The CS5 Peptide Is a Second Site in the IIICS Region of Fibronectin Recognized by the Integrin $\alpha_4\beta_1$

INHIBITION OF $\alpha_4\beta_1$ FUNCTION BY RGD PEPTIDE HOMOLOGUES*

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The alternatively spliced type III connecting segment (IIICS) region of fibronectin contains two distinct sites that support the adhesion of melanoma cells. These sites are contained within the synthetic peptides CS1 and CS5 (residues 1-25 and 90-109 of the IIICS, respectively). Recently, the cellular receptor for the CS1 site has been identified as the integrin heterodimer $\alpha_4\beta_1$. In this report, we have investigated the role of the CS5 sequence in melanoma cell adhesion and the identity of its receptor. Adhesion to CS5, when presented to cells as an immobilized IgG conjugate, was blocked by antifunctional monoclonal antibodies directed against either the $\alpha_4$ or $\beta_1$ integrin subunits, but not by antibodies against other subunits, implying that $\alpha_4\beta_1$ is also the receptor for CS5. In peptide inhibition experiments, CS5 was inhibitory for melanoma cell spreading on both CS5-IgG and CS1-IgG conjugates; conversely, CS1 inhibited spreading on both CS1-IgG and CS5-IgG. In both cases, peptide inhibition could be outcompeted by increasing the concentration of substrate-bound conjugate. These results suggest that CS1 and CS5 are recognized by the same or overlapping sites on $\alpha_4\beta_1$. The minimal active sequence within CS5, the tetrapeptide Arg-Glu-Asp-Val (REDV), is somewhat related to the Arg-Gly-Asp-Ser (RGDS) sequence that represents a major active site in the central cell-binding domain (CCBD) of fibronectin. When RGDS peptide homologues were tested for their ability to inhibit spreading of melanoma cells on CS1- and CS5-IgG conjugates, GRGDS, GRGES, and REDV were found to be inhibitory, while GRDGS had no effect. In contrast, spreading on a fibronectin fragment containing the CCBD was inhibited by GRGDS only. GRGDS was also able to elute $\alpha_4\beta_1$ specifically from a CS1 affinity column, confirming directly that $\alpha_4\beta_1$-IIICS interactions are sensitive to peptides containing this recognition motif. Because the minimal active sequence within CS1 is the tripeptide Leu-Asp-Val (LDV; Komoriya et al., manuscript submitted for publication), these findings together define a new adhesive recognition sequence, X-Asp-Y, used by $\alpha_4\beta_1$ for binding to fibronectin. The central aspartate residue in this tripeptide is almost always essential, but some flexibility in the amino acid residues at X (glycine, leucine, or glutamic acid) and Y (serine or valine) is tolerated.

Potential models for the interaction of the IIICS region with $\alpha_4\beta_1$ are discussed.

The interactions of cells with extracellular matrix components are important in determining behavior during cell growth, differentiation, and migration. Many of these interactions are mediated by integrin receptors, which are a family of structurally related, cell surface heterodimers (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Ginsberg et al., 1988; Akiyama et al., 1990; Hemler, 1990). Based on the nature of their $\beta$ subunit, integrins have been classified into at least five groups. For each class, the $\beta$ subunit can complex with a number of different $\alpha$ subunits, and the resulting dimers exhibit different ligand-binding specificities.

The adhesion of cells to fibronectin has been studied extensively, and several regions of the molecule have been shown to support cell attachment (for reviews, see Mosher, 1988; Humphries et al., 1990; Hynes, 1990). One domain that is recognized by a wide variety of cell types is located close to the center of the fibronectin subunit. Within this central cell-binding domain (CCBD), two sequences appear to act synergistically to generate full cell adhesion activity. One is the tetrapeptide RGDS (Ruoslahti and Ruoslahti, 1984a; Yamada and Kennedy, 1984) and the second, located by deletion mutagenesis, lies approximately 20 kDa NH$_2$-terminal to RGDS (Obara et al., 1988). The integrin $\alpha_4\beta_1$ has been identified as a major receptor for the CCBD, although at least five other integrin receptors may also recognize this region (for a review, see Humphries et al., 1990).

A second cell adhesive domain lies in the type III connecting segment (IIICS, also known as V), a region which is subject to complex alternative splicing of pre-mRNA (Kornblith et al., 1985). This domain was identified originally as an attachment site for murine melanoma cells (Humphries et al., 1986), but subsequently it has been shown to support the adhesion of neural crest cells and their derivatives and lymphocytes (Dufour et al., 1988; Humphries et al., 1988; Wayner et al., 1989; Ferreira et al., 1990; Garcia-Pardo et al., 1990). A series of six overlapping synthetic peptides (designated CS1 to CS6) spanning the entire IIICS region have been examined for their ability to promote the spreading of melanoma cells directly and to inhibit spreading on the parent fibronectin molecule. Two peptides, CS1 and CS5, were found to possess significant activity, although CS1 was about 2 orders of magnitude more potent than CS5.

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1 The abbreviations used are: CCBD, central cell-binding domain of fibronectin; IIICS, type III connecting segment; mAb, monoclonal antibody.
active than CS5 (Humphries et al., 1986, 1987). CS1 and CS5 are located in separate, independently spliced segments of the IIICS that are expressed in a tissue-specific manner (Schwarz-bauer et al., 1983, 1985; Kornblith et al., 1985; Paul et al., 1986; Sekiguchi et al., 1986; Herschberger and Culp, 1990).

This suggests that post-transcriptional modification of fibronectin pre-mRNA may regulate the adhesive activity of the mature protein. The minimal active sites within CS1 and CS5 have been narrowed down to the peptides LDV and REDV, respectively (Humphries et al., 1986). The REDV sequence is present as RGDV in both rat and bovine fibronectins (Schwarz-bauer et al., 1983; Skorstengaard et al., 1986), and hence it is notable that both cell-binding domains in fibronectin contain at least one active site based on an RGD-type motif and a second site that apparently is unrelated.

Recent studies have identified the adhesion receptor for the CS1 sequence (Wayner et al., 1989; Guan and Hynes, 1990; Mould et al., 1990). Using affinity chromatography on a matrix containing the CS1 peptide, both the human melanoma cell (Mould et al., 1990) and murine lymphocyte (Guan and Hynes, 1990) receptors recognizing this sequence have been isolated and identified as the integrin αβ1. In a complementary approach, monoclonal antibodies (mAb) directed against either α or β, blocked melanoma cell (Mould et al., 1990) or lymphocyte (Wayner et al., 1989; Guan and Hynes, 1990) adhesion to peptide or protein ligands containing the CS1 sequence. In this report, we now demonstrate that αβ1 is also the adhesion receptor for the CS5 sequence in the IIICS, and we provide evidence that CS1 and CS5 may be recognized by the same or mutually exclusive sites on this integrin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human plasma fibronectin was purchased from the Blood Products Laboratory, Elstree, Hertfordshire, United Kingdom. 110-kDa cell-binding and 38-kDa heparin-binding fragments of fibronectin were prepared by thermolysin and trypsin digestion, respectively, using established procedures (Zardi et al., 1985; Garcia-Pardo et al., 1987). The synthetic peptides CS1 (Asp-Glu-Leu-Pro-Gln-Leu-Val-Thr-Leu-Pro-His-Pro-Leu-Gly-Ile-Glu-Pro-Asn-Leu-Gly-Ile-Glu-Pro-Arg-Ala-Asn-Leu-Pro-Gln-Leu-His-Pro-Leu-Val-Pro-Gln-Leu-His-Pro-Leu-Val-Pro-Ser-Thr), KKT-CS1-VQK (Lys-Lys-Thr-Asp-Glu-Leu-Pro-Gln-Leu-Val-Thr-Leu-Pro-His-Pro-Leu-Gly-Ile-Glu-Pro-Arg-Ala-Asn-Leu-Pro-Gln-Leu-His-Pro-Leu-Val-Pro-Gln-Leu-His-Pro-Leu-Val-Pro-Ser-Thr), KKT-CS1-VQK-Sepharose, KKT-CS1-VQK-Sepharose-4B were prepared as described (Humphries et al., 1987). The GRDGS was purchased from Bachem Inc. (Saffron Walden, Essex, Seattle, WA), (c) mAbl6 and mAbl3 IgG (recognizing rat CD8; from V.L. Woods, University of California San Diego, d) 12F1 IgG (recognizing mouse CD8; from S. K. Akiyama and S. S. Yamada, Howard University, Washington, DC), (e) mAb16 and mAb13 IgG (recognizing α and β1, respectively; from S. K. Akiyama and S. S. Yamada, Howard University, Washington, DC), (f) P3E3 ascites (recognizing α from E.A. Wayne, Oncogen, Seattle, WA), (g) mAbG10 and mAbG13 IgG (recognizing α and β1, respectively; from J. S. Aikiyama and S. S. Yamada, Howard University, Washington, DC), (h) 50% X-100, 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MnCl2, 1 mM CaCl2, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 5 mg/ml bovine serum albumin. McIl was included in all extraction and chromatography buffers because of its reported ability to enhance the affinity of interaction between integrin receptors and their ligands (Gailit and Ruoslahti, 1988). Bovine serum albumin was included as a carrier. The A375-3SM extract was centrifuged at 40,000 × g for 30 min, and the supernatant solution was preincubated with 2 ml of packed, derivatized Sepharose 4B for 30 min and then rotated end over end overnight at 4°C with 3 ml of KKT-CS1-VQK-Sepharose beads. The suspension was packed into a 1.6-cm diameter chromatography column and washed at 4°C with 60 ml of 1% octyl β-D-glucopyranoside, 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MnCl2, 1 mM CaCl2, 2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin (column buffer) at 3 ml/h. The column was eluted sequentially with 6 ml of 0.5 mg/ml GRDGS and 6 ml of 0.5 mg/ml GRDGS dissolved in column buffer. The column was also washed with 4 ml of column buffer after each peptide elution. 2.36 fractions were collected and precipitated with 10% trichloroacetic acid, redisolved in Laemmli sample buffer (Laemmli, 1970), and subjected to electrophoresis in the absence of reducing agent on a 7.5% polyacrylamide gel.

**RESULTS AND DISCUSSION**

The Receptor for CS5 Is Integrin αβ1.—Of the two sites in the IIICS that support cell attachment and spreading, CS1 was found to be the stronger by at least 2 orders of magnitude in previous studies with B16-F10 melanoma cells (Humphries et al., 1986, 1987). Similarly, CS1 was also much more active than CS5 in promoting the spreading of the human melanoma cell line, A375-3SM. The maximal levels of spreading were approximately 80% of CS1 and 30% of CS5 conjugates (data not shown). A control conjugate, CS2-IgG, did not support spreading. The concentrations of conjugates required for half-maximal spreading were 5 and 30 μg/ml for CS1-IgG and CS5-IgG, respectively. Thus, although CS5 is a weaker adhesive site for human melanoma cell adhesion compared to CS1, it does support significant spreading, and the difference in activity between the two peptides does not appear to be as great as that reported previously for mouse melanoma cell adhesion.

The identification of the adhesion receptor for the CS1 sequence has been achieved recently by affinity chromatography on the immobilized peptide (Guan and Hynes, 1990; Mould et al., 1990). However, when these experiments were...
Sites on \(\alpha\beta_1\)—To investigate further the relationship between CS1 and CS5 in binding to \(\alpha\beta_1\), we next examined the ability of each peptide to inhibit spreading on CS1-IgG and CS5-IgG (Fig. 2). As expected, both peptides were able to autoinhibit spreading on their respective IgG conjugate. However, both peptides were also cross-inhibitory, i.e., CS1 peptide inhibited spreading on CS5-IgG and CS5 peptide inhibited spreading on CS1-IgG. Control peptide CS6 had no effect on spreading on either conjugate (Fig. 2). The concentrations of peptides required for half-maximal inhibition of spreading were 10 \(\mu\)g/mL CS1 on CS5-IgG, 100 \(\mu\)g/mL CS1 on CS1-IgG, and 1.5 mg/mL CS5 on either conjugate.

The cross-inhibition by CS1 and CS5 suggests that they may be recognized (i) by the same site on \(\alpha\beta_1\), (ii) by mutually exclusive, overlapping sites, or (iii) by distant, but allosterically connected sites. To test these possibilities further, the ability of substrate-bound CS1 or CS5 (as IgG conjugates) to outcompete the inhibition of spreading by the opposing CS peptide was examined (Table I). This approach has been used previously to analyze the noncompetitive inhibition of fibronectin-mediated cell adhesion caused by soluble collagen (Nagata et al., 1985). At high concentrations of substrate-bound CS1 or CS5, the levels of cell spreading approached those obtained in the absence of CS5 or CS1 inhibitor. The inhibition of spreading by CS1 on CS5-IgG was never fully competed, however, perhaps due to the extreme sensitivity of CS5-IgG-mediated spreading to CS1 (see Fig. 2). If CS1 and CS5 did bind to spatially separate sites on \(\alpha\beta_1\), it would not be expected that the inhibition of adhesion induced by one peptide would be overcome by an excess of the other. The competitive nature of the inhibition by CS1 and CS5, therefore, suggests instead that the two peptides either share a common binding site or bind at two overlapping sites on \(\alpha\beta_1\).


\[ \alpha_\beta_1 \text{ Interactions with CS1 and CS5 Are Inhibited by RGD}\]

**FIG. 1. Effects of anti-\(\alpha_4\) (A) and anti-\(\beta_1\) (B) antibodies on A375-SM cell spreading on CS1-IgG (open circles) and CS5-IgG (closed circles).** The antibodies used were P3E3 (ascites recognizing \(\alpha_4\)) and mAb13 (IgG recognizing \(\beta_1\)). Controls were 12F1 (open triangle, IgG recognizing \(\alpha_2\) used at 100 \(\mu\)g/mL), 439-9B (closed triangle, ascites recognizing \(\beta_1\) used at 1:20 dilution), OX8 (open square, control ascites recognizing rat CD8 used at 1:20 dilution), and normal rat IgG (closed square, used at 100 \(\mu\)g/mL). For clarity, the effects of control antibodies on CS1-IgG- and CS5-IgG-mediated spreading are shown in either A or B, respectively, but not in both panels. Error bars = S.D. C = controls. The coating concentrations of conjugates were 125 \(\mu\)g/mL for CS1-IgG and 150 \(\mu\)g/mL for CS5-IgG. In a separate experiment, mAb16 (anti-\(\alpha_4\)), when tested at concentrations up to 100 \(\mu\)g/mL, was found to have no significant inhibitory effect on CS1-IgG- or CS5-IgG-mediated spreading (for CS1-IgG, control spreading was 81 ± 6%, and spreading in the presence of mAb16 was 27 ± 1%, and spreading in the presence of mAb16 was 32 ± 5%).

repeated with immobilized CS5, no radiolabeled receptor material was retained by the column. This was true even when the chromatography was performed in the presence of Mn

As an alternative approach to identifying the CS5 receptor, however, we investigated the ability of antifunctional monoclonal antibodies directed against integrin subunits to inhibit A375-SM spreading on CS5-IgG compared with CS1-IgG (Fig. 1). Spreading on both conjugates was sensitive to monoclonal antibodies P3E3 and mAb15 (directed against \(\alpha_4\) and \(\beta_1\), respectively). The other anti-integrin antibodies tested were without effect (anti-\(\alpha_5\), anti-\(\alpha_6\), and anti-\(\beta_4\); Fig. 1). These results strongly suggest that CS5, like CS1, is recognized by the integrin \(\alpha\beta_1\).

**CS1 and CS5 Share Common or Mutually Exclusive Binding**

\[^{a}\text{In further experiments, two other monoclonal antibodies directed against }\alpha_6\text{, B5G10 (from M.E. Hemler) and 8F2 (from C. Morimoto), also inhibited spreading on both conjugates (data not shown).}\]

**Table I**

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Inhibition by 0.5 (\mu)g/mL CS on CS1-IgG</th>
<th>Inhibition by 5 (\mu)g/mL CS on CS6-IgG</th>
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<tbody>
<tr>
<td>(\mu)g/mL</td>
<td>%</td>
<td>%</td>
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<tr>
<td>0.2</td>
<td>67</td>
<td>N.D.*</td>
</tr>
<tr>
<td>0.9</td>
<td>57</td>
<td>N.D.</td>
</tr>
<tr>
<td>2.7</td>
<td>15</td>
<td>N.D.</td>
</tr>
<tr>
<td>9.0</td>
<td>9</td>
<td>62</td>
</tr>
<tr>
<td>27.0</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>90.0</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>270.0</td>
<td>1</td>
<td>18</td>
</tr>
</tbody>
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\[^{a}\text{N.D., not determined.}\]
Peptide Homologues—RGD peptides have been reported previously to inhibit spreading of B16-F10 melanoma cells on fibronectin completely (Humphries et al., 1986). Because these cells recognize the IIICS region almost exclusively, this suggests that IIICS-IIICS receptor interactions are sensitive to RGD peptides. To investigate further the nature of the CS1 and CS5 binding sites on \( \alpha_5 \beta_1 \), the ability of a series of RGD peptide homologues to inhibit A375-SM cell spreading on CS1-IgG and CS5-IgG was tested (Fig. 3). The pattern of inhibition on both conjugates was essentially identical; GRGDS and GRGES were both inhibitory (half-maximal inhibition at approximately 200 \( \mu \)g/ml), while GRGDS was without effect. The peptide REDV, which represents the minimal active site in CS5, was moderately inhibitory for spreading on both conjugates. This pattern of inhibition is the same as that previously reported for B16-F10 cell spreading on either fibronectin or unconjugated CS5 (Humphries et al., 1986). To ensure that the peptides were not causing nonspecific inhibition of cell spreading, we also compared the pattern of inhibition of spreading by RGD-containing peptides on two protease fragments of fibronectin, a thermolysin fragment of 110 kDa containing the CCBD (Zardi et al., 1985) and a 38-kDa tryptic fragment containing the COOH-terminal heparin-binding domain and the first 67 residues of the IIICS (i.e. CS1-CS3; Garcia-Pardo et al., 1987). This experiment allows direct comparison of the peptide sensitivity of CCBD receptors (principally \( \alpha_5 \beta_1 \)) with the receptor for the IIICS (\( \alpha_5 \beta_3 \)).

As shown in Fig. 4, the pattern of inhibition of spreading on the 38-kDa fragment and CS1-IgG was identical and the same as in Fig. 3, but distinctly different from that on the 110-kDa fragment. GRGDS was a potent inhibitor of spreading on the 110-kDa fragment with half-maximal inhibition at approximately 30 \( \mu \)g/ml (60 \( \mu \)M); the other peptides tested were either noninhibitory (GRGDS) or only slightly inhibitory (GRGES and REDV). The specific requirement for the (G)RGDS sequence to inhibit CCBD receptors has been shown previously (Pierschbacher and Ruoslahti, 1984, a and b, 1987; Yamada and Kennedy, 1985). The results in Fig. 4 demonstrate that the effects of GRGDS, GRGES, and REDV on A375-SM cell adhesion are specific and suggest that the ability of these peptides to inhibit spreading on IIICS ligands is due to inhibition of the \( \alpha_5 \beta_3 \) complex. Although RGD-containing peptides disrupt adhesion to the IIICS, a similar cross-inhibition is not seen with larger, more active polypeptide reagents from the CCBD (Humphries et al., 1986). Similarly, the high activity CS1 peptide has no effect on CCBD-dependent adhesion (Fig. 4, legend). Taken together, these findings are consistent with the conclusion that both cell-binding domains employ similar mechanisms of interaction with their respective receptors (since generic RGD-containing peptides block the function of both regions), but that in their native state in the parent molecule the active sites in each domain do not compete with each other for receptor recognition.

As a final test of the involvement of an RGD-type motif in
with a number of studies suggesting that the IIICS receptor that these previous results were due to inhibition of CS1-a4PI. This affinity matrix were applied to a KKT-CS1-VQK-Sepharose column, unpeptide or peptide or.

explanation that, since IIICS receptor-ligand interactions are caused significant elution of the receptor, thereby confirming that the interaction of CS1 is sensitive to RGD-containing peptides.

The ability of RGD peptide homologues to inhibit a6b4 binding to the IIICS, we investigated whether the prototype RGD-containing peptide, GRGDS, could elute a6b4 from a CS1 affinity column. Although A375-SM cells contain a number of integrins, only a6b4 is detected after elution of this affinity matrix (a) with the specific competitor CS1 peptide or (b) with EDTA which disrupts the interaction of all known integrins with their ligands (Mould et al., 1990).^ Triton X-100 extracts of surface-iodinated A375-SM cells were applied to a KKT-CS1-VQK-Sepharose column, unbound material was removed by washing, and the column eluted sequentially with 0.5 mg/ml GRGDS and 0.5 mg/ml GRGDS. As shown in Fig. 5, when the control peptide GRGDS was added to the elution buffer, no receptor was eluted above background. In contrast, elution with GRGDS caused significant elution of the receptor, thereby confirming that the interaction of a6b4 with CS1 is sensitive to RGD-containing peptides.

In summary, although our data favor a model in which CS1 and CS5 to cross-inhibit each other’s biological activity. Possible models include: model I, in which CS1 and CS5 interact with exactly the same site on a6b4; model II, CS1 and CS5 recognize different but overlapping (hence mutually exclusive) sites on a6b4; and model III, CS1 and CS5 recognize different sites on a6b4 but each site can be inhibited in a cis fashion by the opposing peptide. Further experiments will be necessary to distinguish unequivocally between these possibilities. However, model III can probably be discounted because CS1 and CS5 appear to be competitive inhibitors of each other’s activity (Table I). At the present time, we favor model I because (a) three different monoclonal antibodies directed against a4 block recognition of both CS1 and CS5 by a6b4; (b) a6b4 interactions with CS1 and CS5 have the same pattern of inhibition with RGD peptide homologues (Fig. 3); if different sites were involved in binding CS1 and CS5, the pattern of inhibition would be expected to differ and (c) the minimal active site in CS1, LDV, is related in sequence to REDV (the active site in CS5); the similarity between these two active peptides suggests that they could be recognized by the same site on a6b4. The ability of RGD peptide homologues to inhibit binding of CS1 and CS5 to this site would then be explained by their ability to mimic the active LDV/REDV sequences. In summary, although our data favor a model in which CS1 and CS5 are recognized by the same site on a6b4, we cannot currently exclude the possibility that CS1 and CS5 bind to overlapping sites (model II).

The essential structural feature common to both ligands and inhibitors of a6b4 is the presence of a central aspartate residue (with the sole exception of GRGES peptide) within a tripept of amino acids (i.e. X-Asp-Y). The flanking X and Y residues are less well conserved, and some flexibility in these amino acids is tolerated (e.g. X can be leucine, glycine, or glutamic acid, and Y can be valine or serine). This situation contrasts markedly with the motif recognized by a6b4 and a number of other integrins (including the vitronectin receptor and platelet glycoprotein IIb/IIIa) which have a strict requirement for the RGD tripeptide (Ruoslathi and Pierschbacher, 1987; Ginsberg et al., 1988).

Studies employing a chemical cross-linking approach have recently localized binding sites for RGD peptides on both the a and b subunits of the vitronectin receptor and platelet glycoprotein IIb/IIIa (D’Souza et al., 1988, 1990; Smith and Cheresh, 1988, 1990). In the case of the a subunits (a and a6b4), the cross-linked sites were close to putative divalent cation-binding sites which bear strong homology to an EF-hand-type repeat (D’Souza et al., 1990; Smith and Cheresh, 1990). Interestingly, the binding sites on the b subunit of these two receptors (b3) also bear homology to an EF-hand structure, although these sequences do not fit the consensus as well as the sites in the a subunits (D’Souza et al., 1988; Smith and Cheresh, 1988). Recent analyses of a mutant a6b4

^ A. P. Mould and M. J. Humphries, unpublished work.

**Fig. 5.** Affinity chromatography of detergent-extracted, surface-iodinated A375-SM cells on KKT-CS1-VQK-Sepharose. Fractions 15-17 represent material eluted with control GRGDS peptide, and fractions 20-22 are material eluted with active GRGDS peptide. The two bands observed correspond to the subunits of the a6b4 integrin dimer.
integrin derived from patients with the Cam variant of Glanzmann's thrombasthenia have confirmed the importance of these putative cation-binding sites (Loftus et al., 1990). The mutant receptor lacks both ligand recognition and interaction with divalent cations, a phenotype caused by a point mutation in the β2 subunit that converts one of the aspartate residues in the EF-hand consensus to tyrosine. Taken together, these results have now led to the hypothesis that interaction with receptor-bound cations may be a common mechanism for ligand binding to integrins (Edwards et al., 1988; Loftus et al., 1990).

Comparison of the sequence of the α subunit divalent cation-binding sites with the EF-hand consensus reveals an interesting difference. The amino acid occupying the important z coordination position is always a small hydrophobic residue in integrin α subunits, whereas the consensus for a functional EF-hand is either aspartate or glutamate (Kretsinger, 1980). This suggests that the aspartate residue in adhesive recognition signals such as RGD, REDV, LDV, and LGGAKQAGDV (an antiadhesive peptide found in fibrinogen, the aspartate of which is reported to be critical for function; Hawiger et al., 1982; Ruggeri et al., 1986) may play a functional role in cell adhesion by providing the final coordination group for integrins to chelate divalent cation. Presumably the conformation of the EF-hand repeats in different integrin subunits combined with the conformation of the different aspartate-containing active sites in adhesion proteins will be important in determining the adhesive specificity of various receptors. The LGGAKQAGDV and RGDG peptides have already set a precedent for two aspartate-containing peptides binding to the same or mutually exclusive binding sites on an integrin since each competes for the binding of the other to platelet glycoprotein Iib/IIIa (αMβ2; Lam et al., 1987; Santoro and Lawing, 1987; D’Souza et al., 1990). The same now appears to be true for the interaction of LDV (in CS1) and REDV (in CS5) with α5β1.

The dual interaction of CS1 and CS5 with α5β1 is of particular interest because both sequences are located in independently spliced segments of the IIICS and fibronectin variants containing either site alone or both sites have been identified (Kornblihtt et al., 1984; Bernard et al., 1985; Sekiguchi et al., 1986; Oyama et al., 1989; Herschberger and Cupp, 1990). If both sequences do interact with divalent cation-binding sites in α5β1, this raises the interesting question of whether both sequences bind to exactly the same EF-hand repeat or whether they interact with neighboring repeats. A fuller understanding of the molecular basis of the interaction of the IIICS with α5β1 may also provide insight into the functional relevance of the CS5 sequence. Since the adhesive activity of CS5 is so much lower than that of CS1, it is currently unclear what its contribution is to the activity of the whole IIICS region. Intriguing possibilities are that binding of α5 to CS1 or CS5 might transduce different signals to the cell interior or alternatively that interaction with the low affinity CS5 site might allow rapid migration while interaction with the stronger CS1 site facilitates immobilization.

In addition to studying IIICS-α5β1 interactions, it will also be important to identify the binding sites for other sequences known to interact with α5β1. These include sites in the COOH-terminal heparin-binding domain of fibronectin (Wayner et al., 1989) and the endothelial cell surface molecule VCAM-1 (Elices et al., 1990). In both cases, the peptide sequences responsible for α5β1 binding activity have yet to be determined, although VCAM-1 does contain the LDV tripeptide in its extracellular domain (Osborn et al., 1989).

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Integrin αβ₁-Fibronectin IIICS Interactions