125) contained the RGD motif. Some of the heptapeptides contained an NGR motif. As the NGR sequence occurs in the cell-binding region of the fibronectin molecule, this sequence could contribute to the specific recognition of fibronectin by $\alpha_5\beta_1$. Selection for high affinity peptides for $\alpha_5\beta_1$ surprisingly yielded a sequence RRETAWA that does not bear obvious resemblance to known integrin ligand sequences. The synthetic cyclic peptide GACRRETAWACGA (*CRRETAWAC*) was a potent inhibitor of $\alpha_5\beta_1$-mediated cell attachment to fibronectin. This peptide is nearly specific for the $\alpha_5\beta_1$ integrin, because much higher concentrations were needed to inhibit the $\alpha_v\beta_1$ integrin, and there was no effect on $\alpha_v\beta_3$- and $\alpha_v\beta_5$-mediated cell attachment to vitronectin. The peptide also did not bind to the $\alpha_{IIb}\beta_3$ integrin. *CRRETAWAC* appears to interact with the same or an overlapping binding site in $\alpha_5\beta_1$ as RGD, because cell attachment to *CRRETAWAC* coated on plastic was divalent cation dependent and could be blocked by an RGD-containing peptide. These results reveal a novel binding specificity in the $\alpha_5\beta_1$ integrin.

FIBRONECTIN is the only known protein ligand for the $\alpha_5\beta_1$ integrin (Pytela et al., 1985). This binding is mediated by the RGD sequence in the 10th type III repeat of fibronectin (Pierschbacher and Ruoslahti, 1984). Studies with mutated forms of fibronectin have suggested that regions in the 8th and 9th type III repeats are also needed for the full adhesive activity of fibronectin (Obara et al., 1988; Aota et al., 1991; Nagai et al., 1991). These synergistically acting regions may contribute to the specific recognition of fibronectin by $\alpha_5\beta_1$.

The $\alpha_5\beta_1$ integrin is important in promoting the assembly of fibronectin matrix and initiating cell attachment to fibronectin (Akiyama et al., 1989; Zhang et al., 1993). Moreover, $\alpha_5\beta_1$ also appears to play crucial roles in cell migration (Bauer et al., 1992, 1993; Zhang et al., 1993) as well as tumor invasion and metastasis (Gehlsen et al., 1988; Humphries et al., 1986; Saiki et al., 1989; Seftor et al., 1993). Probing of $\alpha_5\beta_1$ function with RGD-containing peptides has suffered from the drawback that these peptides also bind to several other integrins such as the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ vitronectin receptors and the $\alpha_{IIb}\beta_3$ fibrinogen receptor of platelets (Ruoslahti and Pierschbacher, 1987).

Peptide libraries (Smith and Scott, 1993) offer a way of searching for peptides with improved binding affinities and selectivities for integrins. In previous experiments, we searched for peptides binding to $\alpha_5\beta_1$ from a hexapeptide library expressed on the surface of filamentous phage (Koivunen et al., 1993). Among the several phage sequences that bound to $\alpha_5\beta_1$ was the cyclic peptide GA*CRGDC*LGA. This peptide, while not specific for $\alpha_5\beta_1$, has a 10-fold higher affinity for $\alpha_5\beta_1$ than the standard linear RGD peptides such as GRGDSP.

The hexapeptide library, even though capable of presenting peptides containing disulfide bonds, seemed to have only a limited repertoire of such peptides. We, therefore, constructed a heptapeptide library in which a random heptapeptide insert is flanked by a cysteine residue on each side. A disulfide bond formed by the cysteines cyclized the random sequences, and as a result the library expresses conformationally constrained peptides capable of high-affinity interactions with receptors. Potent ligands for the $\alpha_{IIb}\beta_3$ integrin have been isolated from such a library based on a cyclic hexapeptide (O'Neil et al., 1992). We describe here novel $\alpha_5\beta_1$-binding sequences selected from the cyclic heptapeptide library.

Materials and Methods

Materials

The fUSE 5 virions (Scott and Smith, 1990) and *Escherichia coli* strains

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K91kan (Smith and Scott, 1993), K802, and MC1061 were obtained from Dr. G. Smith (University of Missouri, Columbia, MO). Human plasma fibronectin was from the Finnish Red Cross and was iodinated as described (Morla and Ruoslahti, 1992). A 110-kD fragment of fibronectin was prepared as previously described (Pierschbacher et al., 1981). Vitronectin was purified from human plasma as described (Yatohgo et al., 1988). Glutaraldehyde was from Sigma Immunochemicals (St. Louis, MO). Peptides were synthesized on a Applied Biosystems model 430A synthesizer (Foster City, CA) by standard Merrifield solid phase synthesis protocols and *t*-butoxycarbonyl chemistry. Peptides were cyclized by oxidizing with 0.01 M $K_3Fe(CN)_6$ at pH 8.4 and purified by reverse-phase HPLC. To relinearize cyclic peptides, the disulfide bond was reduced and alkylated as described (Koivunen et al., 1993).

The $\alpha_v\beta_3$ integrin was isolated from human placental extracts made in TBS buffer containing 0.1 M octyl glucoside, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and proteinase inhibitors (Pytela et al., 1987). The $\alpha_5\beta_1$ integrin was isolated from the same extract using immunoaffinity chromatography with polyclonal antibodies against the cytoplasmic tail of α_5 subunit (Giancotti and Ruoslahti, 1990). $\alpha_5\beta_1$ bound to the affinity column was eluted with 0.1 mg/ml of the immunizing cytoplasmic tail peptide (Giancotti and Ruoslahti, 1990) in TBS buffer containing 25 mM octyl glucoside. The integrin was purified further using wheat germ agglutinin-Sepharose as described (Pytela et al., 1987). The $\alpha_{IIb}\beta_3$ integrin was isolated from outdated platelets (Pytela et al., 1986).

Construction of Heptapeptide Library

A heptapeptide library displaying cyclic sequences was constructed on fUSE 5 vector essentially as described (Scott and Smith, 1990). The peptides are expressed at the NH_2 terminus of phage surface protein III in this vector. Single stranded DNA was prepared from fUSE 5 virions and transfected into K802 cells by electroporation. Double-stranded fuse 5 DNA was extracted from K802 cells with a plasmid preparation kit (Qiagen Inc., Chatsworth, CA) and further purified by CsCl density gradient centrifugation. The insert was prepared by polymerase chain reaction amplification and BglI digestion from synthetic oligonucleotide (Scott and Smith, 1990) that contained a core sequence $TGT(NNK)_7TGT$ (N = equal molar mixture of A, C, G, T; K = G or T). The degenerate oligonucleotide was purified from 15% polyacrylamide gel and ligated to fUSE 5 vector previously cleaved with SfiI. About 24 μg of the ligated vector was transformed into MC1061 cells by 263 separate electroporations with BRL electroporator and booster according to the instructions of the manufacturer (GIBCO-BRL, Gaithersburg, MD). After culturing the bacteria for 24 h, phage were prepared from 3.2 liter of culture medium by precipitating twice with polyethylene glycol. The procedure yielded a primary library containing 4.5×10^9 clones. Sequencing of DNA extracted from a small aliquot of the library indicated the expected distribution of bases.

Selection of $\alpha_5\beta_1$-binding Phage

An aliquot of the library containing 2.5×10^{12} transducing units (TU)[1] was screened with $\alpha_5\beta_1$ coated on microtiter wells as described (Koivunen et al., 1993). In the first and second panning the coating concentration of the integrin was 5 μg/well. To increase the stringency of the panning, the wells were coated with decreasing concentrations of the integrin. Phage were selected for further amplification from the well with the lowest integrin concentration that showed phage binding over background. In the third and fourth panning, the concentrations were 10 and 1 ng/well, respectively. To rescue the bound phage, the wells were eluted with a 0.1 M glycine buffer, pH 2.2, containing 1 mg/ml bovine serum albumin and 0.1 mg/ml of phenol red. Phage were sequenced from randomly selected clones as described (Koivunen et al., 1993).

Phage Attachment Assay

Binding of individual cloned phage to integrins was studied essentially as described (Koivunen et al., 1993) except that the entire assay was done in microtiter wells. Phage were incubated for 1 h at room temperature in microtiter wells coated with $\alpha_5\beta_1$ or $\alpha_v\beta_3$. The wells were washed five times and 20 μl of concentrated K91kan bacteria (Smith and Scott, 1993), diluted 1:5 in Terrific broth media (Sambrook et al., 1989), was added into the wells and incubated for 10 min at 37°C. After adding 100 μl of Terrific broth containing 0.2 μg/ml of tetracycline and 100 μg/ml of kanamycin, the incubation was continued for an additional 30 min at 37°C. Another 100 μl of Terrific broth containing 50 μg/ml of tetracycline and 100 μg/ml of kanamycin was added and the microtiter plate was incubated at room temperature overnight with shaking. The absorbance indicative of bacterial growth was read at 600 nm with an ELISA reader.

Fibronectin-binding Assay

The binding of ^{125}I-labeled fibronectin to $\alpha_5\beta_1$ coated on plastic was performed as described (Koivunen et al., 1993).

Cell Attachment Assay

Cell lines expressing different integrins were used in cell attachment assays to examine peptide inhibition of integrin function. A human melanoma cell line C8161 (Seftor et al., 1993), a fibroblast cell line WI-38 (Vogel et al., 1990), and an osteosarcoma cell line MG-63 (Pytela et al., 1985) attach to fibronectin through the $\alpha_5\beta_1$ integrin, as do B2/α27, a CHO cell line transfected with human α_5, and B2/C1, the control parental CHO line (Bauer et al., 1992). CHO cells C11 and NIH 3T3 cells express the endogenous Chinese hamster and mouse $\alpha_5\beta_1$ integrins (Solowska et al., 1989; Giancotti and Ruoslahti, 1990). The human melanoma cells A375-M attach to fibronectin through both the $\alpha_5\beta_1$ and $\alpha_4\beta_1$ integrins (Mould et al., 1990). The $\alpha_v\beta_1$ integrin-expressing cell line studied was a CHO cell line B2/v7 (Zhang et al., 1993). The vitronectin-binding integrins $\alpha_v\beta_5$ and $\alpha_v\beta_3$ were assayed using the cell lines HT-29 and IMR-90, respectively (Koivunen et al., 1993). Microtiter wells were coated with various concentrations of fibronectin or vitronectin. In some assays fixed concentrations that resulted in 60% maximum attachment for the test cells were used. Peptide was coated on plastic by incubating for 2 h at 37°C in phosphate buffered saline containing 0.25% glutaraldehyde to crosslink the peptide. Any free binding sites on plastic were blocked with bovine serum albumin. Approximately 1×10^5 cells per well were allowed to attach for 1 h in the presence or absence of competing peptides and the bound cells were determined by staining with 0.1% amido black (Ruoslahti et al., 1982).

Results

Phage Selected by $\alpha_5\beta_1$ Integrin Binding Display RGD and NGR Motifs

A majority (69 of 125) of the phage bound to $\alpha_5\beta_1$ from the cyclic heptapeptide library contained the RGD sequence in the insert (Table I). The RGD sequences were found in three positions of the heptapeptide, in the middle, next to the NH_2-terminal cysteine, or one residue removed from it. Selection of high affinity clones by decreasing the coating concentration of $\alpha_5\beta_1$-favored clones that contained a glycine residue after RGD. Moreover, the glycine was followed by an aromatic residue, tryptophan or phenylalanine, in those sequences. No enrichment of any particular amino acids was noted at the positions preceding the RGD.

Another $\alpha_5\beta_1$-binding motif detected was the RGD analogue NGR, and its variation, NGH (Table II). NGR was found in eight peptides. One NGR-containing clone displayed a sequence VLNGRME which is quite similar to the sequence ALNGREE present in the 9th type III repeat of human fibronectin (Kornblihtt et al., 1985). The sequence ASVNGHT in which the arginine of NGR was replaced by histidine was detected in four clones.

Other Non-RGD-type Sequences

The other $\alpha_5\beta_1$-binding non-RGD sequences derived from the cyclic heptapeptide library were more heterogeneous, but could be classified into five groups (Table II). Represen-

1. *Abbreviations used in this paper*: *CRGDC*, cyclic GACRGDCLGA peptide; *CRRETAWAC*, cyclic GACRRETAWACGA peptide; IC_{50}, half-maximal inhibitory concentration; TU, transducting unit.

Table I. RGD-containing Sequences in Phage Isolated by $\alpha_5\beta_1$ Binding for a Cyclic (CX_7C) Phage Display Library

Integrin coating concentration for plastic					
50,000 ng/ml (2nd panning)	1,000 ng/ml (3rd panning)	100 ng/ml (3rd panning)	10 ng/ml (4th panning)	10 ng/ml (5th panning)	1 ng/ml (5th panning)
LSRGDTP	IPRGDGW (5)	IPRGDGW (3)	ELRGDGW (2)	IPRGDGW (2)	IPRGDGW
DRRGDGF	RSRGDFP	YRRGDGH	EYRGDGF	VARGDGW	MTRGDGF
FTRGDAP	VARGDGW	ELRGDGW	VARGDGW	QTRGDGW	LFRGDGW
TSRGDMP	TRGDGWF (3)	VARGDGW		LFRGDGW	RGDGFGS
QLRGDGW	FRGDGFK (2)	TRGDGWF (3)		LSRGDGW	
EGRGDWH	FRGDFPE	ERGDLRM (2)		FRGDGFV	
TLRGDNH	RRGDGWE	TRGDMQW		RGDGFGS	
HLRGDGW	RGDWPNY	SRGDGWI			
MLRGDSF		LRGDGFL			
MPRGDGF		YRGDHLL			
SRGDGFS (2)		LRGDARF			
FRGDHVR		TRGDGWP			
GRGDSVP		GRGDRPQ			
SRGDGFR		KRGDGFW			
GRGDNLP		RGDFSYM			
RGDLRFN					

Selection and sequencing of phage bound to the $\alpha_5\beta_1$ integrin were performed as described in Materials and Methods. The number of clones encoding the same peptide is shown in parentheses.

tatives of four of these were only present in the initial steps of screening and were eliminated in the high affinity screening steps. They each appeared to have some degree of homology to sequences from the type III repeats in the cell-binding region of fibronectin (Kornblihtt et al., 1985). One group contained two serines and a basic residue, a structure that is analogous to the sequence VPGSKST in the 10th type III repeat of fibronectin (Table II). Two phage sequences in this group contained arginine and asparagine residues; they may be homologous to the VPHSRNS sequence in the 9th type III repeat. The third group consisted of heterogeneous sequences that had a serine or phenylalanine residue or both; many of these sequences were hydrophobic. This structure appears to resemble the ANSFTVH sequence of the 9th type III repeat. We also found one sequence resembling the LVD motif of fibronectin that is recognized by the $\alpha_4\beta_1$ (Mould et al., 1991). That sequence, STSDVGG, is similar to the EILDVPST sequence of the alternatively spliced CS1 region and TVSDVPR sequence of the 10th type III repeat.

To determine the relative affinity of the novel non-RGD sequences toward $\alpha_5\beta_1$, we studied the attachment of individual phage clones to microtiter wells coated with $\alpha_5\beta_1$. The phage clones displaying the peptides VFSIAHE, VRLNSLA, TLVPSRS and STSDVGG each bound to $\alpha_5\beta_1$ significantly over the background (not shown). However, their avidity was severalfold lower than that of RGD or NGR-containing

Table II. Non-RGD Sequences in CX_7C Phage Bound to $\alpha_5\beta_1$ and Their Comparison to Fibronectin Sequences

Type III repeat					
9th	TGLDSPT	ANSFTVH	VPHSRNS	ALNGREESP	?
10th	TVSDVPR	PTSLLIS	VPGSKST	AVTGRGDSP	
Phage sequences	STSDVGG (3)	LNTNLGF (2)	EIVKSSS	WANGRSH (3)	RRETAWA (15)
		PELFVES	GPCSGKS	FVNGRSF (2)	RGAPRAW
		FAGSLLV	NLTLSVS	FANGRHS	
		RFGSHVP	TLVPSRS	VLNGRME	
		SRPSTFL		YVNGRVS	
		SVANSVV	VRLNSLA (2)	WLNGRIN	
		ASFFAVQ	VNVEYRN	MANGRLL	
		HVLASAF		LNGRGLM	
		VFSIAHE		ASVNGHT (4)	
		LVASMTP			
		IGTFHHN			
		AFYQGLP			
		QNAFGYS			
		LGEFAFA			

Phage bound to the $\alpha_5\beta_1$ integrin were isolated as described in Materials and Methods. The amino acid residues that are common in each group are highlighted by bold. The number of sequences encoding the same peptide is indicated in parentheses. The fibronectin sequences are shown in the order as they occur in type III repeats, from NH_2-terminus to COOH terminus. The sequences at analogous positions of the 9th and 10th repeats are aligned. The residues underlined show homology to phage sequences.

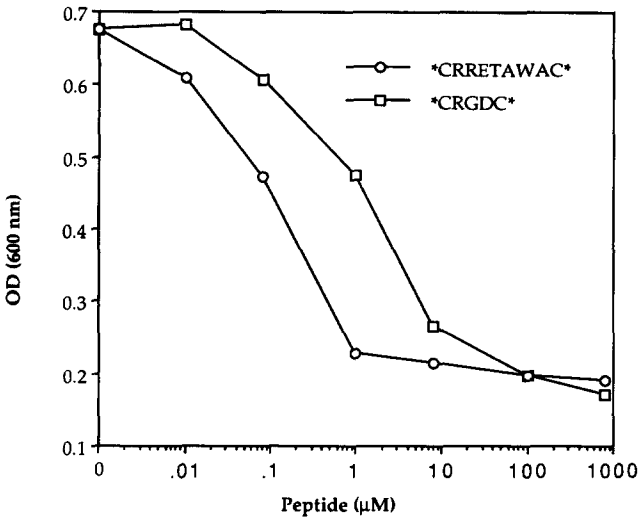

Figure 1. Inhibition of binding of RRETAWA-displaying phage to $\alpha_5\beta_1$ integrin by RRETAWA and RGD-containing peptides. Phage carrying the insert RRETAWA (10^{11} TU/well) were incubated for 1 h in the presence of various concentrations of the cyclic peptides *CRRETAWAC* and *CRGDC* in microtiter wells coated with the $\alpha_5\beta_1$ integrin and the phage remaining bound were quantitated as described in Materials and Methods. The data show means from duplicate wells.

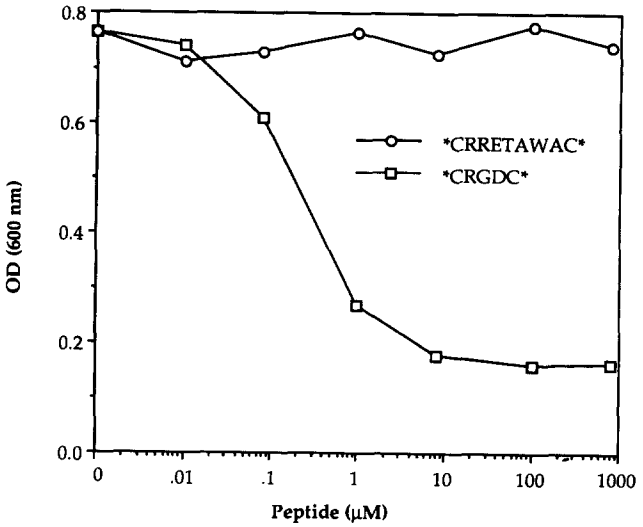

Figure 3. Effect of *CRRETAWAC* and *CRGDC* peptides on binding of RGD-displaying phage to $\alpha_v\beta_3$ integrin. The cyclic peptides *CRRETAWAC* and *CRGDC* were allowed to compete for the attachment of *CRGDC*-phage to the $\alpha_v\beta_3$ integrin coated on microtiter wells and phage binding was measured as in Fig. 1. The data show means from duplicate wells.

phage, indicating that the sequences have a low affinity towards $\alpha_5\beta_1$.

A Novel High-affinity Peptide Ligand for $\alpha_5\beta_1$

Surprisingly, the most common sequence detected after the high affinity selection was not an RGD peptide but a peptide with a sequence RRETAWA. The RRETAWA sequence was progressively enriched when the binding conditions for phage were made more stringent by decreasing the amount of integrin available on plastic. The sequence was detected in 4 of 38, 1 of 7, and 10 of 25 total sequences obtained in the third, fourth, and fifth pannings, respectively. The same nucleotide insert encoded the peptide, suggesting a clonal origin.

We synthesized the cyclic peptide GACRRETAWACGA (*CRRETAWAC*) and compared its activity to GA*CRGDC-*LGA (*CRGDC*) that had thus far been the most avid peptide binder of $\alpha_5\beta_1$ (Koivunen et al., 1993). *CRRETA-

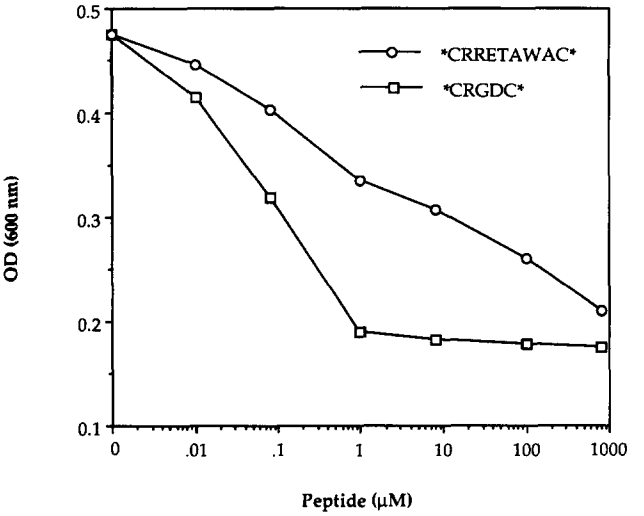

Figure 2. Inhibition of binding of RGD-displaying phage to $\alpha_5\beta_1$ integrin by RRETAWA and RGD-containing peptides. Phage carrying the insert *CRGDC* (10^{11} TU/well) were added together with various concentrations of the cyclic peptides *CRRETAWAC* and *CRGDC* into microtiter wells coated with the $\alpha_5\beta_1$ integrin and phage binding was measured as in Fig. 1. The data represent means from duplicate wells.

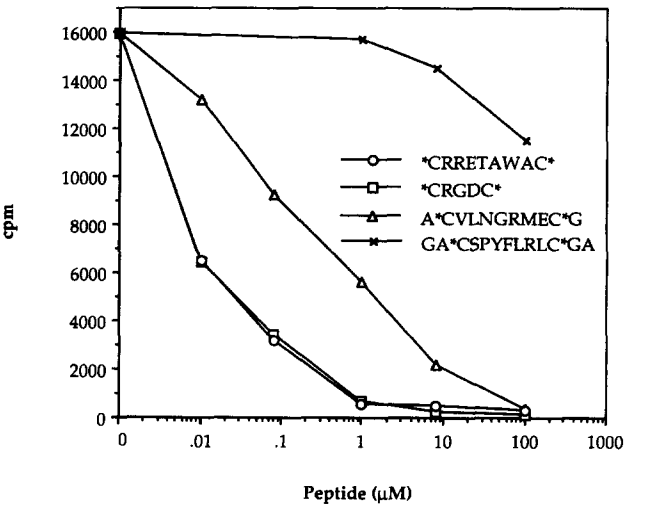

Figure 4. Inhibition of binding of ^{125}I-labeled fibronectin to $\alpha_5\beta_1$ integrin. ^{125}I-labeled fibronectin was incubated for 1 h in the presence of competing peptides in microtiter wells coated with the $\alpha_5\beta_1$ integrin. The wells were washed four times and the radioactivity remaining bound was measured. The data represent means from duplicate wells.

WAC* inhibited the binding of RRETAWA-containing phage to $\alpha_5\beta_1$ 10-fold better than *CRGDC* (Fig. 1). A control peptide GRGESP had no effect (not shown). *CRRETAWAC* also inhibited the binding of *CRGDC*-containing phage to $\alpha_5\beta_1$, but in this assay the *CRGDC* peptide was a better inhibitor than *CRRETAWAC* (Fig. 2). In contrast, the binding of *CRGDC*-containing phage to the $\alpha_v\beta_3$ integrin could not by inhibited by *CRRETAWAC* (Fig. 3). Consistent with this result, the phage displaying RRETAWA bound only weakly to microtiter wells coated with $\alpha_v\beta_3$, whereas it bound avidly to $\alpha_5\beta_1$ under the same conditions. RRETAWA-phage also did not bind to wells coated with the RGD-directed integrin $\alpha_{IIb}\beta_3$ (not shown).

The *CRRETAWAC* peptide inhibited the binding of fibronectin to $\alpha_5\beta_1$ (Fig. 4). Half maximal inhibition (IC_{50}) for both the *CRRETAWAC* and *CRGDC* peptide was seen at 8×10^{-9} M. We also synthesized and tested one NGR-containing peptide A*CVLNGRMEC*G. This sequence was selected because of its similarity to the fibronectin sequence. The NGR-containing peptide with an IC_{50} of 2×10^{-7} M was clearly less active than *CRRETAWAC* or *CRGDC* (Fig. 4). However, this cyclic NGR peptide was a much more potent binder of $\alpha_5\beta_1$ than the linear peptide NGRAHA (Koivunen et al., 1993). An unrelated cyclic peptide, GA*CSPYFLRLC*GA, used as a control had no significant effect.

*The *CRRETAWAC* Peptide Inhibits Cell Attachment to Fibronectin*

The high potency and specificity of *CRRETAWAC* for $\alpha_5\beta_1$ inferred from the integrin-binding assays was confirmed by results from cell attachment assays. *CRRETAWAC* inhibited $\alpha_5\beta_1$-mediated cell attachment to fibronectin at an IC_{50} of 3×10^{-5} M, showing similar or slightly higher potency than the *CRGDC* peptide. A control pep-

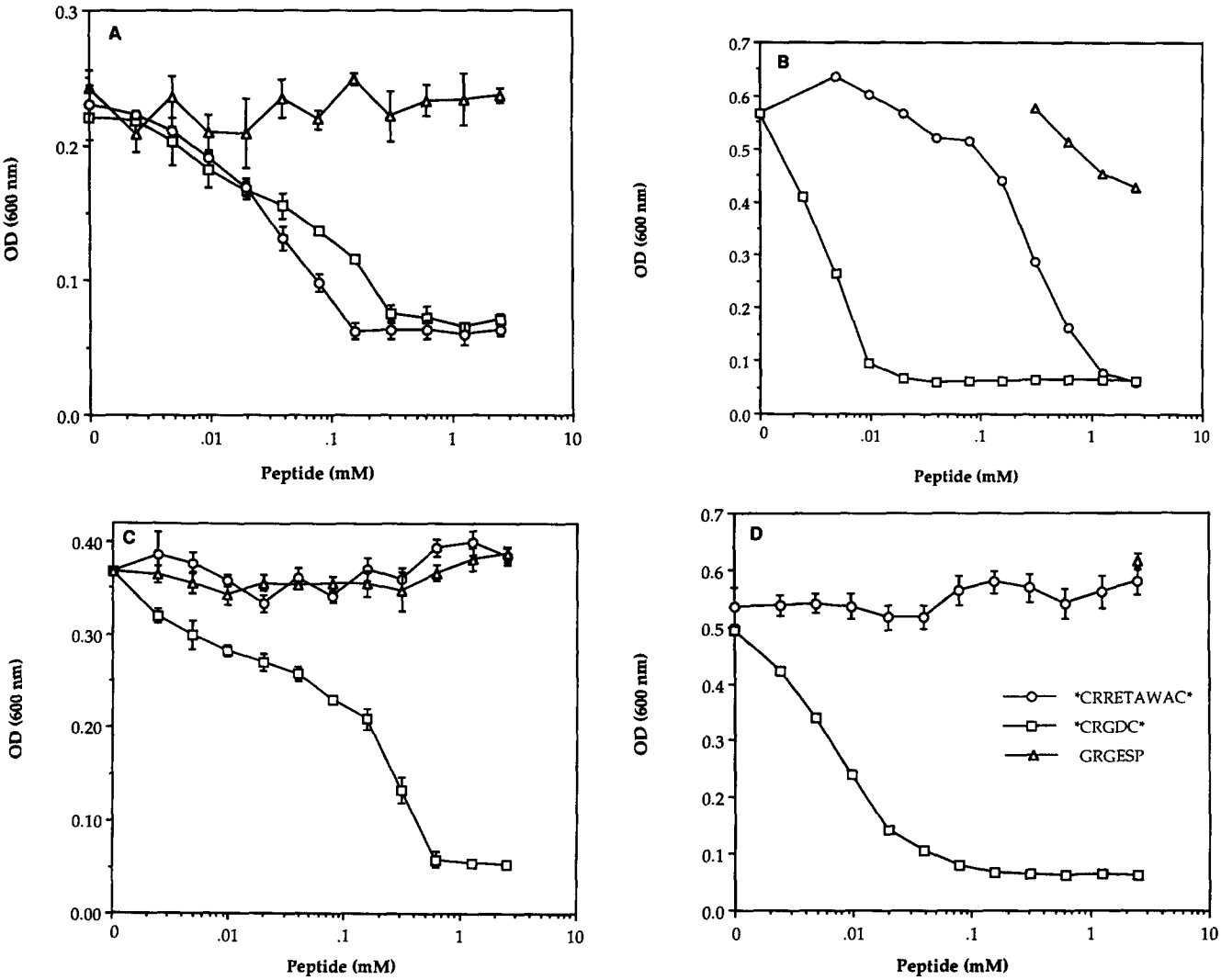

Figure 5. Effect of *CRRETAWAC* peptide on cell attachment to fibronectin and vitronectin. *A*, B2/α27 cells ($\alpha_5\beta_1$); *B*, B2/v7 cells ($\alpha_v\beta_1$); *C*, IMR-90 cells ($\alpha_v\beta_3$); *D*, HT-29 cells ($\alpha_v\beta_5$). Cells were allowed to attach for 1 h in microtiter wells coated with fibronectin (*A* and *B*) or vitronectin (*C* and *D*). The cells that attached were counted as described in Materials and Methods. The data represent means from duplicate wells.

tide, GRGESP, had no effect. Fig. 5 *A* shows the results for a CHO cell line (B2/α27) that expresses the human α_5 subunit from transfected cDNA (Bauer et al., 1992). Similar results were obtained with other $\alpha_5\beta_1$-expressing human cell lines studied, C8161, MG-63, and WI-38. The attachment of A375-M cells was only partially inhibited by *CRRETAWAC*, possibly, because this cell line expresses other fibronectin-binding integrins, including $\alpha_4\beta_1$ (Mould et al., 1990). The *CRRETAWAC* peptide also blocked cell attachment to a 110-kD fragment of fibronectin that contains the cell-attachment domain (not shown). Surprisingly, *CRRETAWAC* could not block the attachment of CHO C11 cells or mouse NIH 3T3 to fibronectin (not shown), indicating that the peptide may be species specific. The disulfide bond was important for the activity of *CRRETAWAC*, as reduction and alkylation of the peptide decreased its activity about 50-fold (not shown). The GACRRETAWACGA peptide that was not cyclized by oxidation also had a reduced activity.

CRRETAWAC inhibited $\alpha_v\beta_1$-mediated cell attachment to fibronectin, but high concentrations were needed and its activity was more than 100-fold lower than that of *CRGDC* (Fig. 5 *B*). *CRRETAWAC* showed no inhibition of $\alpha_v\beta_3$- or $\alpha_v\beta_5$-mediated cell attachment to vitronectin, whereas these interactions were readily inhibited by the *CRGDC* peptide (Fig. 5, *C* and *D*).

Cell lines expressing the $\alpha_5\beta_1$ integrin attached onto plastic coated with the *CRRETAWAC* peptide (Fig. 6). The $\alpha_v\beta_1$-expressing cells also bound, but to a lesser extent (result not shown). A peptide-coating concentration of 1 mg/ml was needed to produce significant attachment, confirming the weaker interaction of *CRRETAWAC* with $\alpha_v\beta_1$ than with $\alpha_5\beta_1$ indicated by the cell attachment inhibition results presented above. In contrast, cell lines that do not express these integrins (CHO-B2/C1, HT 29) or express nonhuman

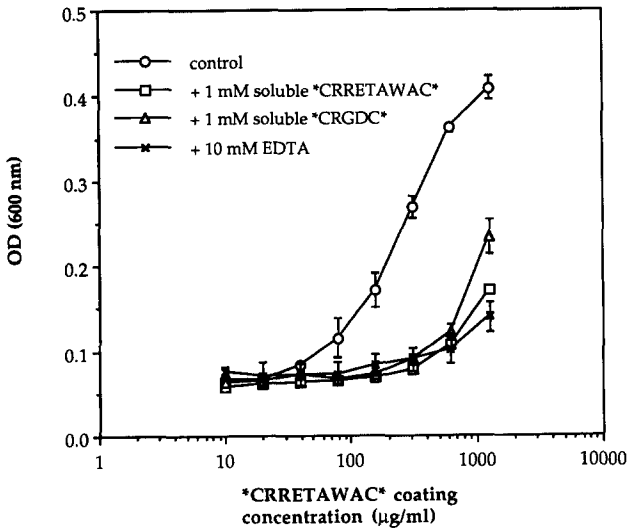

Figure 6. Binding of $\alpha_5\beta_1$-expressing cells to *CRRETAWAC* peptide coated on plastic. B2/α27 cells were incubated for 1 h in microtiter wells coated with various concentrations of the *CRRETAWAC* peptide. The soluble competing peptides or EDTA were added at the concentrations indicated. The cells that attached were quantitated as described in Materials and Methods. The data represent means from duplicate wells.

$\alpha_5\beta_1$ (CHO C11) did not attach to immobilized *CRRETAWAC* (not shown). The cell attachment to *CRRETAWAC* was inhibited by the soluble *CRRETAWAC* and *CRGDC* peptides, and also by EDTA.

Discussion

Our screening of a phage display library that was designed to express a random heptapeptide sequence flanked on each side by cysteine residues has resulted in the isolation of a number of new $\alpha_5\beta_1$-binding peptides. These include the heterogeneous group of non-RGD–containing sequences that may be homologous to sequences present in the cell-binding region of fibronectin. Our results also define the first specific peptide ligand for $\alpha_5\beta_1$.

The results presented here indicate that the peptides selected from the cyclic heptapeptide library for binding to $\alpha_5\beta_1$ are more variable than those selected from a linear hexapeptide library. With only a few exceptions, the peptides derived from the linear hexapeptide library we used earlier were RGD peptides independent of whether the phage were eluted by RGD-containing peptide, EDTA, or a low pH buffer (Koivunen et al., 1993). In the present study, we consistently used a low pH buffer as an elution method to not omit any tight binding phage. That many $\alpha_5\beta_1$-binding non-RGD sequences were found in the present study is apparently due to the longer insert in the present library and, more importantly, the cyclic structure of the peptides displayed by it.

Conformationally constrained peptides are known to have an improved affinity for integrins (Pierschbacher and Ruoslahti, 1987; Samanen et al., 1991; Cardarelli et al., 1992; Gurrath et al., 1992; Imura et al., 1992). The importance of the disulfide bond in the sequences we isolated is indicated by the fact that the activity of *CRRETAWAC* peptide was greatly diminished after reduction of the disulfide bond and alkylation of the cysteines. The NGR sequence also had a higher activity when presented in a cyclic peptide. Peptides containing a cysteine pair can also appear in high affinity screens of the linear peptide library (Koivunen et al., 1993). These results and those of O'Neil et al. (1992) suggest that peptides containing two cysteines, when fused to the NH_2-terminus of phage protein III are capable of forming the expected disulfide bridge and that the presence of such a bond can greatly enhance the integrin-binding activity of the peptide.

O'Neil et al. (1992) used a CX_6C library to isolate cyclic hexapeptides to the $\alpha_{IIb}\beta_3$ platelet integrin and found sequences that contained either the RGD or KGD motif. The number of sequences described is small, but there are some notable differences as compared to the RGD sequences we selected for the $\alpha_5\beta_1$ integrin. First, we did not see any KGD sequences among the $\alpha_5\beta_1$-binding clones. That sequence is known to bind only to the $\alpha_{IIb}\beta_3$ integrin and is found in some snake venoms (Scarborough et al., 1991). Second, the residue COOH-terminal to RGD often was a large hydrophobic one in the $\alpha_{IIb}\beta_3$-binding sequences, whereas we found a strong preference for a glycine residue in the $\alpha_5\beta_1$-binding clones. The glycine was followed by an aromatic tryptophan or phenylalanine. Our recent studies with CX_6C and CX_5C libraries have similarly indicated that the sequence RGDGW/F is highly enriched in the $\alpha_5\beta_1$-binding phage (our unpublished results). These results show

that phage display libraries are able to reveal minor differences in the binding specificities of related integrins.

The $\alpha_5\beta_1$-binding sequences that did not contain the RGD, NGR, or RRETAWA motif could be categorized into four main groups. Each group showed possible homologies to sequences present in the 9th and 10th type III repeats of fibronectin (Table II). The homologous sequences occur at analogous positions in the two type III repeats. Interestingly, all these sequences are likely to reside in exposed loop regions based on the known structure of the 10th type III repeat (Main et al., 1992). Only the serine- and phenylalanine-containing hydrophobic sequence may locate partially in a β strand. The sequences we identified in this manner in the 9th type III repeat could represent the synergistically acting region of fibronectin that has been deduced from site-directed mutagenesis and antibody mapping studies of fibronectin (Obara et al., 1988; Aota et al., 1991; Nagai et al., 1991). These findings suggest a model of fibronectin interaction with $\alpha_5\beta_1$ that is mediated by cumulative binding of several loop regions of the type III repeats. The interaction at the RGD loop is undoubtedly the strongest, but the NGR and other loops may provide the signal for specific recognition of fibronectin by $\alpha_5\beta_1$.

The identification of the *CRRETAWAC* peptide as a high affinity ligand for $\alpha_5\beta_1$ was surprising in that this sequence bears no obvious similarity to fibronectin sequences or other known ligand sequences for $\alpha_5\beta_1$ or other integrins. Smith and colleagues have also described isolation of peptides from a phage display library that are entirely different from known epitopes for the proteins studied (Smith and Scott, 1993; Smith et al., 1993). Because of its divergent sequence, we were interested to clarify whether *CRRETAWAC* might interact with some other site in $\alpha_5\beta_1$ than RGD. Four different approaches, phage attachment assay, fibronectin-binding assay, cell attachment to fibronectin, and cell attachment to the *CRRETAWAC* peptide, indicated that *CRRETAWAC* and RGD compete with each other for binding to $\alpha_5\beta_1$. Moreover, the binding of cells to immobilized *CRRETAWAC* peptide was inhibited by EDTA, indicating that the interaction is divalent cation dependent. However, since the attachment of RRETAWA-bearing phage was inhibited better by the *CRRETAWAC* peptide than by the *CRGDC* peptide and the reverse was true for the *CRGDC*-carrying phage, it is probable that *CRRETAWAC* does not bind at exactly the same site in $\alpha_5\beta_1$ as RGD, but rather at an overlapping site.

Because we found only one variation of the RRETAWA sequence among the $\alpha_5\beta_1$-selected clones and because the phage carrying that sequence, RGAPRAW, bound weakly to $\alpha_5\beta_1$, we do not know which amino acids in *CRRETAWAC* are important for its activity. Since this was the only high-affinity sequence isolated, it is possible that the entire sequence has to remain invariable. The combination of positive and negative charges of *CRRETAWAC* is likely to be important, and the same may be true of the tryptophan residue, because tryptophan was enriched in the RGD peptides selected by high-affinity screening. In any event, the *CRRETAWAC* peptide seems to have an appropriate disulfide-dependent conformation to fit in the binding site of $\alpha_5\beta_1$.

In conclusion, we have found a novel binding specificity in the $\alpha_5\beta_1$ integrin. Because of its selectivity and high affinity for $\alpha_5\beta_1$, the *CRRETAWAC* peptide may be useful to discriminate cell adhesion that is mediated by $\alpha_5\beta_1$. Immobilized *CRRETAWAC* peptide can selectively promote the attachment of $\alpha_5\beta_1$-expressing cells. Finally, this work illustrates the potential value of phage display libraries for providing information on the possible contact sites between a ligand and its receptor.

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