Evidence for the location of a binding sequence for the $\alpha 2\beta 1$ integrin of endothelial cells, in the $\beta 1$ subunit of laminin

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To date no specific location on laminin 1 for the binding of $\alpha 2\beta 1$ integrin has been described, although recent evidence supports a location in the E1XNd fragment of the cross region. We have identified a peptide sequence from this region, in the $\beta 1$ chain of laminin 1, YGYYGDALR, which inhibits the adhesion of endothelial cells to laminin 1 and type-IV collagen. A structurally related sequence from the CNBr-cleaved fragment CB3 of the $\alpha 1$ chain of collagen type IV, FYFDLR, inhibits endothelial cell adhesion to both collagen types I and IV and laminin 1. The CB3 fragment containing the FYFDLR sequence has been shown to contain binding sites for both $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins. Present experiments with anti-integrin antibodies indicate that the $\alpha 2\beta 1$ integrin on endothelial cells can account for all the cell binding to collagen types I and IV, and that this integrin makes a major contribution towards the adhesion of these cells to laminin 1. We therefore propose that the peptide FYFDLR participates in $\alpha 2\beta 1$ binding to collagen type IV and that the putatively structurally similar peptide, YGYYGDALR, participates in $\alpha 2\beta 1$ binding to laminin 1. This is the first account of structurally related peptide sequences from laminin 1 and type-IV collagen which show reciprocal inhibition of cell adhesion to either ligand and which might form part of a common integrin-binding site, as well as the first suggestion of a precise location contributing to the $\alpha 2\beta 1$ integrin binding site on laminin 1.

INTRODUCTION

Tissue cells interact with the extracellular matrix (ECM) via specific receptors on the cell surface. These receptors are often members of a large family of transmembrane glycoprotein heterodimers called integrins, or may be other specific transmembrane or peripheral glycoproteins or proteoglycans (for reviews see [1,2]).

Identification of the specific binding sites on individual ECM molecules for some cellular receptors has been achieved by enzymic and chemical fragmentation of the ECM ligand into smaller biologically active fragments or by synthesis of peptides corresponding to regions suspected of having biological activity. The biological activity of such reduced sequences can be tested for direct cell adhesiveness, inhibition of adhesion to the parent molecule or specific elution of ligand or receptor from a complex. The first sequence to be identified as important for integrin binding was a short peptide (RGD in fibronectin [3]), recognized by the specific fibronectin receptor, $\alpha 5\beta 1$ integrin. Subsequently this sequence has also been identified as the binding site in vitronectin for the $\alpha v\beta 3$ integrin [4]. Many different sequences in laminin 1 and types-I and -IV collagen have been claimed to have cell-adhesive activity (e.g. [5–10]). Although RGD sequences have been found in laminin 1 and collagens their role in cell adhesion is arguable. It is thought that they may be hidden in the native structure or held in inappropriate conformations, which may become available upon denaturation or dissolution of the ECM [11,12]. There is no apparent consensus motif among the reported adhesive sequences in laminin 1 and collagen types I and IV, which do not contain RGD. Some bind heparin [7,10] and may interact with cell-surface proteoglycans, while others have been shown to interact with specific integrins or other surface glycoproteins [13].

During studies investigating the relative expression of fibronectin and vitronectin receptors on the cell surface of endothelial cells we used the epidermal growth factor (EGF) C-terminal peptide (WWELR) as a negative control. We discovered that this peptide inhibited endothelial cell adhesion to laminin 1 and collagen types I and IV while having no activity on fibronectin or vitronectin. Consequently we used a consensus sequence of two aromatic residues followed by an acidic residue, a hydrophobic residue and a basic residue, to search the sequences of these three proteins for similar peptides. The present paper describes the biological activity of these peptides and identifies a putative new integrin-binding site in laminin 1.

EXPERIMENTAL

Materials

Polystyrene tissue-culture plasticware was from Nunc. The 96-well microtitration plates, not treated for tissue culture, were from Flow (Linbro/Titertek, cat no. 76-232-05) and polyvinyl U-shaped 96-well ELISA plates were from Dynatec. Bovine fibronectin, collagen I, mouse Engelbreth–Holm–Swarm sarcoma (EHS) collagen IV and EHS laminin were obtained from Sigma. EHS laminin (laminin 1) has the form $\alpha 1\ \beta 1\ \gamma 1$ (see [14] for laminin nomenclature). Chicken fibronectin was purified from fresh plasma by affinity chromatography on gelatin–Sepharose (Pharmacia) as described previously [15]. Bovine vitronectin was affinity-purified from serum using a monoclonal antibody (mAb) affinity column as described previously [16].

Abbreviations used: ECM, extracellular matrix; EGF, epidermal growth factor; mAb, monoclonal antibody; BCE, bovine corneal endothelial; T/EDTA, 0.125% trypsin/0.02% EDTA; HUVEC, human umbilical vein endothelial cell; FCS, fetal-calf serum.

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Table 1 Peptides synthesized for the present experiments

Based on the C-terminal sequence of EGF a consensus sequence (Ar)ArArAHB (see Experimental section) was used to search the sequences of collagen IV (col IV), collagen I (col I) and laminin (LM). Small spacers such as a glycine or alanine were allowed in the consensus. Underlined residues in the Table represent the consensus (scrambled in the case of LM peptide C and separated by a long spacer in B). The col I peptide shown (E) was the closest to the consensus found in the whole sequence of α1(I) and α2(I).

Peptide	Source protein	Subunit	Sequence of peptide	Residue nos.	Reference
	Mouse EGF	C-terminus	WWELR	49–53	[20]
E	Mouse col IV	α1 CNBr III	FYFDLR	553–558	[21]
	Bovine col I	α1 N-telopeptide	YGYDEK	4–9	[22]
A	Mouse LM	β1	QYGYYGDALR	992–1001	[23]
B	Mouse LM	α	YHWVTVTLDLR	87–97	[24]
C	Mouse LM	α	YYYSIKD	240–246	[24]
D	Mouse LM	α	HFMFDLGK	2758–2766	[24]

These ECM molecules were tested for purity by a combination of SDS/electrophoresis with protein staining and Western blotting and sensitive ELISA assays using mAbs or polyclonal antisera which displayed cross-species reactivity. No cross-contamination of ECM molecules was found except for a trace of collagen IV present in the collagen I preparation. All ECM molecules were stored in aliquots at −70 °C. All other chemicals were of Analar grade. mAb RMAC 11 was a generous gift from Dr. A. D'Apice, Department of Nephrology, Royal Melbourne Hospital, Melbourne, Australia. This IgG2a mAb was raised against the $\alpha2\beta1$ integrin of human umbilical vein endothelial cells (HUVECs) and recognizes the $\alpha2$ subunit [17].

Peptides

The cell adhesion peptide, RGDS, was purchased from Peptide Technology Pty. Ltd. (Dee Why, NSW, Australia). The C-terminal EGF peptide, WWELR, was synthesized by a combination of chemical and enzymic methods as described elsewhere [18]. Other peptides were synthesized on an Applied Biosystems 430A peptide synthesizer using the N^{α}-9-fluorenylmethoxycarbonyl dimethyl formamide/1-hydroxybenzotriazole ester technique [19]. These included a negative control, RDGS; the laminin 1 peptide, YIGSR [5]; and the collagen I peptide with extended glycines, GRGDTPGG [8]. Laminin 1, collagen IV and collagen I peptides with the consensus sequence (Ar)ArArAHB, where Ar represents an aromatic amino acid, and A, H and B represent acidic, hydrophobic and basic amino acids respectively, were also synthesized using this method and are shown in Table 1. Peptides were dissolved at 4–10 mg/ml in serum-free medium 199 (Gibco, Life Technologies Inc.) containing 1% BSA and brought to neutral pH with 0.5 M NaOH. Dissolved peptides were stored at −70 °C. Some peptides were conjugated to polystyrene wells coated with BSA [25].

Cell culture

Primary cultures of bovine corneal endothelial (BCE) cells were prepared from steer eyes as described previously [26]. Cultures were maintained on uncoated tissue-culture dishes in McCoy's 5A medium or medium 199 (Gibco, Life Technologies Inc.) containing 10–20% fetal-calf serum (FCS, Gibco or PA Biologicals), 1×10^{-5} M thymidine, 100 units/ml penicillin, 100 μg/ml streptomycin sulphate and 5 μg/ml Fungizone. Cells were passaged at a 1:2 split ratio after disaggregation with 0.125% trypsin/0.02% EDTA (T/EDTA). Cells were used between passages 6 and 11. Primary HUVECs were isolated from fresh umbilical cords delivered by Caesarian section at the Royal North Shore Hospital, Sydney, as described elsewhere [27] using 0.1% collagenase (Sigma C6885). Cells were grown on tissue-culture plastic precoated for 2 h at 37 °C with chicken fibronectin at 10 μg/ml (5 ml per 75 cm^2 flask), in phosphate-buffered saline (PBS). The culture medium was medium 199 with Earle's salts (Gibco), containing 20% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin sulphate, 100 μg/ml heparin (Sigma H3149) and 2% bovine brain extract prepared as described elsewhere [28]. Cells were disaggregated with T/EDTA, passaged at a 1:3 split ratio and used between passages 6 and 9.

Cell adhesion assays

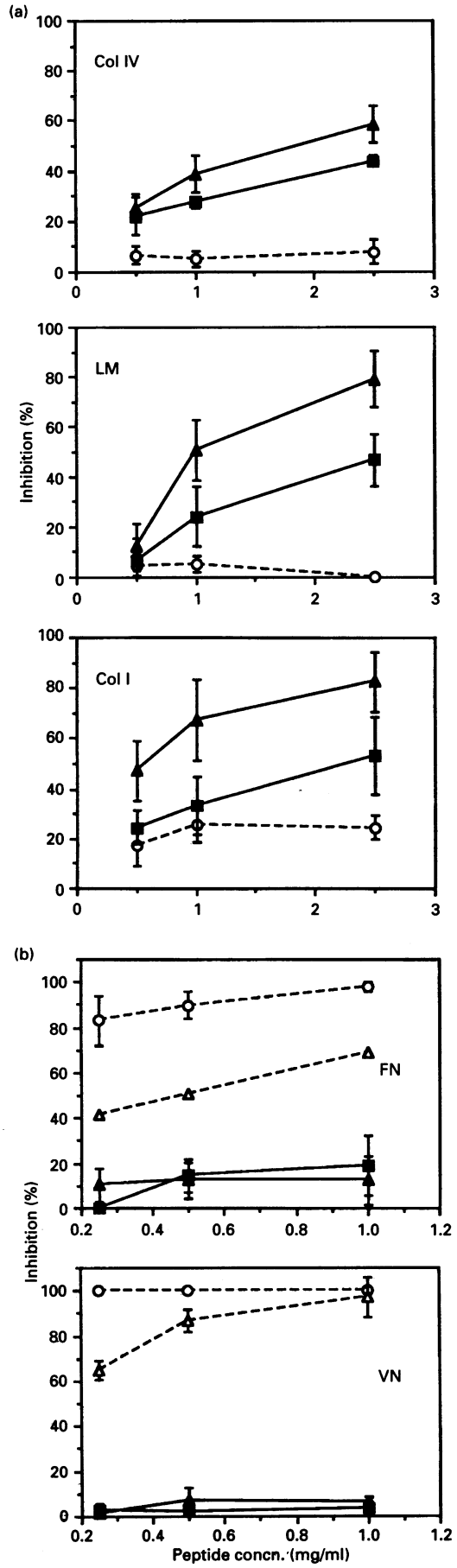
Adhesive ligands were coated in 50 μl aliquots in 96-well polystyrene microtitration plates for 2 h at 37 °C. Cell adhesion was done as for the standard assay previously described [29]. After 1–2 h incubation, adherent cells were fixed with 10% formalin in PBS (formol saline) and stained with Methylene Blue (Gurr no. 34048) as described [30]. Absorbance at 655 nm was read on a Bio-Rad 3550 plate reader (test wavelength (T) 655 nm, reference wavelength (R) 450 nm). Inhibition by synthetic peptides or mAbs was assayed by pre-incubation of disaggregated cells with inhibitors for 15 min at 37 °C before seeding on to the coated wells. Antibodies and/or peptides were kept in the reaction mixture for the duration of the cell adhesion assay.

[^{3}H]Heparin binding assays

Wells of Dynatec polyvinyl ELISA plates were coated with laminin 1 or collagen IV at various concentrations in PBS for 2 days at 4 °C. Heparin binding was assayed as described previously [31] using 10–20 μg/ml of [^{3}H]heparin (NEN) without further purification. Incubations were done at room temperature on an orbital shaker. Inhibition of heparin binding by unlabelled heparin was assayed by pre-incubation of coated wells with 50 μl of inhibitor for 2 h at room temperature, while synthetic peptides were pre-incubated with labelled heparin for the same period. Inhibitor and labelled heparin incubations and subsequent washes were done using PBS containing 1% BSA.

ELISAs for β1 integrins

HUVECs were seeded in growth medium at 3×10^4 cells per well into wells coated as for adhesion with laminin 1 or collagen IV. After incubation at 37 °C for 24 h the adhered cells were washed in PBS containing 0.1 mM Ca^{2+} and $MgCl_2$ and then fixed in formol saline. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed with PBS and incubated with 1% BSA



in PBS to block any unoccupied protein-binding sites on the wells. Primary mAbs at various concentrations and biotinylated secondary antibodies (Amersham, 1/1000 in 1% BSA in PBS) were incubated sequentially in 50 μl volumes on a plate shaker (Titertek, ICN-Flow) for 1.5 h. Peroxidase-conjugated streptavidin (Amersham, 1/500 in 1% BSA in PBS) was similarly incubated for 45 min. The peroxidase substrate was 2 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (Sigma) made up in 0.5 M citrate buffer, pH 4.5, containing 1/5000 of 30% H_2O_2. All reagents were at room temperature. The reaction steps were each separated by 3–4 washes in PBS. Absorbances were read at 405 nm on a Bio-Rad 3550 plate reader (T405 and R490 nm). Anti-integrin mAbs were as follows. Anti-α2 was RMAC-11 as described above. Anti-α3 clone P1B5 (subclass G1) and anti-α5 clone P1D6 (subclass G3) were obtained from Telios (Life Technologies Inc.). Anti-α6 MAB 1972 (rat) was obtained from Chemicon International Inc. Mouse antibody subclass negative controls were derived from subclass pools of anti-influenza mAbs and rat serum was used as the control for MAB 1972.

Statistical treatment of data

Individual experiments were repeated at least twice. Statistical analyses of data were done using Student's t test, analysis of variance and Student–Newman–Keuls' test where applicable.

RESULTS

Effects of synthetic peptides on BCE adhesion

Figure 1 shows the effects of various synthetic peptides upon BCE cell adhesion to ECM substrates. The EGF peptide WWELR and its most closely matched counterpart in collagen type IV, FYFDLR, both demonstrated concentration-dependent inhibition of BCE cell adhesion to collagen types I and IV and to laminin 1, albeit less than 100% (Figure 1a). In contrast the RGD-containing peptide GRGDTPGG was without effect except for a small degree of inhibition on collagen I, from which it is derived (Figure 1a). Quite a different picture was obtained with BCE cell adhesion to fibronectin and vitronectin (Figure 1b). The two RGD-containing peptides, particularly the longer one, were very efficient inhibitors on these substrates, with inhibition reaching 100%. In contrast, no significant inhibition of adhesion to these two substrates was observed with either WWELR or FYFDLR. The laminin β1-chain peptide YIGSR did not affect BCE cell adhesion to any substrate including laminin 1 (data not shown).

Interference of synthetic peptides with heparin binding

The sequence FYFDLR forms part of a larger peptide which has been described to display both cell adhesive and heparin-binding properties [10]. In order to test the hypothesis that inhibition of BCE cell adhesion to laminin 1 and collagen IV by FYFDLR and WWELR was due to binding of these peptides to a cell-

Figure 1 Effect of synthetic peptides on adhesion of BCE cells to ECM substrates

(**a**) Percentage inhibition of BCE cell adhesion to collagen IV, laminin 1 and collagen I in the presence of GRGDTPGG (○), FYFDLR (■) and WWELR (▲). Substrates were coated at concentrations giving optimal sensitivity of cell adhesion to inhibitors. For collagen IV, laminin 1 and collagen I these were 6.4, 4.0 and 1.0 μg/ml respectively. Cells were allowed to adhere for 1 h at 37 °C. Points are the means of three experiments ± S.E.M. (**b**) Percentage inhibition of BCE cell adhesion to fibronectin (FN) and vitronectin (VN). Substrates were coated at 4.0 and 1.6 μg/ml respectively. Conditions and symbols as for (**a**) with the addition of RGDS (△).

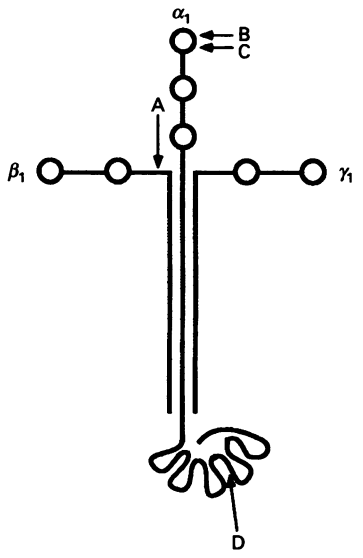


Figure 2 Schematic model of the structure of laminin 1 showing the locations of synthetic peptides with the consensus sequence (Ar)ArArAHB

The $\alpha 1$, $\beta 1$ and $\gamma 1$ chains of laminin 1 are depicted. Location of the four novel peptides synthesized for the present study is shown by the letters A–D as identified in Table 1.

surface heparin-like proteoglycan, their ability to inhibit the binding of [^{3}H]heparin to surface-coated laminin 1 and collagen IV was tested. The binding of [^{3}H]heparin to surface-coated laminin 1 and collagen IV was not inhibited by either peptide over a wide range of peptide concentrations, whereas unlabelled heparin readily inhibited the binding of the radiolabel (data not shown). It is unlikely therefore that heparin-like cell-surface proteoglycans are important contributors to BCE cell adhesion to laminin 1 or collagen IV.

Peptides with the consensus sequence

To investigate further the mechanism of inhibition of cell adhesion by WWELR-type peptides, and the role of integrins, human endothelial cells were used. A search of the available amino acid sequence information for bovine collagen I and mouse collagen IV and laminin 1 using the consensus sequence (Ar)ArArAHB yielded the set of peptides shown in Table 1. Laminin peptides B and C were completely insoluble and N-terminal extension of these by two further amino acids failed to solubilize them. Cell adhesion studies with HUVECs were therefore done using laminin peptides A and D and collagen I peptide E. This latter peptide was the closest to the consensus in collagen I but lacks the hydrophobic residue in the consensus sequence, which is replaced by an acidic residue. The approximate positions of the laminin peptides are shown on a diagram of laminin 1 (Figure 2).

Effects of synthetic peptides and anti-integrin mAb on HUVEC adhesion

The effects of peptides A, D, E and of anti-($\alpha 2$ integrin) mAb upon HUVEC adhesion to laminin 1 and collagen types I and IV are shown in Table 2. Peptide A and anti-$\alpha 2$ mAb each significantly inhibited the adhesion of these cells to laminin 1 and were most effective at low laminin coating concentrations. Peptide D showed a small degree of inhibition but this was not statistically significant and peptide E was completely ineffective. mAb to $\alpha 2$ integrin was very effective at inhibiting HUVEC adhesion to all coated concentrations of collagen types I and IV, the extent of inhibition approaching 100%. Peptide D showed low, but non-significant, inhibition of adhesion on each of the collagen substrates and peptide E was ineffective on either. Peptide A significantly inhibited HUVEC adhesion to collagen IV and similar levels of inhibition (40–50%) were observed on all collagen IV coating concentrations, unlike the situation with laminin 1, where the inhibitory effect was enhanced at low laminin coating concentrations. On collagen I, over the range of coating concentrations used, similar levels of inhibition of HUVEC adhesion were observed with peptide A (20–30%). The response of the HUVECS to this peptide on the collagen I substrate was more variable between experiments than observed with the other substrates, resulting in an overall lack of statistical significance. In summary, the anti-($\alpha 2$ integrin) mAb inhibited HUVEC adhesion to laminin 1 and collagen types I and IV but was most effective on the collagen substrates. Peptide A most effectively inhibited HUVEC adhesion to laminin 1, significantly

Table 2 Effects of synthetic peptides and anti-($\alpha 2$ integrin) mAb upon adhesion of HUVECs to laminin 1, collagen IV and collagen I

Entries are the percentage inhibition of cell adhesion compared with control treatments (peptide RDGS for peptide treatments and mouse non-specific G2a antibody for anti-$\alpha 2$). Peptides were preincubated with cells at 2 mg/ml and antibodies at 1/50 dilution of ascites as indicated in the Experimental section. Cells were allowed to adhere to substrata for 2 h. Values are the means of 3–5 experiments for laminin, 3–4 experiments for collagen I and 2–3 experiments for collagen IV ± standard errors. * indicates statistically significant inhibition of adhesion compared with controls ($P < 0.05$, analysis of variance and Student–Newman–Keuls' test).

Coating of substratum (μg/ml)	Peptide A	Peptide D	Peptide E	Anti-$\alpha 2$ mAb
Laminin (5)	62.8 ± 11.8*	20.9 ± 18.2	−5.5 ± 13.6	66.4 ± 9.1*
Laminin (10)	31.9 ± 10.0*	9.1 ± 14.6	−3.6 ± 12.7	34.6 ± 1.8*
Laminin (20)	39.1 ± 6.4*	16.4 ± 6.4	0.0 ± 10.9	20.0 ± 6.4*
Collagen IV (2.5)	45.6 ± 17.4*	19.5 ± 9.9	−7.6 ± 6.5	95.5 ± 2.2*
Collagen IV (5)	43.4 ± 4.3*	10.9 ± 26.0	−4.4 ± 2.2	93.3 ± 2.2*
Collagen IV (10)	43.4 ± 6.5*	6.5 ± 28.2	−6.5 ± 13.0	91.1 ± 2.2*
Collagen I (2.5)	21.7 ± 17.4	0.0 ± 17.4	−6.5 ± 13.1	91.1 ± 7.6*
Collagen I (5)	33.6 ± 21.7	6.5 ± 15.2	−5.4 ± 14.1	91.1 ± 3.3*
Collagen I (10)	32.6 ± 26.0	8.7 ± 13.0	−4.3 ± 10.8	93.3 ± 4.3*

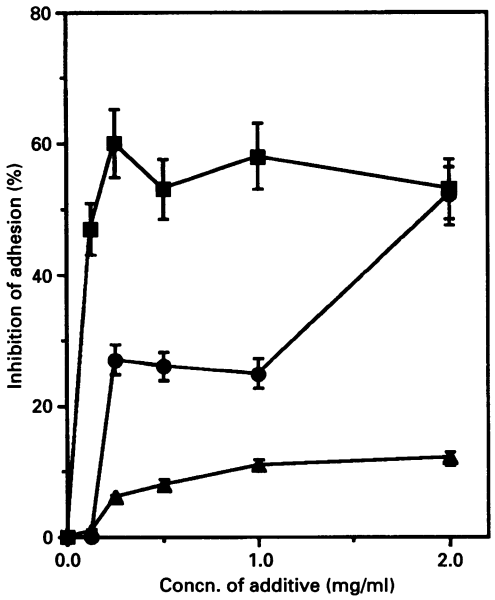


Figure 3 Titration of the inhibitory activity of peptide A and anti-(α2 integrin) mAb upon HUVEC adhesion to laminin 1

Values are the mean percentage inhibition of adhesion compared with control treatments, with standard errors of four replicates. Conditions as for Figure 1. Wells were coated with laminin 1 at 5 μg/ml. On the abscissa the 2 mg/ml concentration of peptides corresponds to a 1/50 dilution of anti-α2 ascites. Inhibition by: ●, peptide A; ▲, peptide E and ■, anti-α2 mAb.

inhibited adhesion to collagen IV and showed weak inhibition of adhesion to collagen I.

Peptides A, D and E at 1 mg/ml had no effect on the adhesion of HUVECs to fibronectin or vitronectin, whereas RGDS at the same concentration inhibited adhesion of HUVECs to vitronectin by 90% (results not shown). Similarly anti-(α2 integrin) mAb failed to inhibit HUVEC adhesion to fibronectin or vitronectin. These results demonstrate the ligand specificity of the inhibitory effects of peptide A and anti-(α2 integrin) mAb.

The additive effects of anti-(α2 integrin) mAb and peptide A on HUVEC adhesion to laminin 1 coated at 10 μg/ml were investigated. Peptides were used at 2 mg/ml and mAbs at 1/50 dilution of ascites. Peptide E and non-specific mouse IgG2a were taken as negative controls. Combination of these latter two does not inhibit HUVEC adhesion. Peptide A and anti-(α2 integrin) mAb significantly reduced HUVEC adhesion by 25 and 33% respectively in this experiment ($P < 0.05$). When the two inhibitors were combined a significant additive effect was observed (Student–Newman–Keuls' test, $P < 0.05$) resulting in 60% inhibition. This combined effect appeared to be simply additive rather than synergistic, suggesting that the two inhibitors were affecting a common adhesion mechanism rather than complementary ones. The effectiveness of peptide A and anti-(α2 integrin) mAb as inhibitors of HUVEC adhesion to laminin 1 was determined by titrating them in order to measure maximal levels of inhibition. The results are shown in Figure 3. The anti-(α2 integrin) mAb clearly saturates at an inhibition level well below 100%, indicating the presence of an additional adhesion mechanism to laminin 1 in the HUVECs over and above α2 integrin. This is in contrast to the inhibition observed with this mAb on collagen types I and IV (see Table 2) where inhibition approached 100%, indicating that on these substrates α2 integrin is the sole mechanism of HUVEC adhesion. It was impossible to test peptide A concentrations much above 2 mg/ml due to problems with solubility, but in most experiments the degree of inhibition on laminin 1 at this concentration matched the maximal inhibition observed with the anti-(α2 integrin) mAb (Figure 3, Table 2).

Table 3 Expression of integrins in HUVECs grown for 24 h on laminin 1 or collagen IV

Wells were coated with laminin at 20 μg/ml or collagen IV at 10 μg/ml as for adhesion experiments. HUVECs were seeded at 3×10^4 cells per well in complete growth medium. After 24 h incubation at 37 °C adherent cells were washed with PBS, fixed, solubilized and used in an ELISA to detect the presence of β1 integrins as described in the Experimental section. Entries are the mean ELISA absorbances at 405 nm ± S.E.M. of four replicates corrected for the appropriate controls. α2 mAb, α3 mAb, and α5 mAb were used at 1/500 dilution of ascites and α6 mAb at 2 μg/ml. Where peptide A was included it was incubated with the fixed cells for 1 h before addition of α2 mAb to 1/500. ND, not determined.

Integrin mAb	Laminin 1	Collagen IV
α2	0.79 ± 0.06	0.96 ± 0.05
α3	0.15 ± 0.03	0.08 ± 0.03
α5	0.35 ± 0.05	0.51 ± 0.03
α6	0.19 ± 0.02	0.31 ± 0.01
α2 + peptide A	0.85 ± 0.06	ND

Expression of integrins by HUVECS

The expression of specific integrins on the surface of HUVECs was investigated by a cell ELISA. The results are shown in Table 3. While an ELISA cannot strictly speaking be used for quantitative comparisons of different antibodies without reference to antigen standards, the absorbance endpoints shown in Table 3 were not increased by increasing the primary antibody concentrations above the levels shown in the Table. This indicates that the specific antibodies were of sufficiently high affinity to saturate the antigen at the antibody concentrations used and suggests that the results can be taken as roughly quantitative. An indication that this is the case is given by different relative ELISA absorbance profiles of these antibodies with other human cell types reported to be rich in expression of different members of the integrin family (results not shown). It is clear from Table 3 that HUVECs in culture express an abundance of α2 integrin and significant amounts of α5 (fibronectin receptor). The other laminin 1-binding integrins, α3 and α6, appeared to occur in lesser abundance but were nevertheless detectable. The presence of peptide A did not inhibit the binding of α2 mAb to laminin 1 (Table 3) (at this or lower concentrations of mAb), indicating that if peptide A binds to the α2 integrin on the fixed cells it does so at a different epitope location from the mAb. It is still possible for peptide A and the mAb to inhibit the same cell:laminin 1 binding mechanism even if they bind to the cell receptor at different locations.

DISCUSSION

The present work identifies a peptide sequence within the β1 chain of laminin 1 which inhibits the attachment of endothelial cells to both laminin 1 and type-IV collagen. This sequence (YGYYGDALR) shows homology to a peptide FYFDLR in type-IV collagen which is part of a larger sequence previously

described as having cell-attachment activity [32], mediated by $\alpha1\beta1$ and $\alpha2\beta1$ integrins. This is the first account of related peptide sequences from laminin and type-IV collagen which show reciprocal inhibition of cell adhesion to either ligand and which might form part of a common integrin-binding site.

Several α integrin types have been described which can bind to laminin 1. These include $\alpha6$, which is a relatively specific laminin receptor [33]. The presence of $\alpha6$ has been detected in some endothelial cells [34], but only in low amounts in HUVECs [35], which is in agreement with our findings. A second α integrin type which binds to laminin 1, $\alpha3$, has also been described to bind to collagens [1,36] and has been located on the surface of some endothelial cells [36], although at low levels on HUVECs [35], which also agrees with our present findings. The laminin-binding sites of each of these integrins has been mapped to the long arm of the laminin 1 molecule [11,36,37]. The $\alpha6$ integrin has also been implicated in the binding to the RGD site in the short arm of the $\alpha1$ chain [38]. Since RGD-containing peptides were completely ineffective in blocking endothelial cell adhesion to laminin 1 in the present study, $\alpha6$ integrin interaction with this site on laminin 1 in our assay system is unlikely. Given the large distance between the major binding locations of $\alpha3$ and $\alpha6$ (α chain long arm) and peptide A on the laminin 1 molecule (see Figure 2), it is unlikely that peptide A is inhibiting interaction with these integrins. Two further integrin α types have been described which can bind to both laminin 1 and collagens, $\alpha1$ and $\alpha2$ [17,39–41]. There is ample evidence in the literature for abundant expression of $\alpha2$ integrin on HUVECs in culture, and for a major role of this integrin in attachment of these cells to laminin 1 and collagen [17,35,40,42,43]. In contrast , while $\alpha1$ integrins have been detected in endothelial cells in tissues, they do not appear to be expressed in cultured cells [34]. Antibodies to $\alpha1$ integrins were not available to us so we could not formally test for the presence of this integrin but in the light of these reports its presence on our HUVECs appears to be unlikely.

Our finding that anti-$\alpha2$ mAb inhibited the binding of HUVECs to collagen types IV and I at levels approaching 100% suggests that $\alpha2$ integrin was the only cellular receptor responsible for adhesion to these ligands. The collagen IV peptide, FYFDLR, forms the C-terminus of the cyanogen bromide-cleaved fragment CB3 of collagen IV which has also been reported to contain all the cell-binding activity of HT1080 cells [32]. Both $\alpha1\beta1$ and $\alpha2\beta1$ integrins have been isolated from these cells by affinity chromatography on the CB3 fragment. These authors demonstrated that full cell-adhesive capacity of this fragment required both an N-terminal portion included between residues 376 and 433 and a C-terminal portion included between residues 517 and 558. The last six residues of this C-terminal portion are FYFDLR. More recent work [41] has identified the C-terminal portion of the CB3 fragment as a minor binding site for platelet integrin $\alpha2\beta1$. This portion of the CB3 fragment is not required for placental $\alpha1\beta1$ binding. Other evidence suggests that the CB3 fragment is the only fragment of collagen IV with an $\alpha2\beta1$-binding site [44]. A 13-amino-acid sequence from CB3, containing FYFDLR, has been described which can induce cell adhesion in a number of cell types [10], but the integrin(s) involved were not investigated. In the present study FYFDLR was effective in inhibiting BCE cell adhesion to collagen types IV and I and a little less effective on laminin 1. The structurally homologous laminin $\beta1$ peptide A was effective in inhibiting HUVEC adhesion to laminin 1 and collagen IV and only weakly effective on collagen I. These results suggest that similar peptides may be capable of inhibiting the binding of one integrin type to a number of different ligands, but also that different particular peptide sequences may be optimal in each different ligand for binding the same integrin. There is no sequence close to FYFDLR in collagen type I. The closest sequence, found in the $\alpha1$ N-telopeptide, YGFDEK, replaces the penultimate hydrophobic residue with an acidic one, and was entirely without effect on cell adhesion. A sequence important in adhesion of platelets and breast carcinoma cells to collagen type I has been identified as DGEA, from the cyanogen bromide-cleaved fragment CB3 of collagen I, and binding of the $\alpha2\beta1$ integrin to the fragment has been reported to be inhibited by this peptide [9]. Type-III collagen has a similar sequence, DGES [41]. Sequences of this type are not found in collagen IV or laminin 1, yet the peptide DGEA was reported to inhibit $\alpha2\beta1$-mediated breast carcinoma cell adhesion to both collagen I and laminin 1 [9].

The $\alpha1\beta2$ integrin has some very interesting properties in that when expressed in some cell types it binds exclusively to collagen, while in other cell types it will bind to both collagen and laminin 1, even though the integrin derived from these different cell types appears to be biochemically and immunologically identical [39,40]. A recent elegant study [45] has demonstrated that transfection of different cell types with the same $\alpha2$ integrin cDNA can yield different binding specificities. These could be further altered by the matrix environment of the cell or by binding antibody to the integrin $\beta1$ chain. These authors concluded that ligand specificity of $\alpha2$ integrin was not controlled at the DNA level, but by the cellular environment and binding events causing conformational changes in the integrin. The results we have reported, together with discussions in the literature on the features of $\alpha2$ integrin binding, suggest that this integrin has a broad spectrum of ligand structures to which it can bind; the specificity of binding depending on the conformational presentation of both integrin and ligand. We propose that the peptide FYFDLR participates in $\alpha2\beta1$ binding to collagen IV and that the putatively structurally similar peptide YGYYGDALR participates in $\alpha2\beta1$ binding to laminin 1. The cell-adhesion inhibitory activity of both these peptides was specific but of relatively low affinity, requiring high concentrations for maximal activity, and neither peptide was sufficient to support HUVEC adhesion when conjugated to coated albumin. Study of the activities of extended peptides, combinations with laminin peptides in the vicinity of peptide A and specific elution of $\alpha2\beta1$ integrin from laminin 1 columns was beyond the scope of this work but should yield more information on the mechanism of endothelial cell $\alpha2\beta1$ integrin binding to laminin. A recent report [46] has mapped the binding site of the $\alpha2\beta1$ integrin (from platelets/placenta) to laminin 1 fragment E1XNd, in the cross region of the molecule, consistent with the location of peptide A. Although the evidence presented suggested that the $\alpha1$ chain portion of this fragment was essential for integrin binding, the E1XNd fragment also contains peptide A, and contribution of the $\beta1$ chain was not determined.

The inhibition of endothelial cell adhesion to collagen types I and IV by anti-$\alpha2$ mAb was complete; however, inhibition of adhesion to laminin 1 did not exceed 70%, suggesting contribution of a second adhesion receptor for laminin in these cells. This was unlikely to be the 67 kDa receptor described to bind to the peptide YIGSR [47], as in our hands this peptide was ineffective as an inhibitor of endothelial cell adhesion to laminin 1. The additional contribution to endothelial cell adhesion to laminin could be provided by either $\alpha3$ or $\alpha6$ integrins as has been suggested by others [35]. Our demonstration of the expression of these integrins in HUVECs, although at low levels, supports this suggestion.

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REFERENCES

1 Akiyama, S. K., Nagata, K. and Yamada, K. M. (1990) Biochim. Biophys. Acta **1031**, 91–110
2 Ruoslahti, E. (1991) in Cell Biology of Extracellular Matrix, 2nd edn. (Hay, E. D., ed.), pp. 343–363, Plenum Publishing Corp, NY
3 Pierschbacher, M. D. and Ruoslahti, E. (1984) Nature (London) **309**, 30–33
4 Ruoslahti, E. and Pierschbacher, M. D. (1986) Cell **44**, 517–518
5 Graf, J., Iwamoto, Y., Sasaki, M., Martin, G. R., Kleinman, H. K., Robey, F. A. and Yamada, Y. (1987) Cell **48**, 989–996
6 Grant, D. S., Tashiro, K., Segui-Real, B., Yamada, Y., Martin, G. R. and Kleinman, H. K. (1989) Cell **58**, 933–943
7 Skubitz, A. P. N., Letourneau, P. C., Wayner, E. and Furcht, L. T. (1991) J. Cell Biol. **115**, 1137–1148
8 Dedhar, S., Ruoslahti, E. and Pierschbacher, M. D. (1987) J. Cell Biol. **104**, 585–593
9 Staatz, W. D., Fok, K. F., Zutter, M. M., Adams, S. P., Rodriguez, B. A. and Santoro, S. A. (1991) J. Biol. Chem. **266**, 7363–7367
10 Koliakos, G. G., Kouzi-Koliakos, K., Furcht, L. T., Reger, L. A. and Tsilibary, E. C. (1989) J. Biol. Chem. **264**, 2313–2323
11 Sonnenberg, A., Linders, C. J. T., Modderman, P. W., Damsky, C. H., Aumailley, M. and Timpl, R. (1990) J. Cell Biol. **110**, 2145–2155
12 Davis, G. E. (1992) Biochem. Biophys. Res. Commun. **182**, 1025–1031
13 Yamada, K. M. (1991) J. Biol. Chem. **266**, 12809–12812
14 Burgeson, R. E., Chiquet, M., Deutzmann, R., et al. (1994) Matrix Biol. **14**, 209–211
15 Ruoslhati, E., Hayman, E. G., Pierschbacher, M. and Engvall, E. (1982) Methods Enzymol. **82**, 803–831
16 Underwood, P. A. and Bennett, F. A. (1989) J. Cell Sci. **93**, 641–649
17 O'Connell, P. J., Faull, R., Russ, G. R. and D'Apice, A. J. F. (1991) Immunol. Cell Biol. **69**, 103–110
18 Widmer, F., Bayne, S., Houen, G., Moss, B. A., Rigby, R. D., Whittaker, R. G. and Johansen, J. T. (1985) in Peptides 1984 (Ragnarsson, U., ed.), pp. 193–200, Almquist & Wiksell, Stockholm
19 Konig, W. and Geiger, R. (1970) Chem. Berichte **103**, 788–798
20 Gray, A., Dull, T. J. and Ullrich, A. (1983) Nature (London) **303**, 722–725
21 Brazel, D., Pollner, R., Oberbaumer, I. and Kuhn, K. (1988) Eur. J. Biochem. **172**, 35–42
22 Glanville, R. W., Breitkreutz, D., Meitinger, M. and Fietzek, P. P. (1983) Biochem. J. **215**, 183–189
23 Sasaki, M., Kato, S., Kohno, K., Martin, G. R. and Yamada, Y. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 935–939
24 Nissinen, M., Vuolteenaho, R., Boothandford, R., Kallunki, P. and Tryggvason, K. (1991) Biochem. J. **276**, 369–379
25 Price, M. R., Sekowski, M., Hooi, D. S. W., Durrant, L. G., Hudecz, F. and Tendler, S. J. B. (1993) J. Immunol. Methods **159**, 227–281
26 McAuslan, B. R., Reilly, W. and Hannan, G. N. (1979) J. Cell. Physiol. **100**, 87–94
27 Jaffe, E. A., Nachman, R. L., Becker, C. G. and Minick, C. R. (1973) J. Clin. Invest. **52**, 2745–2756
28 Maciag, T., Cerundolo, J., Ilsley, S., Kelly, P. R. and Forand, R. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 5674–5678
29 Underwood, P. A., Steele, J. G., Dalton, B. A. and Bennett, F. A. (1990) J. Immunol. Methods **127**, 91–101
30 Oliver, M. H., Harrison, N. K., Bishop, J. E., Cole, P. J. and Laurent, G. J. (1989) J. Cell Sci. **92**, 513–518
31 McCarthy, J. B., Skubitz, A. P. N., Zhao, Q., Yi, X. Y., Mickelson, D. J., Klein, D. J. and Furcht, L. T. (1990) J. Cell Biol. **110**, 777–787
32 Vandenberg, P., Kern, A., Ries, A., Luckenbilledds, L., Mann, K. and Kuhn, K. (1991) J. Cell Biol. **113**, 1475–1483
33 Aumailley, M., Timpl, R. and Sonnenberg, A. (1990) Exp. Cell. Res. **188**, 55–60
34 Albelda, S. M. and Buck, C. A. (1990) FASEB J. **4**, 2868–2880
35 Languino, L. R., Gehlsen, K. R., Wayner, E., Carter, W. G., Engvall, E. and Ruoslahti, E. (1989) J. Cell Biol. **109**, 2455–2462
36 Gehlsen, K. R., Dickerson, K., Argraves, W. S., Engvall, E. and Ruoslahti, E. (1989) J. Biol. Chem. **264**, 19034–19038
37 Gehlsen, K. R., Sriramarao, P., Furcht, L. T. and Skubitz, A. P. N. (1992) J. Cell Biol. **117**, 449–459
38 Tashiro, K., Sephel, G. C., Greatorex, D., Sasaki, M., Shirashi, N., Martin, G. R., Kleinman, H. K. and Yamada, Y. (1991) J. Cell. Physiol. **146**, 451–459
39 Elices, M. J. and Hemler, M. E. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 9906–9910
40 Kirchhofer, D., Languino, L. R., Ruoslahti, E. and Pierschbacher, M. D. (1990) J. Biol. Chem. **265**, 615–618
41 Kern, A., Eble, J., Golbik, R. and Kuhn, K. (1993) Eur. J. Biochem. **215**, 151–159
42 Giltay, J. C., Brinkman, H. M., Modderman, P. W., von dem Borne, A. E. G. and Mourik, J. A., v. (1989) Blood **73**, 1235–1241
43 Lampugnani, M. G., Resnati, M., Dejana, E. and Marchisio, P. C. (1991) J. Cell Biol. **112**, 479–490
44 Gullberg, D., Gehlsen, K. R., Turner, D. C., Ahlen, K., Zijenah, L. S., Barnes, M. J. and Rubin, K. (1992) EMBO J. **11**, 3865–3873
45 Chan, B. M. C. and Hemler, M. E. (1993) J. Cell Biol. **120**, 537–543
46 Pfaff, M., Gohring, W., Brown, J. C. and Timpl, R. (1994) Eur. J. Biochem. **225**, 975–984
47 Graf, J., Ogle, R. C., Robey, F. A., Sasaki, M., Martin, G. R., Yamada, Y. and Kleinman, H. K. (1987) Biochemistry **26**, 6896–6900

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