

# Identification of the Arg-Gly-Asp sequence in laminin A chain as a latent cell-binding site being exposed in fragment P1

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A single RGD-containing sequence present within an epidermal growth factor-like repeat of the short arms of laminin is shown by peptide inhibition to block integrin receptors recognizing a latent cell-binding site of laminin. Based on proteolysis data it is proposed that masking occurs by folding of the globular domain IVa over the cell-binding site in the adjacent rod-like structures of laminin A chain.

Basement membrane; Cell attachment; Integrin; Proteolytic fragment; Synthetic peptide

## 1. INTRODUCTION

Fragment P1 generated from laminin by pepsin treatment was the first domain of the protein shown to be involved in cellular interactions [1,2]. Various cells have been found which bind fragment P1 with high affinity ( $K_D \sim 1\text{--}4\text{ nM}$ ) to an apparently homogenous class of receptors [3–5]. It was later shown that this binding site is latent both within laminin and within a larger P1-related fragment produced by cleavage with elastase [6]. The P1 fragment (about 200 kDa) comprises the inner rod-like structure of the short arms of laminin and contains some 28 EGF-like repeats contributed by the A, B1 and B2 chains of laminin [7–9]. Its A chain segment also possesses the only RGD (Arg-Gly-Asp) sequence present in mouse laminin [9]. Similar sequences are involved in cell binding to several other proteins including fibronectin, vitronectin and collagens [10]. Here, we show by peptide and antibody inhibition and by proteolytic inactivation that the laminin RGD sequence very likely corresponds to the latent cell-binding site identified previously [6], and is recognized by cellular receptors of the integrin family.

## 2. EXPERIMENTAL

The mouse EHS tumor laminin-nidogen complex [11] and its large tryptic fragment T1 [12] were prepared as previously described. Both

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*Abbreviations:* EGF, epidermal growth factor; EHS, Engelbreth-Holm-Swarm tumor; HPLC, high performance liquid chromatography; TLCK, tosyl lysyl chloromethane

components were cleaved in 0.1 M glycine-HCl, pH 1.9 with pepsin (24 h, 25°C, enzyme-substrate ratio 1:100) to generate fragment P1 which was then purified on a molecular sieve [12]. Some small peptides in the pepsin digest were bound to heparin-Sepharose (Pharmacia) equilibrated in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.4 and eluted with 0.5 M NaCl. This material was separated into 25 distinct peaks by reversed phase HPLC and individual peptides were identified by Edman degradation [13]. Trypsin inactivation of P1 some synthetic peptides was performed at 37°C in 0.2 M  $\text{NH}_4\text{HCO}_3$  (enzyme-substrate ratio 1:100) and the reaction was stopped by adding TLCK. Laminin fragment P1 was also isolated from human placenta [14]. Human plasma fibronectin (Behringwerke) was further purified on heparin-Sepharose. Synthetic peptides GRGDS (Promega) and RGES (Peninsula) were from commercial sources. Other RGD-containing peptides (see fig. 1) were synthesized according to sequences of mouse [9] and human [15] laminin A chain, mouse laminin B1 chain [16] and mouse nidogen [17] and kindly supplied by Drs J. Knolle, S. Henke (Hoechst AG) and W. Stüber (Behringwerke). They were purified by HPLC and their structure was confirmed by Edman degradation.

Cell attachment was determined in microtiter wells coated with optimal substrate concentrations and adherent cells were analyzed after staining with Crystal violet by a colorimetric assay [18]. In the inhibition assays cells ( $1\text{--}5 \times 10^5/\text{ml}$ ) were mixed with synthetic peptides ( $1\text{--}500\text{ }\mu\text{g}/\text{ml}$ ) or various antibody dilutions and plated for 30 min at 37°C on coated microtiter wells. Cultured cell lines are identified in table 1 and were those used in previous studies [5,18,19]. A monoclonal rat antibody against the  $\alpha 6$  subunit of integrin VLA-6 [20] and a rabbit antiserum against human fibronectin receptor [21] were used for inhibition. The latter was a kind gift from Dr G. Tarone (University of Torino).

## 3. RESULTS AND DISCUSSION

Examination of the adhesion of ten different cell lines (table 1) to laminin fragment P1 showed typical dose-response profiles [5,18] with 30–80% of the cells bound in the plateau region. Comparable attachment profiles were observed for fibronectin and, with two exceptions (A375, SCII), for the laminin-nidogen com-

Table 1

Inhibitory capacity ( $IC_{50}$  = 50% inhibition) of GRGDS peptide for cell adhesion on laminin fragment P1 and fibronectin (Fn)

Cell lines		$IC_{50}$ ( $\mu$ g/ml) on	
		P1	Fn
HT1080, fibrosarcoma	H	< 10	500
SAOS-2, osteosarcoma	H	< 10	> 500
A375, melanoma	H	< 10	nt
HBL100, mammary epithelia	H	< 10	> 500
RN22, Schwannoma	R	< 10	> 500
B16F10, melanoma	M	< 10	nt
A431, epidermoid	H	100	200
SCII, epithelial Ca	H	> 500	nt
SCII, epithelial Ca	H	320	nt
Rugli, glioblastoma	R	50	250

H, human; R, rat; M, mouse; Ca, carcinoma; nt, not tested

plex. Three more teratocarcinoma cell lines (mouse F9, PYS-2, human Tera-2) showed strong attachment on fibronectin but none on fragment P1 substrates. This indicates the unique nature of the P1 substrate whose cellular recognition is not necessarily correlated to that of laminin and fibronectin.

Adhesion of six cell lines to fragment P1 could be clearly inhibited by less than 10  $\mu$ g/ml (20  $\mu$ M) required for 50% inhibition (fig.1a) of synthetic GRGDS peptide (table 1) which corresponds to fibronectin cell-binding sequence [10]. About 20–50 times higher peptide concentrations were needed to achieve this inhibition for the other four cell lines. This low sensitivity for GRGDS inhibition was generally observed when the same cells were exposed to fibronectin substrates (table 1). Peptide GRGDS (10–500  $\mu$ g/ml) was completely inactive in inhibiting adhesion of HT1080, SAOS-2 and B16F10 cells to laminin or laminin-nidogen (data not shown). This is in agreement with the previous observation [6] that the P1 binding site is not recognized by HT1080 cells in intact laminin.

Specificity of peptide inhibition of cell adhesion to fragment P1 was studied by using the variant RGEs structure which was inactive up to 500  $\mu$ g/ml for HT1080 (fig.1a), B16F10 and SAOS-2 cells. Similar observations have been reported for fibronectin [22], collagen type VI [18] and several other cell adhesion substrates. Also, no inhibitory activity for HT1080 (fig.1a) and A375 cells was found for the synthetic CDPGYIGSR-amide peptide which was designed according to a B1 chain sequence [16] present in fragment P1. This peptide has been reported [23] to block cell adhesion to laminin by binding to a 68 kDa receptor.

The RGD-containing sequence of laminin A chain [9] was synthesized as the 18-mer oligopeptide CQAGTF-ALRGDNPQGCSP-amide and used in cyclic, disulfide-linked and linear, alkylated forms for inhibition. Both peptides were of equivalent inhibitory capacity when compared with GRGDS on a molar basis for cells

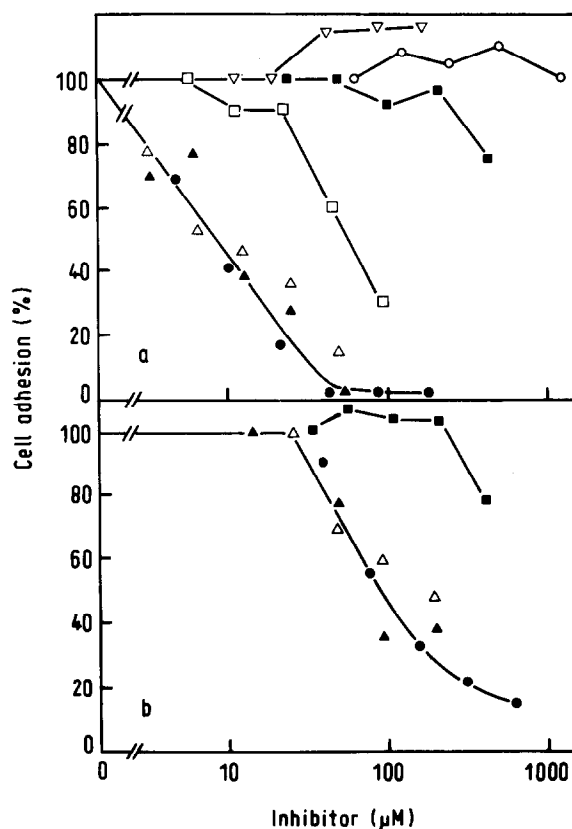


Fig.1. Peptide inhibition of attachment of HT1080 cells (a) and of A431 cells (b) on a laminin fragment P1 substrate. Inhibitors used were GRGDS (●), RGEs (○), CQAGTFALRGDNPQGCSP-amide corresponding to a mouse laminin A chain sequence which was used either in cyclic, disulfide-linked (△) or linear, S-carboxyamided from (▲), GDVEKRGDREEA from a human laminin A chain sequence (■) SIGFRGDGQT from a mouse nidogen sequence (□), and CDPGYIGSR-amide (▽) from a mouse laminin B1 chain sequence.

highly sensitive to peptide inhibition as shown for HT-1080 cells (fig.1a). Similar studies with cells where GRGDS is of low inhibitor potency against P1 also showed no better inhibitory activity for the authentic laminin peptides (fig.1b). This indicates a small binding sequence, unlike that indicated by observations with fibronectin [22,24] where larger peptides were the better inhibitors. It also indicates that the disulfide-bonded loop structure supposed to exist in the EGF-like repeats of laminin [25] is not important for activity. Another RGD sequence is found in the C-terminal globule of human laminin A chain [15] which is changed in mouse laminin [9] to AGG. A synthetic peptide corresponding to the human sequence GDVEKRGDREEA was found to be of very low inhibitory potency for cell adhesion to fragment P1 (fig.1). A somewhat better activity was observed for the peptide SIGFRGDGQT which corresponds to a sequence in mouse nidogen possibly involved in cell adhesion [17]. This suggests that amino acid residues adjacent to the RGD sequence determine its inhibitory activity in the P1 adhesion assay.

Whether the active RGD sequence as shown here is conserved in laminin A chain of other species or in isoforms [8] of the chain is so far not known. We have therefore used laminin fragment P1 prepared from pepsin digests of human placenta [14] in cell adhesion assays. Four cell lines (A375, A431, HBL-100, SAOS-2) showed attachment up to 60–100% of the plateau levels achieved with mouse laminin P1. The attachment of A375 cells to human laminin P1 could be inhibited by GRGDS with a similar sensitivity when compared to a mouse laminin P1 substrate (data not shown). It indicates conservation of this particular RGD sequence in laminin of human and possibly other species.

The latent nature of the P1 cell-binding site was previously shown in radioligand assays with HT-1080 cells [6]. We now examined the general nature of this masking effect by using a tryptic laminin fragment T1 which is larger than fragment P1 [12] and was an inactive adhesion substrate for the cell lines listed in table 1. Progressive pepsin treatment of fragment T1 led to increased attachment activity reaching eventually the levels of authentic P1 (fig.2a). After exposure by pepsin treatment this RGD sequence becomes sensitive to trypsin, which reduces cell attachment activity to negligible levels (fig.2b). In agreement with this, trypsin treatment for 4 h reduced the inhibitory capacity of the cyclic 18-mer RGD-peptide of laminin (see above) to less than 5% (not shown).

A structural explanation of these observations is proposed in fig.3, indicating that the EGF-like repeats present in the rod-like domains IIIa and IIIb in laminin A chain are connected to each other by a disulfide bond despite their separation in the primary sequence [9] by a 196-residue globular domain IVa, lacking cysteine residues. This would predict folding of IVa over adjacent portions of IIIa/IIIb, making the RGD sequence in intact laminin and fragment T1 inaccessible to cellular receptors and trypsin. There are several facts supporting this prediction. The EGF-like repeats of IIIa/IIIb bordering the IVa sequence are both incomplete, but nevertheless complement each other to a full length repeat with 8 cysteine residues which should [25] bring domains IIIa and IIIb in close proximity (fig.3). Unmasking of the RGD could then occur by extensive cleavage within domain IVa by pepsin without separating the link between domains IIIa and IIIb. This is supported by our sequence analysis of various small peptides obtained from pepsin digests of laminin-nidogen identifying two peptides with either 12 or 13 residues which both share the N-terminal sequence IKGG starting at position 1243 in domain IVa (fig.3). It has been also observed that fragment T1 or similar adhesion-inactive fragments still possess globular domains of the short arms [22] which are no longer visible by electron microscopy after pepsin treatment [7,26]. Yet, the rods in fragment P1 still have a length of

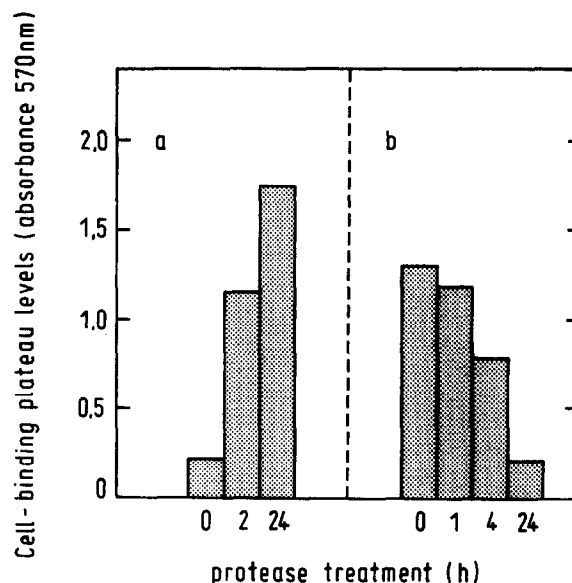


Fig.2. Activation (a) and inactivation (b) of the cell-binding structure in laminin fragment P1 by protease treatment for the indicated periods of time. In (a) the tryptic laminin fragment T1 was treated with pepsin and in (b) the pepsin fragment P1 was treated with trypsin. Cell-binding properties of starting materials (0 h) and of digested materials were then determined with A375 cells by constructing dose-response attachment profiles using a colorimetric assay [18]. Plateau levels of attachment are indicated by the bars.

26–37 nm while a 10–12 nm long rod [9,25] would have to be observed if pepsin cleavage separates domains IIIa and IIIb.

Recognition of RGD sequences in some adhesive proteins is due to cellular receptors of the integrin type [10,27]. To examine this possibility for the P1 substrate we used an antiserum against human fibronectin recep-

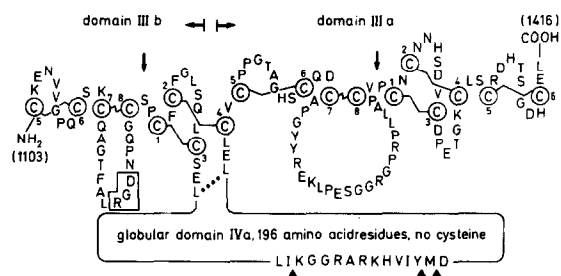


Fig.3. Model of the possible disulfide bond junction between two incomplete EGF-like repeats in domains IIIa and IIIb of mouse laminin A chain to explain masking of the RGD cell-binding site by folding of the inserted domain IVa over adjacent regions in IIIa and IIIb. The model shows part of the amino acid sequence between positions 1103–1416 of the A chain [9] with cysteine encircled. Numbering of the eight cysteines and disulfide bonding pattern (C1–C3, C2–C4, C5–C6, C7–C8) is indicated as predicted [25] from the similarity to EGF. Borders of the predicted EGF-like repeat contributed by IIIa and IIIb are marked by arrows. A normal size of the loop in the postulated EGF-like repeat actually occupied by domain IVa is indicated by dots. The RGD sequence is boxed. Arrowheads in the domain IVa sequence mark identified pepsin cleavage sites.

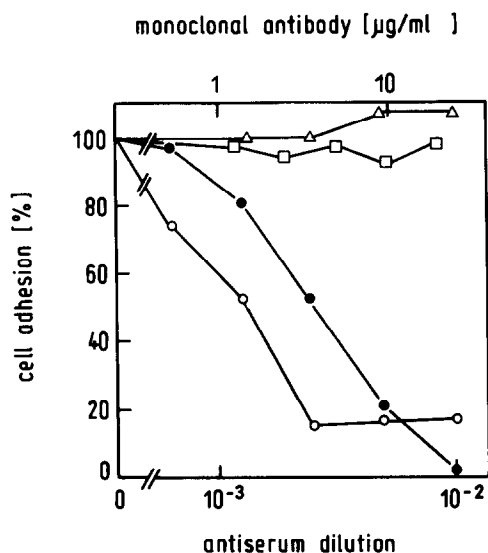


Fig.4. Inhibition of attachment of HT1080 cells (full symbols) or A431 cells (open symbols) on a fragment P1 substrate by anti-receptor antibodies. Inhibitors were rabbit antiserum against human fibronectin receptor (○,●), a purified rat monoclonal antibody against the  $\alpha 6$  subunit of integrin VLA-6 (□) and for control normal rabbit serum (Δ).

tor [21] which was previously shown to react with both its  $\beta 1$  and  $\alpha 5$  subunits. This antiserum showed complete and specific blocking of P1 adhesion of two cell lines (fig.4) which differ considerably in their sensitivity to inhibition by GRGDS peptide (table 1). A monoclonal antibody (GoH3) specific for the  $\alpha 6$  subunit of integrins was previously found to inhibit platelet adhesion to laminin [20]. This antibody was incapable of preventing cell adhesion to laminin fragment P1 (fig.4) when tested in a concentration range completely blocking cell adhesion of the same HT1080 cells to laminin or its major cell-binding fragment E8 [19]. These data indicate that cell adhesion to fragment P1 is mediated by integrin receptors but since the  $\beta 1$  subunit is shared by several integrins [10,27] it does not suggest the exclusive involvement of the fibronectin receptor.

Our data provide evidence that the single RGD sequence present in the A chain component of laminin fragment P1 is involved in a latent cell-binding site with binding activity for integrin receptors. While apparently recognized by a large variety of cells it may not represent the only cell-binding site in P1 since the sequence YIGSR was also found to be active [23] and to prevent metastasis [28]. Mitogenic activity [29] and stimulation of pheochromocytoma PC12 cells (G. Tarone, personal communication) shared by laminin and its fragment P1 are insensitive to RGD and YIGSR peptide inhibition and therefore appear not to be masked indicating further independent cell-binding sites. The latent nature of the RGD binding site in fragment P1 raises the question on its biological relevance, which would necessitate proteolytic activation in situ, for example, during base-

ment membrane remodelling. Proteolysis is a common mechanism in interacting protein systems but usually does not involve proteases such as pepsin working at acidic pH. Recent data ([30,31] and our unpublished results) with endothelial cells interacting with laminin substrates at neutral pH show interference by RGD peptides suggesting that these cells may possess a functional activation mechanism. The elucidation of such mechanisms will be crucial in understanding the role of this cell adhesion reaction.

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