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Identification of a novel recognition sequence for the integrin $\alpha4\beta1$ in the COOH-terminal heparin-binding domain of fibronectin

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The type III connecting segment of fibronectin contains two cell binding sites, represented by the peptides CS1 and CS5, that are recognized by the integrin receptor $\alpha4\beta1$. Using assays measuring the spreading of A375-SM human melanoma cells, we now report that the adhesion promoting activity of a 29 kDa protease fragment of fibronectin containing the COOH-terminal heparin-binding domain (HepII), but lacking CS1 and CS5, is completely sensitive to anti-$\alpha4$ and anti-$\beta1$ antibodies, suggesting that HepII contains a third $\alpha4\beta1$-binding sequence. Examination of the primary structure of HepII revealed a sequence with homology to CS1. A 19mer peptide spanning this region (designated H1) was found to support cell spreading to the same level as the 29 kDa fragment. H1-dependent adhesion was completely sensitive to anti-$\alpha4$ and anti-$\beta1$ antibodies. When soluble peptides were tested for their ability to block cell spreading on the 29 kDa fragment, a 13mer peptide comprising the central core of H1 was found to be completely inhibitory. The active region of H1 was localized to the pentapeptide IDAPS, which is homologous to LDVPS from the active site of CS1. Taken together, these results identify a novel peptide sequence in the HepII region of fibronectin that supports $\alpha4\beta1$-dependent cell adhesion.

Key words: adhesion/fibronectin/heparin/integrins/peptides

Introduction

The interaction of cells with their surrounding matrix plays an important role in determining cell differentiation, growth and migration (Edelman *et al.*, 1989; Hay, 1991; Humphries *et al.*, 1991). Adhesion to extracellular matrix components is mediated by cell surface receptors, many of which belong to the integrin gene family (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Ginsberg *et al.*, 1988; Akiyama *et al.*, 1990; Hemler, 1990; Mecham and McDonald, 1991). Integrins are $\alpha\beta$ heterodimers that have been classified into seven different groups according to their β subunit. Depending on the identity of the α and β subunits present in an integrin, the resulting dimer exhibits a characteristic ligand-binding specificity (Humphries, 1990; Ruoslahti, 1991). A second class of molecules capable of mediating cell–matrix interactions are the cell surface heparin sulfate proteoglycans (HSPGs): these recognize the heparan-binding sequences that are a common feature of matrix molecules (Gallagher, 1989; Bernfield and Sanderson, 1990).

Cell adhesion to fibronectin has been studied extensively and several regions of the molecule have been shown to support cell attachment, spreading and migration (Mosher, 1989; Hynes, 1990). The central cell-binding domain (CCBD) contains the cell recognition sequence RGDS (Pierschbacher and Ruoslahti, 1984; Yamada and Kennedy, 1984), as well as two regions that function synergistically with this peptide (Obara *et al.*, 1988; Aota *et al.*, 1991; Nagai *et al.*, 1991). The integrin $\alpha5\beta1$ has been identified as a major receptor for the CCBD (Pytela *et al.*, 1985), although several other integrins also recognize this domain (Humphries, 1990; Ruoslahti, 1991). In addition to its adhesive function, the CCBD also appears to contribute to the assembly of extracellular fibronectin matrices in concert with site(s) in the NH_2-terminal domain of the molecule (McDonald *et al.*, 1987; Fogerty *et al.*, 1990).

A second fibronectin domain capable of promoting cell adhesion is contained within the type III connecting segment (IIICS; also known as V), which is present in some, but not all fibronectin isoforms due to complex alternative splicing of this region from mRNA (Hynes, 1985). The inclusion of segments of the IIICS appears to account for the size difference between the A ($IIICS^{high}$) and B ($IIICS^{low}$) subunits of the fibronectin dimer (Paul *et al.*, 1986). The IIICS is recognized by a limited number of cells types, such as neural crest cells and their derivatives, lymphocytes and monocytes (Humphries *et al.*, 1989). Two active sites for adhesion have been identified in the IIICS, represented by the synthetic peptides CS1 and CS5 (residues 1–25 and 90–109 of the IIICS, respectively; Humphries *et al.*, 1986, 1987). The minimal active sequences within CS1 and CS5 are LDV (Komoriya *et al.*, 1991) and REDV (in human fibronectin; Humphries *et al.*, 1986) or RGDV (in bovine or rat fibronectins), respectively. Both of these sites are recognized specifically by the integrin $\alpha4\beta1$ (Wayner *et al.*, 1989; Garcia-Pardo *et al.*, 1990; Guan and Hynes, 1990; Mould *et al.*, 1990, 1991). In contrast to the integrin-binding promiscuity of the CCBD, only cells possessing $\alpha4\beta1$ appear to be capable of recognizing the IIICS.

The heparin-binding domains of fibronectin have also been implicated in cell adhesive events (Rogers *et al.*, 1985, 1987; McCarthy *et al.*, 1986; Bernardi *et al.*, 1987; Mugnai *et al.*, 1988; Liao *et al.*, 1989); in particular they synergize with classical cell-binding fragments in promoting the formation of focal contact structures (Izzard *et al.*, 1986; Woods *et al.*, 1986). The COOH-terminal heparin-binding domain (HepII) contains at least two sequences, represented by the synthetic peptides FN-C/H I and FN-C/H II, that support heparin-dependent adhesion of melanoma and neuroblastoma cells (McCarthy *et al.*, 1988, 1990; Haugen *et al.*, 1990). Adhesion to these sequences is probably mediated by cell surface HSPGs (Drake *et al.*, 1990). A large 58 kDa fragment of fibronectin containing both the HepII and FibII domains, but lacking the IIICS, has also been reported to support the adhesion of T lymphocytes (Wayner *et al.*,

1989). However, adhesion to this fragment was completely blocked by antibodies directed against either the α4 or β1 integrin subunits. Since the FibII domain is inactive in cell adhesion assays, these findings provide indirect evidence for the existence of a site in HepII recognized by α4β1. The identity of this site and its relationship to other sequences in HepII that are capable of supporting cell adhesion is not known.

In this report, we demonstrate that the human melanoma cell line A375-SM is able to spread on a fragment of fibronectin that contains only HepII. Spreading is blocked by antibodies directed against α4 or β1, but not by antibodies against α5 or by heparin. We have also identified a novel α4β1 recognition sequence in HepII (designated H1), which is distinct from FN-C/H I and FN-C/H II. H1 contains an IDAPS sequence that appears to function as an active homologue of the LDVPS-containing active site in the CS1 segment of the IIICS.

Results

Melanoma cells spread on a 29 kDa fibronectin HepII fragment

Fibronectin fragments from the COOH-terminal portion of the fibronectin monomer that contain HepII have been shown previously to support α4β1-dependent lymphocyte attachment (Wayner *et al.*, 1989; Garcia-Pardo and Ferreira, 1990; Garcia-Pardo *et al.*, 1990). A 38 kDa tryptic fragment of the A chain of fibronectin, containing HepII and the first 67 amino acids of the IIICS (i.e. including the major CS1 active site; Garcia-Pardo *et al.*, 1987), has also been shown to promote A375-SM melanoma cell spreading (Mould *et al.*, 1991). To investigate if melanoma cells could recognize the HepII domain alone, a 29 kDa thermolytic fragment, comprising HepII and only the first five amino acids of the IIICS (Pande *et al.*, 1987), i.e. lacking the active site of CS1, was tested for its ability to support A375-SM cell spreading.

As shown in Figure 1a, the 29 kDa fragment was active, but the maximal level of spreading was ~20% compared to ~80% for the 38 kDa fragment. A similar level of activity to that of the 29 kDa fragment has been reported previously for the low affinity CS5 site in the IIICS (Mould *et al.*, 1991), suggesting that cell interactions with HepII may also be of low affinity. In accord with previous studies using larger fragments from the COOH-terminus of fibronectin (Wayner *et al.*, 1989), cell spreading on the 29 kDa fragment was completely abolished by treatment with anti-functional anti-α4 (P3E3) and anti-β1 (mAb13) antibodies (Figure 1b), but was not affected by an anti-α5 antibody (mAb16) or by heparin (100 μg/ml). The difference between the adhesion-promoting activities of the 29 kDa and 38 kDa fragments was probably not due to poorer adsorption of the 29 kDa fragment onto the wells of the tissue culture plate since even at very high coating concentrations no increase in the level of spreading was observed. A more likely explanation is that the 38 kDa fragment contains the potent CS1 adhesive sequence, whereas the 29 kDa fragment does not. Nevertheless, these results do confirm that A375-SM cells recognize one or more low affinity α4β1-binding sites in the HepII domain.

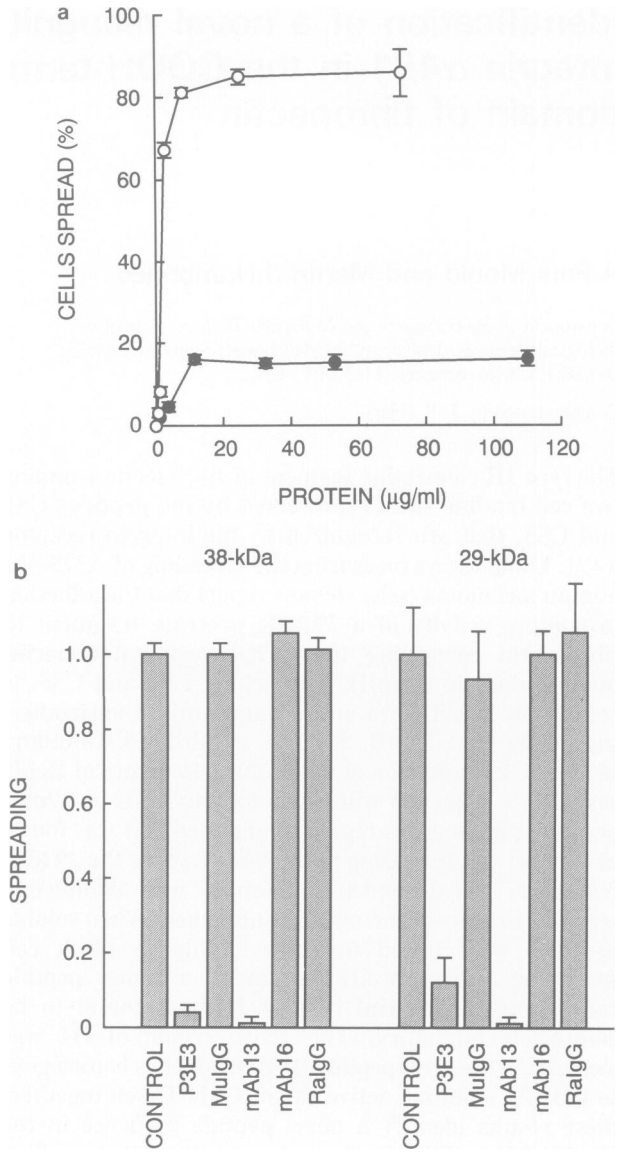

Fig. 1. a. Spreading of A375-SM melanoma cells on the 38 kDa (open circles) and 29 kDa (closed circles) heparin-binding fragments of fibronectin. The abscissa shows the coating concentration of the fragments. Error bars = standard deviation. **b.** Effects of anti-integrin antibodies on 38 kDa and 29 kDa fragment-mediated spreading. Results are expressed relative to spreading in the absence of antibodies which was 68 ± 2% (38 kDa fragment) or 16 ± 2% (29 kDa fragment). Coating concentrations were 7.5 μg/ml for the 38 kDa fragment and 30 μg/ml for the 29 kDa fragment. Immunoglobulin controls were either mouse IgG (for P3E3) or rat IgG (for mAb16 and mAb13). Error bars = standard deviations. In a separate experiment, addition of heparin (100 μg/ml) was found to have no significant effect on 29 kDa fragment-mediated spreading (control = 17 ± 2%; + heparin = 14 ± 2%).

Identification of an α4β1-binding site in HepII

In previous studies, we reported that melanoma cell spreading on the 38 kDa fragment of fibronectin was completely blocked by RGD peptide homologues such as GRGDS and GRGES (Mould *et al.*, 1991), of which the latter is a diagnostic inhibitor of α4β1−IIICS interactions (Humphries *et al.*, 1986). This suggested that a low affinity α4β1-binding site present in HepII may share sequence similarity with either the LDV or REDV/RGDV active sites

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                                   CS1
                         ─────────────────────────
Partial IIICS:  KKTDELPQLVTLPHPNLHGPEI[LDVPS]TVQKTPFVT
                 .. . :   . .. ..:.:: . .  :..
Partial HepII:  YLYTLNDNARSSP-VVIDASTA[IDAPS]NI-R--FLA
                                        ─────────────
                                             H1
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Fig. 2. Identification of a peptide from HepII showing homology with the CS1 site in the IIICS. Partial sequences of each region of fibronectin are shown and matches indicated by ':' and conservative changes by '.'. The locations of the CS1 and H1 peptides are indicated and their most homologous regions, LDVPS and IDAPS, are shown boxed.

in CS1 and CS5, respectively. Comparison of the sequence of HepII with CS1 and CS5 revealed no site related to REDV or RGDV, but instead one peptide that bears homology to the LDV-containing region of CS1 (Figure 2). Although this region possesses substantial homology to CS1, it is notable that it contains two conservative substitutions that convert the LDV active site tripeptide to IDA.

A 19mer peptide (designated H1) which spanned the most homologous part of this region of HepII was synthesized, conjugated to IgG, and tested as a substrate for cell spreading (Figure 3a). The peptide was active, and supported spreading to a similar maximal level as the 29 kDa HepII fragment. Two other peptides from HepII, the heparin-binding FN-C/H I and FN-C/H II, were inactive in this assay (maximal spreading $\leq 2\%$). As for the 29 kDa and 38 kDa fragments, spreading on H1 – IgG was completely blocked by treatment with anti-functional anti-$\alpha 4$ and anti-$\beta 1$ antibodies, but was not affected by anti-$\alpha 5$ or heparin (Figure 3b). Taken together, these results indicate that H1 contains the recognition site for $\alpha 4\beta 1$ in the HepII domain of fibronectin.

H1 is functionally active in HepII and IDAPS is its active site

H1 contains two IDA sequences (Figure 2), either of which could conceivably function as a potential homologue of LDV, although only the flanking sequences of the COOH-terminal sequence are homologous to the active site in CS1. To define the active site in H1, sub-peptides were synthesized and tested for cell spreading activity (Table I). Peptide H1a, containing the central 13 amino acids of H1 and the COOH-terminal IDA sequence, retained activity. In contrast, peptide H1b, which contained the NH_2-terminal IDA sequence, was inactive. As a further test of specificity, a peptide was synthesized in which the D residue in the IDA of H1a was substituted by an E residue (peptide H1a[E8]). This conservative substitution rendered H1a almost inactive (Table I), thereby demonstrating the specificity of its adhesive activity and indicating that this peptide might function in a similar manner to previously characterized RGD and LDV sites which also have an absolute dependency on their D residues.

To determine if the H1 sequence was functionally active in the 29 kDa fragment, peptides were tested for their ability to inhibit melanoma cell spreading on this fragment. Since H1 was only soluble at low concentrations at neutral pH, H1a was used instead and compared with peptides H1a[E8], H1b, FN-C/H I and FN-C/H II. As shown in Figure 4, H1a completely inhibited spreading, whereas the D to E substituted peptide (H1a[E8]) was only inhibitory at high concentration and the other peptides were completely

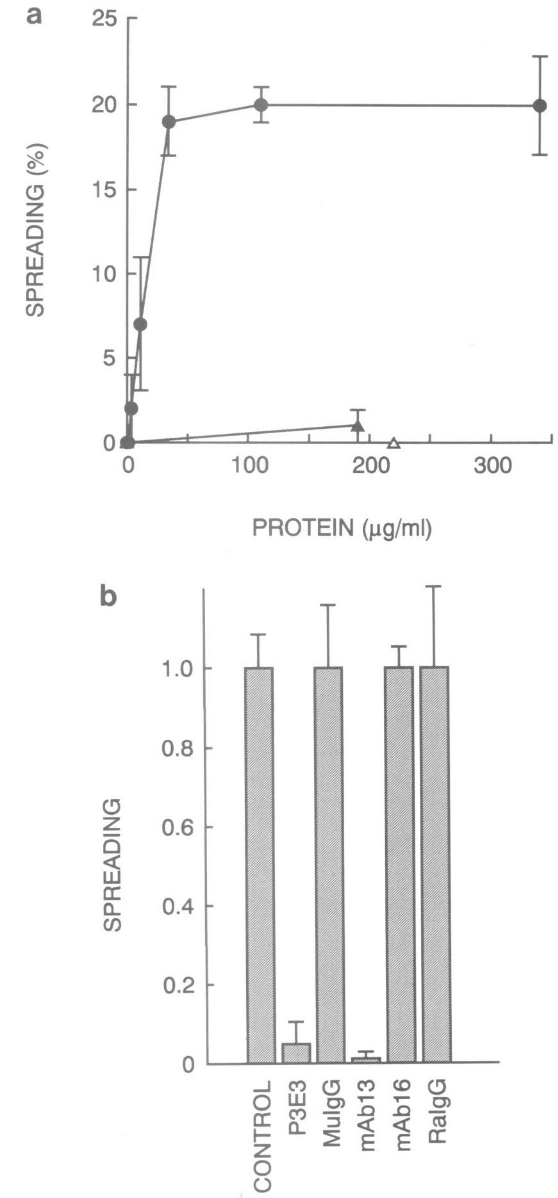

Fig. 3. a. Spreading of A375-SM cells on H1 (closed circles), FN-C/H I (closed triangles) and FN-C/H II (open triangles) peptide – IgG conjugates. Peptide sequences were: H1, VVIDASTAIDAPSNLRFLA; FN-C/H I, YEKPGSPPREVVPRPRPGV; FN-C/H II, KNNQKSEPL-IGRKKT. The abscissa shows the coating concentration of the conjugates. Error bars = standard deviation. **b.** Effects of anti-integrin antibodies on H1 – IgG-mediated spreading. Results are expressed relative to spreading in the absence of antibodies which was $19 \pm 2\%$. The coating concentration of H1 – IgG was 34 μg/ml. Immunoglobulin controls were either mouse IgG (for P3E3) or rat IgG (for mAb16 and mAb13). Error bars = standard deviation. In a separate experiment, addition of heparin (100 μg/ml) was found to have no significant effect on H1 – IgG-mediated spreading (control = $22 \pm 2\%$; + heparin = $21 \pm 2\%$).

inactive. In addition, in other experiments, H1a and H1a[E8] were found to have no significant inhibitory effect on cell spreading mediated by the 110 kDa CCBD fragment of fibronectin.

Further peptides were tested for their ability to inhibit spreading on the 29 kDa fragment; these results are summarized in Table II. A pentapeptide from the putative

Table I. Spreading of A375-SM cells on H1− and H1−sub-peptide−IgG conjugates

Peptide	Sequence	Spreading (%)
H1	VVIDASTAIDAPSNLRFLA	37 ± 4
H1a	DASTAIDAPSNLR	15 ± 2
H1a[E8]	DASTAIEAPSNLR	3 ± 1
H1b	TLNDARSSPVVIDASTAI	1 ± 1

The percentage of cells spread represent the maximal, plateau value obtained at high coating concentrations of conjugate. The two IDA sequences in H1 are underlined.

active region of H1, IDAPS, was found to be sufficient to inhibit cell spreading, although it was less active on a molar basis than H1a. A similar loss of activity upon truncation has been observed in previous studies comparing the specific activities of LDV and its parent CS1 peptide (Komoriya *et al.*, 1991): this probably reflects the requirement for flanking sequences to maintain an active peptide conformation. The mutated pentapeptide IEAPS was also inhibitory, although its activity was lower than IDAPS. This finding is in agreement with the low residual activity found for H1a[E8] (Figure 4 and Table I), and suggests that the D to E mutation, although greatly reducing activity, does not completely abrogate it. It also appears that the shorter the peptide, the smaller the difference in activity between the D and E analogues. Together with the results in Table I, these data demonstrate that IDAPS represents the major active site in H1 and that H1 functions as a cell recognition sequence within HepII.

Relationship of H1 to other α4β1 recognition sites in fibronectin

Both CS1 and CS5 inhibited cell spreading on the 29 kDa fragment (Table II) and on H1−IgG, although, consistent with previous studies (Mould *et al.*, 1991), CS1 was 15 to 20-fold more inhibitory than CS5. Conversely, peptide H1a inhibited spreading on CS1−IgG and CS5−IgG conjugates, and exhibited similar activity to soluble CS5 (results not shown). The D to E substituted peptide (H1a[E8]) had little effect. The level of cell spreading on H1−IgG was consistently similar to or slightly less than that on CS5−IgG (Mould *et al.*, 1991). Taken with previous results (Mould *et al.*, 1991), these data suggest that CS1, CS5, and H1 may compete for a common, or mutually exclusive, binding site(s) on α4β1: CS1 represents the highest affinity site in fibronectin, while CS5 and H1 are of comparable affinity (~20-fold less active than CS1).

Discussion

Our main findings in this report are: (i) That fragments of fibronectin containing the HepII proteoglycan-binding domain alone are able to support the spreading of human melanoma cells; (ii) that this spreading is mediated by the integrin receptor α4β1; (iii) that the HepII domain contains a key cell recognition site represented by the synthetic peptide H1 and; (iv) that the active site within H1 is a short, aspartate-containing motif, IDA(PS), homologous to the LDV(PS) active site in the CS1 segment of the IIICS.

In previous studies, a 38 kDa fragment of fibronectin containing HepII and CS1−CS3 has been shown to possess potent adhesive activity for lymphocytes and melanoma cells (Wayner *et al.*, 1989; Garcia-Pardo and Ferreira, 1990; Garcia-Pardo *et al.*, 1990; Mould *et al.*, 1991). A 58 kDa fragment comprising the HepII and FibII domains also supports attachment, but is much less active (Wayner *et al.*, 1989). Lymphocyte adhesion to each of these fragments was completely inhibited by antibodies directed against the α4

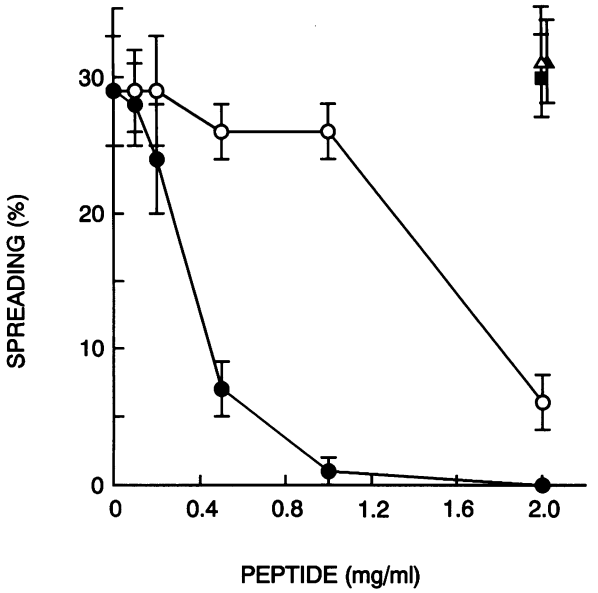

Fig. 4. Inhibition of A375-SM cell spreading on the 29 kDa fibronectin fragment by HepII peptides. The abscissa shows the concentration of H1a (closed circles), H1a[E8] (open circles), H1b (open triangle), FN-C/H I (closed triangle), and FN-C/H II (closed square). In other experiments, none of the peptides had any significantly inhibitory effect on cell spreading on a 110 kDa fragment from the central cell-binding domain.

Table II. Effects of HepII and IIICS peptides on 29 kDa fragment-mediated cell spreading

Peptide	IC_{50} (mM)
FN-C/H I	NI
FN-C/H II	NI
H1a	0.26
H1a[E8]	1.09
H1b	NI
IDAPS	1.93
IEAPS	3.06
CS1	0.034
CS5	0.57

The coating concentration of the 29 kDa fragment was 30 μg/ml. The IC_{50} values show the concentration of peptides required to produce 50% inhibition of control spreading in the absence of peptide inhibitors (29 ± 3%). In other experiments, none of the peptides had any significant inhibitory effect on cell spreading on the 110 kDa CCBD fragment of fibronectin. NI = did not achieve 50% inhibition.

or β1 integrin subunits (Wayner *et al.*, 1989). Hence it has been suggested that the 58 kDa and 38 kDa fragments share a low affinity binding site in the HepII domain, and that the 38 kDa fragment was more active at promoting lymphocyte attachment due to the high affinity α4β1-binding site in CS1. We have confirmed and extended this suggestion by comparing the spreading of A375-SM melanoma cells on the 38 kDa fragment with that on a 29 kDa fragment containing HepII alone. Although the 29 kDa fragment supported a lower level of spreading than the 38 kDa fragment, it is clear that this domain is active at promoting adhesion independent of either the IIICS or FibII regions that have complicated previous analyses. Furthermore, spreading on either fragment was blocked by monoclonal antibodies directed against α4 or β1, indicating that the adhesive activity of HepII resulted from its recognition by the integrin α4β1.

The sequence in the HepII domain supporting α4β1-dependent adhesion was initially identified by homology to CS1 and was represented by the peptide sequence designated H1. The region of H1 most similar to CS1 contained a pentapeptide IDAPS which closely resembles the LDVPS active site sequence in CS1. The latter is highly conserved in all published fibronectin sequences [identical in human (Kornblihtt *et al.*, 1985), rat (Schwarzbauer *et al.*, 1983), bovine (Skorstengaard *et al.*, 1986), and chicken (Norton and Hynes, 1987)], and the same is true for H1 (identical with the exception of a conservative substitution in rat and chicken of threonine for alanine at the COOH-terminus of H1). The H1 peptide, after conjugation to IgG, was as active as the 29 kDa fragment at supporting melanoma cell spreading, and as predicted, this activity was completely sensitive to anti-α4 and anti-β1 antibodies. Hence the H1 sequence can fully account for the ability of the 29 kDa fragment to support α4β1-dependent cell spreading. A peptide containing a D to E substitution within the IDAPS region was much less active, thereby demonstrating the specificity of H1 activity. Since IDAPS pentapeptide itself was also able to block the adhesion of cells to HepII, this sequence appears to be the active site within H1.

IDAPS contains two conservative substitutions that convert the known active LDV tripeptide to IDA. However, like LDV, IDA alone was not sufficient to support melanoma cell spreading since a peptide containing the NH_2-terminal IDA sequence of H1 (peptide H1b) was inactive. This suggests that the secondary and tertiary structures of the IDA tripeptide are important for conferring activity. Since there is substantial sequence homology between H1 and CS1 outside of the IDA/LDV tripeptides, it seems likely that the amino acid residues surrounding the second IDA sequence may confer on it a conformation similar to that of LDV in CS1, and suggests that if LDV and its homologues are employed by proteins other than fibronectin for integrin binding, it may be possible to predict further active sites on the basis of sequence homology. In contrast, it is interesting to note that there is no apparent homology between the sequences flanking RGD active sites in different adhesion proteins.

H1 is unrelated to two other sequences from HepII (FN-C/H I and FN-C/H II) that bind heparin and support melanoma and neuroblastoma cell attachment (McCarthy *et al.*, 1988, 1990; Haugen *et al.*, 1990). These peptides did not support A375-SM cell spreading (although in other assays they did support low levels of cell attachment; A.P.Mould and M.J.Humphries, unpublished) and the free peptides did not inhibit A375-SM spreading on the 29 kDa fragment. Hence, for these cells, adhesion to the HepII domain appears to be mediated almost entirely by the integrin α4β1 and not by heparin-dependent mechanisms (e.g. by HSPGs). It has been reported previously that fragments of fibronectin containing the HepII region cooperate with central cell-binding domain fragments in promoting the formation of focal contacts (Izzard *et al.*, 1986; Woods *et al.*, 1986). In the future, it will be interesting to examine the contribution of the H1 sequence to this process.

The CS1 and CS5 adhesion sites lie in independently spliced segments of the IIICS, and either one, both or neither of the sites may be present in fibronectin variants (Schwarzbauer *et al.*, 1983; Kornblihtt *et al.*, 1985). H1, however, lies outside the regions of alternative splicing and is present in all fibronectin isoforms. Hence, in human fibronectin, four different combinations of CS1, CS5 and H1 are possible, each of which may have distinct functional effects on cell behaviour. The ability of CS1, CS5 and H1 to cross-inhibit each other's function competitively (Mould *et al.*, 1991, and this report) suggests two possible mechanisms of interaction with α4β1 which at present have not been distinguished: (i) that they might each share the same binding site or (ii) that they might bind to structurally related, but spatially separate sites. Since the adhesive activities of H1 and CS5 are lower than CS1, their functional relevance is not yet clear. Intriguing possibilities include the following: (i) Since HepII and IIICS are contiguous, and hence closely related spatially, H1 and/or CS5 may co-operate with CS1 in binding to α4β1 and thereby provide supporting site(s) for stabilizing CS1-dependent adhesion. (ii) Adhesion to the lower affinity H1 or CS5 sites may transduce different signals to the cell interior than does adhesion to CS1. Since there are now precedents for integrin ligands affecting receptor conformation subsequent to binding (Frelinger *et al.*, 1990), the effects that binding of CS1, CS5 and/or H1 have on α4β1 conformation warrant future consideration. (iii) Interaction with the H1 or CS5 site may allow more rapid migration, while interaction with the stronger CS1 site may facilitate immobilization. It is also relevant that, in addition to its interaction with the IIICS/HepII region of fibronectin, the integrin α4β1 has also been shown to mediate adhesion to vascular cell adhesion molecule-1 (VCAM-1; Elices *et al.*, 1990), and to be responsible for T lymphocyte cytotoxicity (Clayberger *et al.*, 1987; Takada *et al.*, 1989) and intercellular aggregation (Campanero *et al.*, 1990; Bednarczyk and McIntyre, 1990; Pulido *et al.*, 1991) via an as yet unknown ligand. In the future, it will clearly be important to determine the binding sites for each of these molecules (and their peptide active sites) within α4β1 in order to obtain a clearer picture of the structural and functional significance of the different ligand–receptor interactions.

Finally, the present data further define the X–D–Y triplet of amino acids used by α4β1 in binding to fibronectin (Mould *et al.*, 1991). To date, X can be glycine, leucine, isoleucine or glutamic acid and Y can be valine or alanine. The central aspartate residue in this triplet appears to be crucial since mutation of LDV to LEV (Komoriya *et al.*, 1991) or IDA to IEA (this report) results in a loss of adhesive

activity. It has previously been suggested (Corbi *et al.*, 1987; Edwards *et al.*, 1988), and experimental evidence is now available to support this (Loftus *et al.*, 1990; D'Souza *et al.*, 1991; Kirchhofer *et al.*, 1991), that the aspartate residue in integrin-binding peptides such as RGD and LGGAKQAGDV (an αIIbβ3 recognition site in the γ-chain of fibrinogen; Hawiger *et al.*, 1982) might provide a co-ordination group for divalent cation held in the EF hand-like structures of integrin α (and perhaps β) subunits. Since RGD and LDV appear to be functionally equivalent, it is possible that the D residue in LDV homologues functions in the same way. By extrapolation, the interaction of different conformations of aspartate-containing peptide active sites with specific integrin EF hands may therefore provide a molecular explanation for the generation of adhesive specificity.

Materials and methods

Materials

Human plasma fibronectin was purchased from the Bioproducts Laboratory (Elstree, Hertfordshire, UK). 110 kDa cell-binding and 29 kDa heparin-binding fragments of fibronectin were prepared by thermolysin digestion, and a 38 kDa heparin-binding fragment was prepared by trypsin digestion, using established procedures (Zardi *et al.*, 1985; Garcia-Pardo *et al.*, 1987); the purity of each fragment was >90% as assessed by SDS–PAGE. Both heparin-binding fragments were recognized in ELISA by the anti-HepII monoclonal antibody IST-2 (Sera-Lab, Crawley Down, Sussex, UK). The synthetic peptides CS1 (DELPQLVTLPHPNLHGPEILDVPST: residues 1852–1876 of fibronectin) and CS5 (GEEIQIGHIPREDVDYHLYP: residues 1941–1960) were synthesized using an Applied Biosystems 430A peptide synthesizer and purified as described previously (Humphries *et al.*, 1986, 1987). The following peptides were purchased from BioMac (Department of Biochemistry, University of Glasgow, UK): H1, VVIDASTAIDAPSNLRFLA (residues 1755–1773 of fibronectin); H1a, DASTAIDAPSNLR (residues 1758–1770); H1a[E8], DASTAIEAPSNLR; H1b, TLNDARSSPVVIDASTAI (residues 1745–1763); IDAPS; IEAPS; FN-C/H I, YEKPGSPPREVVPRPRPGV (residues 1797–1815); and FN-C/H II, KNNQKSEPLIGRKKT (residues 1837–1851). All peptides, except IDAPS and IEAPS, were synthesized with an NH_2-terminal cysteine residue to facilitate coupling to IgG (Humphries *et al.*, 1987). Since H1 was minimally soluble at neutral pH, it was dissolved in 10 mM NH_4HCO_3, 0.1% NH_4OH and coupled at pH 8. Heparin (from porcine intestinal mucosa) was purchased from Sigma (Poole, Dorset, UK). The following monoclonal antibodies were obtained as gifts: P3E3 (recognizing α4; from E.A.Wayner, Oncogen, Seattle, WA), mAb16, and mAb13 (recognizing α5 and β1, respectively; from S.K.Akiyama and K.M.Yamada, National Institute for Dental Research, Bethesda, MD).

Cell spreading

A375-SM cells, a human metastatic melanoma cell line (provided by I.J.Fidler, M.D.Anderson Hospital and University of Texas, Houston, TX) were cultured in Eagle's minimal essential medium containing 10% foetal calf serum, minimal essential medium vitamins, non-essential amino acids, sodium pyruvate, and glutamine (all from GIBCO Life Technologies, Paisley, UK) as described (Kozlowski *et al.*, 1984). Cell-spreading assays were performed in 96 well microtitre plates (Costar) essentially as described previously (Humphries *et al.*, 1986). Briefly, wells were coated for 60 min at room temperature with 100 μl aliquots of adhesion factors diluted with Dulbecco's phosphate-buffered saline, and then sites on the plastic for non-specific cell adhesion were blocked for 30 min at room temperature with 100 μl of 10 mg/ml heat-denatured bovine serum albumin (Humphries *et al.*, 1986). A375-SM cells were detached with 0.05% trypsin, 0.02% EDTA, resuspended to 2×10^5/ml of serum-free Dulbecco's minimal essential medium, and allowed to recover for 10 min at 37°C. For experiments examining the effects of antibodies or peptides on spreading, 50 μl aliquots of the cell suspension were added to wells together with 50 μl of antibodies (50 μg/ml) or peptides in Dulbecco's phosphate-buffered saline and were then incubated in a humidified atmosphere of 5% CO_2 for 90 min at 37°C. In some assays, 6% CO_2 was used, since this was found to enhance the sensitivity of the assay slightly. The cells were then fixed with 3% glutaraldehyde and counted, using phase contrast microscopy, for the degree of spreading as described (Humphries *et al.*, 1986). Each point was obtained by counting 300 cells/well from a number of randomly selected fields. No cell spreading was observed on wells coated only with heat-denatured bovine serum albumin.

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