Covalently Immobilized Laminin Peptide Tyr-Ile-Gly-Ser-Arg (YIGSR) Supports Cell Spreading and Co-localization of the 67-Kilodalton Laminin Receptor with α-Actinin and Vinculin*

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Stephen P. Massia†, Sekhar S. Rao‡, and Jeffrey A. Hubbell††
From the †Departments of Chemical Engineering, ‡Mechanical Engineering, and the ††Division of Biological Sciences, University of Texas, Austin, Texas 78712-1062

The laminin-based nonapeptide Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg (CDPGYIGSR) and pentapeptide Tyr-Ile-Gly-Ser-Arg (YIGSR) have been previously demonstrated to support the attachment of several cell types and to competitively bind to the 67-kDa high affinity laminin receptor. Cell attachment, but not spreading, on substrates containing adsorbed CDPGYIGSR or YIGSR was observed. In this report we describe YIGSR-mediated attachment and spreading of a wide variety of cell types. YIGSR-promoted cell spreading and stress fiber formation when it was covalently immobilized into the amino-terminal Gly residue, used as a spacer arm. Spreading was not observed when adsorbed YIGSR peptide was used. Functionally blocking antisera directed against the 67-kDa and related laminin-binding proteins blocked human foreskin fibroblast (HFF) spreading, but not attachment, on covalently grafted YIGSRY substrates. However, functionally blocking antisera directed against the vitronectin receptor, integrin αβ3, and the fibronectin receptor, integrin α5β1, did not affect HFF spreading on these substrates. When HFFs spread on these substrates, the 67-kDa laminin receptor co-localized with the cytoplasmic proteins α-actinin and vinculin into discrete structures. These results suggest that the adhesion ligand YIGSR is solely sufficient for cell spreading when it is conformationally constrained by covalent attachment to a solid substrate, at least when attached via its amino terminus. Furthermore, the role of the 67-kDa laminin receptor in recognition of this ligand and mediating cell attachment is confirmed in this study. This report also provides the first evidence for direct or indirect association of this receptor with vinculin and α-actinin when YIGSR-mediated cell spreading occurs.

Laminin is the major noncollagenous glycoprotein component of basement membranes and is a mediator of cell adhesion, migration, growth, and differentiation (Timpl et al., 1983; Kleinman et al., 1985; Martin and Timpl, 1987; Beck et al., 1990). Engelbreth-Holm-Swarm tumor-derived laminin, the best characterized form of laminin, \(M_r = 800,000\) is composed of three chains, A \(M_r = 400,000\), B1 \(M_r = 210,000\), and B2 \(M_r = 200,000\) which are covalently linked by disulfide bonds to form a cross-like structure (Engel et al., 1981; Cooper et al., 1981; Barlow et al., 1984; Palm et al., 1985; Paulsson 1985; Sasaki et al., 1988). Laminin derived from other tissues and cells have been shown to have distinctly different polypeptide chains, chain compositions, and structures. Although these laminin isoforms have been studied in less detail, such findings suggest that Engelbreth-Holm-Swarm tumor laminin is just one member of a family of related proteins (Beck et al., 1990).

Research efforts focusing on cloning and sequencing of the three laminin chains have led to investigations that define functionally active regions within the laminin molecule (Barlow et al., 1984; Sasaki et al., 1987; Sasaki and Yamada, 1987; Pikkarainen et al., 1987, 1988; Montell and Goodman, 1988). These bio logically active sites within laminin have been defined with proteolytic fragments, antibodies, and synthetic peptides. Several synthetic peptides based on laminin sequences have been described as having biological activities similar to those of the whole laminin molecule. A nonapeptide, Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg (CDPGYIGSR), as well as the pentapeptide Tyr-Ile-Gly-Ser-Arg (YIGSR), from the B chain were shown to promote cell attachment and migration (Graf et al., 1987a, 1987b). Other laminin-based peptides that have biological activity include an Arg-Gly-Asp (RGD) sequence from the short arm of the A chain (Grant et al., 1989; Tashiro et al., 1991) and the Ile-Lys-Val-Ala-Val (IKVAV)-containing peptide from the long arm of the A chain (Tashiro et al., 1989; Sephe et al., 1989). A 20-mer peptide (S9) from the B1 chain was reported to promote cell attachment and bind heparin (Charonis et al., 1988). Besides promoting cell attachment and migration, YIGSR-containing peptides were reported to block angiogenesis and tumor metastases (Grant et al., 1989; Iwamoto et al., 1987; Sakamoto et al., 1991). Additionally, IKVAV-containing peptides were observed to increase neurite outgrowth, tumor metastases, and collagenase production (Kanemoto et al., 1990).

Since laminin has multiple cell adhesion domains, it is not surprising that many laminin-binding proteins have been isolated and described (Mercurio and Shaw, 1991; Mecham, 1991a, 1991b). The first laminin-binding proteins were isolated from tumor cell extracts by affinity chromatography on laminin-Sepharose. A 67-kDa laminin-binding protein \(M_r = 67,000-70,000\) retained a high affinity for laminin after purification (Rao et al., 1983; Malinoff and Wicha, 1983). A wide variety of cell types has been reported to express immunologically related laminin-binding proteins within this size range, including muscle cells (Lesot et al., 1985), macrophages (Huard et al., 1986), neutrophils (Yoon et al., 1987), endothelial cells (Yannariello-Brown et al., 1988), epithelial cells

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† To whom correspondence and reprint requests should be addressed.
plasmic proteins such as vinculin and talin (Burridge, 1986; Tandon et al., 1991). Supporting evidence for the 67-kDa protein functioning as a laminin adhesion receptor lies in its ability to bind to YIGSR-containing adhesion-promoting sequences from the laminin B1 chain with high affinity (Graf et al., 1987a, 1987b). Furthermore, it was demonstrated that the 67-kDa protein is a membrane-associated protein that interacts with the cytoskeleton (Brown et al., 1983, Cody and Wicha, 1986). Other studies have raised questions regarding the identity of the 67-kDa protein as a membrane-bound laminin receptor. Specifically, full-length cDNA clones for this receptor encodes a much smaller protein (32 kDa) that does not contain a membrane-spanning domain (Wewer et al., 1986; Yow et al., 1988). Antisera to a fusion protein, which contains a sequence from the full-length clone for the 32-kDa protein, reacts with the 32-kDa, the 67-kDa, and a 45-kDa protein (Mercurio and Shaw, 1988; Clément et al., 1990). Laminin-binding proteins ranging from 32-36 kDa were reported to actively promote laminin-mediated cell attachment and spreading, suggesting that this group of proteins function as cell surface receptors as well as the 67-kDa proteins (Clément et al., 1990; Woo et al., 1990; Davis et al., 1991). Other reports suggest that the 32-36-kDa proteins are precursors for the 67-kDa class of receptors (Lazar et al., 1988; Castrejón et al., 1991).

Laminin adhesion receptors, other than the 67-kDa and related laminin-binding proteins, include several members of the integrin superfamily, namely α5β1 (Hall et al., 1990), α5β3 (Lengutino et al., 1989; Elices and Hemler, 1989; Lotz et al., 1990), α6β3 (Gehlsen et al., 1989), α6β8 (Sonnenberg et al., 1990; Shaw et al., 1990), αβ1 (Kramer et al., 1989), αβ4 (Sonnenberg et al., 1990), and α6β3 (van Kuppevelt et al., 1989; Kramer et al., 1990). Typically, integrins bind to cell adhesion proteins via RGD and related sequences; however, integrin binding to laminin is generally not RGD-dependent (Mecham, 1991a, 1991b). Another important class of laminin-binding cell adhesion receptors is cell surface, carbohydrate-binding proteins (Mercurio and Shaw, 1991; Shur, 1989).

Receptor-mediated cell adhesion and spreading results in the formation of discrete regions of close apposition and tight adhesion of the cell plasma membrane to the substrate (Abercrombie and Dunn, 1975; Izzard and Lochner, 1976, 1980). These discrete regions, known as focal contacts or adhesion plaques, are formed by clustered membrane-spanning adhesion receptors (typically integrins), which are extracellularly bound to adhesion proteins and internally bound to cytoplasmic proteins such as vinculin and talin (Burridge, 1986; Burridge et al., 1988; Burridge and Fath, 1989). Talin, vinculin, α-actinin, and other cytoplasmic proteins have been demonstrated by immunofluorescent staining to co-localize with integrins in mature focal contacts of spread cells (Geiger, 1979; Wehland et al., 1979; Burridge and Connell, 1983a, 1983b). Furthermore, actin microfilament bundles have been shown to terminate into mature focal contacts (Abercrombie et al., 1971; Heath and Dunn, 1978). F-actin cytoskeletal elements, e.g. stress fibers, interact indirectly with the cytoplasmic domains of integrins via cytoplasmic proteins such as talin and vinculin. Talin has been shown to bind integrins (Horwitz et al., 1986) and vinculin in vitro (Burridge and Mangeat, 1989). Furthermore, vinculin has been shown to associate with the actin-binding protein α-actinin (Wachstock et al., 1987). Together, interactions between these protein components and others potentially form a transmembrane link between the extracellular matrix and the cytoskeleton. This transmembrane linkage is not as well characterized for the 67-kDa laminin receptor as it is for integrins. The 67-kDa laminin receptor has been shown to associate and co-localize with actin microfilament bundles (Brown et al., 1983; Cody and Wicha, 1986; Yannariello-Brown et al., 1988); however, co-localization of this receptor with vinculin and talin has not been demonstrated.

The focus of this paper is the adhesive interactions of cells with the laminin adhesion ligand YIGSR. In previous studies of YIGSR-mediated cell attachment, substrates for adhesion assays were prepared by adsorbing YIGSR-containing peptides to tissue culture wells. These substrates promoted attachment of several cell types; however, cell spreading was not observed (Graf et al., 1987a, 1987b). In this report, we demonstrate YIGSR-mediated attachment and spreading of several cell types upon well-characterized substrates comprising YIGSR peptide covalently attached by the amino-terminal primary amine to otherwise poorly adhesive materials. With these and similar substrates, we previously demonstrated that adhesion and spreading were dominated by the laminin receptor and not immobilized peptides. Therefore, the effects of potentially adsorbing proteins from cellular or culture medium sources were minimal (Massia and Hubbell, 1990, 1992a). Previous studies demonstrated YIGSR-mediated human foreskin fibroblast (HFF) spreading with stress fiber formation on covalently immobilized YIGSR substrates (Massia and Hubbell, 1990). In this study, we examined the YIGSR-mediated cell spreading response that was observed on covalently immobilized peptide, but not on adsorbed YIGSR peptide. All cell types that spread on YIGSR-grafted substrates formed actin microfilament bundles. HFF spreading on these substrates was inhibited by antisera that functionally block the 67-kDa and related receptors. Immunocytochemical studies of spread HFFs on these substrates demonstrated co-localization of the 67-kDa receptor with α-actinin and vinculin.

**EXPERIMENTAL PROCEDURES**

**Materials**—The synthetic heptapeptide YIGSR was obtained from Biosynthesis, Inc. Laminin and fibronectin were purchased from Sigma. Polyclonal antisera against human vitronectin receptor, integrin α3β1, and fibronectin receptor, integrin α5β1, were obtained from Telios Pharmaceuticals, Inc. Monoclonal mouse anti-human vinculin and α-actinin were purchased from ICN Immunobiologicals. The following rabbit antisera preparations were a generous gift from H. K. Kleinman: (a) Antiserum I, directed against the 67-kDa laminin receptor and a 45-kDa laminin-binding protein, and (b) Antiserum II, directed against the 32- and 45-kDa laminin-binding proteins.

**Cell Culture**—Human umbilical vein endothelial cells (HUVECs) were obtained from Endotech Corp., at passage 1-2, and cells from passage 2-5 were used in this study. HUVECs were maintained in Medium 199 supplemented with 20% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 15 μg/ml sodium heparin, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, and 20 μg/ml retinoid-derived growth factor (Endotech Corp.). Human vascular smooth muscle cells were isolated from umbilical veins, as previously described (Hubbell et al., 1991), and maintained in Medium 199 supplemented with 20% fetal calf serum and antibiotics. BT-474 ductal breast carcinoma cells (American Type Culture Collection, ATCC HTB 20), and mouse laryngeal epithelial cells (ATCC CCL 64), MG 63 human osteosarcoma cells (ATCC CRL 1427), C32 human melanotic melanoma cells (ATCC CRL 1585), rabbit corneal epithelial cells (a gift from R. A. Acosta), rat calvarium osteoblasts (a gift from R. Bizos), and HFFs (derived from neonatal tissue as described in Massia and Hubbell, 1990) were maintained in Dulbecco's modification of Eagle's medium supplemented with 10% fetal 1

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1 The abbreviations used are: HFF, human foreskin fibroblast; HUVECs, human umbilical vein endothelial cells; BSA, bovine serum albumin; PBS, phosphate-buffered saline.
calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. All cultures were incubated at 37 °C in a humidified 5% CO2 atmosphere. Media and serum were obtained from GIBCO Bethesda Research Laboratories, and other reagents were purchased from Sigma unless otherwise noted.

Preparation of Adhesive Substrates—A four-step chemical process was employed for the preparation of GYIGSRY-grafted substrates; the chemical scheme is somewhat modified (in the fourth step) from one described by us elsewhere (Massia and Hubbell, 1990). In the first step, glass was rendered relatively poorly cell-adhesive by modification with a silylating agent to produce what is called glycophasse glass. In the second step, the glycophasse glass was activated with treisyl chloride, and in the third step, the activated glycophasse glass was coupled with GYIGSRY via the amino-terminal Gly residue. This amino-terminal residue was included in the synthetic peptide sequence to serve as a structural spacer between the substrate surface and the adhesion ligand sequence of the peptide. The carbonyl-terminal Tyr residue was included to permit radioiodination when desired (not used in this study). In the fourth step, peptide-grafted substrates were subsequently reacted first with mercaptoethanolamine (100 mM in 1 mM HCl at room temperature for 1 h) and second with diglycolic anhydride (10 mM in dry tetrahydrofuran at room temperature for 1 h) to replace unreacted sulfonate chloride groups with nonadhesive moieties (Massia and Hubbell, 1992b). The only changes in the previous protocol (Massia and Hubbell, 1990) were in this fourth step; all other procedures were precisely as described.

Substrates containing adsorbed laminin, fibronectin, or GYIGSRY were prepared by incubating clean glass cover slips with 20 μg/ml protein or peptide for 1 h. Subsequently, these substrates were incubated for 30 min with 1 mg/ml bovine serum albumin (BSA) and rinsed with PBS before cell seeding.

Cell Spreading Assays—Cell spreading was measured as previously described (Massia and Hubbell, 1991). Briefly, nonenzymatically harvested cells suspended in serum-free medium (Medium 199 or Dulbecco’s modified Eagle’s medium with 2 mg/ml BSA) were incubated on substrates and incubated for 6 h at 37 °C. The samples were then washed twice with PBS to remove unattached cells and fixed in 3.5% formaldehyde in PBS for 30 min. The fixed samples were washed and then permeabilized in PBS containing 0.1% Triton X-100 for phase contrast microscopy. Attached cells (spread and nonspread) were counted in 10 fields at 200x magnification for each sample. Spread cells were tallied separately, as well as with the total number of attached cells, and scored by the criteria of polygonal shape and distinct nuclei. From these measurements, the percentage of attached cells that were spread was calculated.

To identify receptors that were involved in prompting attachment and spreading of HFFs to surface-grafted GYIGSRY, antifunctional antisera directed against the 87-kDa laminin receptor and related laminin-binding proteins were employed. The protocol that was employed for this assay has been previously described (Massia and Hubbell, 1991, 1992b). Briefly, nonenzymatically harvested HFFs suspended in serum-free medium (Dulbecco’s modified Eagle’s medium with 2 mg/ml bovine serum albumin) were incubated for 30 min before seeding with increasing concentrations of antibody. In control experiments, cells were replaced by preimmune rabbit serum (GIBCO BRL). Cell suspensions preincubated with antiserum or preimmune serum were seeded on GYIGSRY-grafted substrates for the remainder of the incubation period. The samples were then incubated with the secondary antibodies (diluted 1:100 in BSA-PBS) for 45 min at 37 °C. After the first incubation, samples were washed twice in BSA-PBS for 5 min each wash. The samples were then incubated with the secondary antibodies (diluted 1:50 in BSA-PBS) for 45 min at 37 °C. After two 5-min washes in BSA-PBS, the samples were mounted on glass slides with 1:1 glycerol/PBS and sealed with nail polish. The preparations were viewed on a Leitz Fluovert microscope equipped with a 100X PL Fluotar objective and a 100-watt mercury lamp. These preparations were photographed from a high resolution video monitor after digital contrast enhancement of video images.

RESULTS

Cell Spreading and Cytoskeletal Organization—All cell types examined, except for rat calvarium osteoblasts, were observed to spread on GYIGSRY-grafted substrates (Table I). The extent of cell spreading on GYIGSRY-grafted substrates was quite low for rabbit corneal epithelial cells and the MG 63 human osteosarcoma cell line. Furthermore, cell spreading was markedly increased for these cell types on the laminin- and fibronectin-coated substrates. The extent of cell spreading on GYIGSRY-grafted substrates for the remainder of the cell types tested was generally comparable to that on fibronectin- and laminin-coated substrates (Table II).

Inhibition of Cell Spreading by Laminin-binding Protein

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Attached cells that were spread</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFFs* (fibroblast)</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>HVSMC* (smooth muscle)</td>
<td>85 ± 6</td>
</tr>
<tr>
<td>HUVEC* (endothelial)</td>
<td>76 ± 3</td>
</tr>
<tr>
<td>MG 63* (osteosarcoma)</td>
<td>14 ± 7</td>
</tr>
<tr>
<td>BT 474* (ducal breast carcinoma)</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>C 32* (melanoma)</td>
<td>67 ± 5</td>
</tr>
<tr>
<td>Corneal epithelium†</td>
<td>50 ± 2</td>
</tr>
<tr>
<td>Lung† (epithelial-like)</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>Osteoblasts†</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

* Human species.  
† ND, not determined.  
‡ Rabbit species.  
§ Mink species.  
& Rat species.
TABLE II
Cell spreading on grafted CYIGSRY versus adsorbed laminin or fibronectin control substrates

Cell spreading upon CYIGSRY-grafted substrates expressed as a percentage of that on adsorbed laminin and fibronectin. Values are means ± S.E. and are derived from Table I.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Spreading on CYIGSRY</th>
<th>% Laminin control</th>
<th>% Fibronectin control</th>
<th>% control spreading</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFF</td>
<td>99 ± 2</td>
<td>105 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVSVMC</td>
<td>97 ± 2</td>
<td>93 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HUVEC</td>
<td>89 ± 1</td>
<td>86 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG 63</td>
<td>48 ± 2</td>
<td>28 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BT 474</td>
<td>88 ± 1</td>
<td>70 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C 32</td>
<td>92 ± 1</td>
<td>84 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corneal epithelium</td>
<td>83 ± 1</td>
<td>61 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mink lung</td>
<td>85 ± 1</td>
<td>60 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat osteoblast</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Antisera—To identify the receptors involved in cell spreading on CYIGSRY-grafted substrates, HFFs were incubated with antisera directed against the 67-kDa laminin receptor and related proteins. Cell spreading was assayed after a 6-h incubation period. Antiserum I (directed against the 45-kDa and 67-kDa laminin-binding proteins) and Antiserum II (directed against the 32-kDa and 45-kDa laminin-binding proteins) both inhibited cell spreading by approximately 50% when compared with cells treated with preimmune serum (Fig. 2A). However, Antiseria I and II did not inhibit cell attachment to CYIGSRY-grafted substrates (Fig. 2B). Functionally blocking antisera for integrins αβ1 and αβ2 did not affect cell attachment or spreading on these substrates (Fig. 2, A and B).

Distribution of Laminin-binding Proteins within Spread Cells on CYIGSRY-grafted Substrates—Dual-label immunofluorescence labeling was performed with Antiserum I or II and an antivinculin monoclonal antibody. Antiserum I reacted with proteins in discrete bands in the cell periphery that also stained positively for vinculin (Fig. 3, A and B). Antiserum II stained positively in a diffuse fibrillar pattern throughout the cytoplasmic portion of the cell (Fig. 3C), whereas vinculin localized within dense peripheral structures (Fig. 3D).

Dual-label immunofluorescence labeling with Antiserum I or II and monoclonal antibody directed against α-actinin revealed discrete peripheral regions in spread cells which stained positively for Antiserum I and α-actinin (Fig. 4, A and B). Antiserum I again stained positively in a diffuse fibrillar pattern (Fig. 4C) and did not co-localize with the peripheral staining pattern of α-actinin (Fig. 4D).

DISCUSSION

In a previous study, we observed YIGSR-mediated spreading of HFFs on CYIGSRY-grafted substrates (Massia and Hubbell, 1990). This result was striking, since Graf et al. (1987b) observed attachment, but not spreading of several cell types on substrates containing adsorbed YIGSR peptides. Furthermore, they observed no attachment of human skin fibroblasts to these substrates after a 1-h incubation period. In this study, we observed no cell spreading on adsorbed CYIGSRY substrates at 6 h (Table I). However, cell spreading on covalently immobilized CYIGSRY occurred with every cell type that we tested, with the exception of rat calvarium osteoblasts (Table I). This agrees with the result of Graf et al. (1987b) for this cell type on adsorbed YIGSR substrates. Our results in this study demonstrate that YIGSR-mediated cell spreading is a general response that is induced in many cell types when the ligand is covalently immobilized, and that the covalently immobilized peptide is nearly as effective as intact laminin and fibronectin for most cell types that were examined (Table II). Moreover, adsorbed peptide was demonstrated to be ineffective at supporting cell spreading (Table I).

The particular YIGSR-containing peptide sequence used in this study was CYIGSRY which contains a carboxyl-terminal tyrosyl residue. The tyrosyl residue was added at the carboxyl terminus as a site for radioiodination. The amine-terminal glycy glutamyl residue is the only reactive species in the peptide CYIGSRY, therefore immobilization of the peptide is via the amino-terminal glycy glutamyl residue which serves as a spacer between the YIGSR peptide and the surface. The carboxyl-terminal tyrosyl residue is not reactive with sulfonyl esters; therefore, this residue could not form a covalent linkage with the substrate surface and serve as a spacer between the YIGSR peptide and the surface. Merely the addition of the carboxyl-terminal tyrosyl residue to the CYIGSRY sequence was not sufficient to promote cell spreading since simple...
adsorption, rather than covalent immobilization, of the peptide GYIGSRY supported essentially zero cell spreading (Table I, right-most column). Therefore, the important difference that provided the novel biological activity (promotion of cell spreading) was not the addition of the carboxyl-terminal tyrosyl residue, but rather the covalent immobilization of the peptide.

GYIGSRY-mediated cell spreading occurs exclusively when the adhesion ligand is covalently immobilized, suggesting that conformational constraint of the ligand is necessary to optimize receptor-ligand interaction and consequently the adhesive interactions. The work of Graf et al. (1987b), which first characterized adhesion-promoting activity in the YIGSRY sequence, showed that carboxyl-terminal amide derivatives of YIGSRY-containing synthetic peptides were significantly more active than underivatized peptides. Studies by Kleinman et al. (1989) demonstrated that polymeric and cyclic forms of YIGSRY-containing peptides were biologically more active than linear forms. Both studies suggested that the secondary structure of the pentapeptide YIGSR and the tertiary structure surrounding this sequence within the laminin molecule may be important factors for receptor recognition. A recent molecular modeling study of the pentapeptide YIGSRY with a carboxyl-terminal amide showed a conformation that is stable over a wide range of solvent conditions (McKelvey et al., 1991). This highly stable conformation of YIGSR, described by McKelvey et al. (1991), is a partial right-hand α-helix formed by Tyr (Y), Ile (I), and Gly (G) and is held in place by the side chain of the Arg (R) residue. Since the Arg residue is vital for the stability of the partial right-hand α-helical structure within YIGSR and since substitution of other residues for R essentially abolishes biological activity of the pentapeptide, it was proposed that the partial right-hand α-helical structure is necessary for binding of YIGSRY to laminin receptors (McKelvey et al., 1991). Based on these findings and our observations, we propose that rotational constraints on the YIGSR molecule, imposed by cyclization or by covalent immobilization of YIGSRY-containing peptides through the amino terminus, enhances biological activity of the ligand by increased stabilization of the partial right-hand α-helical structure. A second possibility is that the orientations of the peptide favored by adsorption (e.g., possibly side-on) render the substrate-bound peptide less competent of binding to cellular receptors than to orientations arising from amino-terminal covalent immobilization (e.g., possibly end-on).

**Fig. 2.** Effects of Antiserum I (directed against the 45-kDa and 67-kDa laminin-binding proteins), Antiserum II (directed against the 32-kDa and 45-kDa laminin-binding proteins), anti-vitronectin receptor, and anti-fibronectin receptor on HFF spreading (A) and attachment (B) to GYIGSRY-grafted substrates. The reference controls were preincubated with preimmune rabbit serum. Antibodies were diluted 1:10 in culture medium from purified rabbit antiserum preparations.

**Fig. 3.** Double-label immunofluorescence micrographs of spread HFFs on GYIGSRY-grafted substrates. The 67-kDa laminin-binding protein clustered into dense peripheral bands (A, indicated by white arrows, A and B are matched fields) which also contained high concentrations of the cytoskeleton-binding protein vinculin (B, indicated by white arrows). The 32-kDa and 45-kDa laminin-binding proteins localized in perinuclear, cytoplasmic fibrillar structures (C, C and D are matched fields), whereas vinculin localized within dense peripheral structures (D, indicated by black and white arrow). Scale bar = 20 μm.
In this report, YIGSR-mediated cell spreading on GYIGSRY-grafted substrates was shown to involve the 32-, 45-, and 67-kDa laminin-binding proteins, since antifunctional antisera directed against these proteins (Antisera I and II) effectively inhibited HFF spreading (Fig. 2A). Since integrins αβ1 and αβ3 do not recognize YIGSR and were shown not to promote cell attachment or spreading on these substrates, it was concluded that YIGSR was the predominant adhesion-promoting signal on these substrates (Fig. 2, A and B). Furthermore, it was confirmed that cellulary secreted proteins, which include those recognized by integrins αβ1 and αβ3, did not significantly affect cell spreading on these substrates. Antisera I and II did not inhibit cell adhesion on GYIGSRY-grafted substrates; therefore, it was concluded that the 67-kDa and related peptides were not involved with cell attachment on these substrates (Fig. 2B). Other non-integrin laminin-binding proteins (36–38 kDa) which recognize YIGSR have been described for rat hepatocytes (Clément et al., 1990). These proteins have low affinities for laminin and do not react with Antiserum I or II. Although the 36–38-kDa low affinity laminin-binding proteins have not been described for HFFs, it is possible that HFFs express this class of proteins. These proteins could possibly mediate HFF attachment to GYIGSRY-grafted substrates and would not be detected by Antisera I and II.

Immunofluorescence microscopy with Antiserum I (directed against the 45- and 67-kDa proteins) revealed localization of receptor proteins in peripheral bands within spread HFFs on GYIGSRY-grafted substrates (Figs. 3A and 4A). When Antiserum II (directed against the 32- and 45-kDa proteins) was used, localization of proteins into discrete bands was not observed (Figs. 3C and 4C). Therefore, we conclude that the 67-kDa receptor is the predominant protein that localizes into these peripheral bands. Vinculin and α-actinin were observed to co-localize with the 67-kDa receptor into these peripheral bands (Figs. 3, A and B, and 4, A and B), suggesting direct or indirect association of vinculin and α-actinin with the 67-kDa receptor.

Since stress fiber formation (Fig. 1A) and co-localization of the 67-kDa receptor with vinculin and α-actinin (Figs. 3, A and B, and 4, A and B) were observed within spread HFFs on GYIGSRY-grafted substrates, we propose that clustering of the 67-kDa receptor mediates vinculin association and stress fiber formation within spread cells. Yannariello-Brown et al. (1988) also observed clustering of endothelial cell 69-kDa laminin receptors (closely related if not identical with the fibroblast 67-kDa receptors) into dense peripheral bands within spread cells on laminin-coated substrates. Furthermore, they noted co-localization of actin microfilament bundles into the peripheral bands and proposed that the 69-kDa receptor associated directly or indirectly with the cytoskeleton. Evidence for the association of the 67-kDa receptor with actin microfilaments has also been documented by other workers (Brown et al., 1983; Cody and Wicha, 1986). From their observations, Mecham et al. (1991a, 1991b) proposed that fetal bovine fibroblast 67-kDa receptors bind laminin and are initially uncoupled from the cytoskeleton, specifically actin microfilament bundles. Subsequently, binding of the receptor to the ligand invokes association of the cytoskeleton with the receptor-ligand complex and movement of these complexes to appropriate sites on the cell, e.g., the dense peripheral bands. We propose that this association of the 67-kDa receptor-ligand complex is indirect, since the cytoplasmic, actin-binding proteins vinculin and α-actinin were shown to be directly or indirectly associated with clustered receptor-ligand complexes (Figs. 3, A and B, and 4, A and B). Evidence is not conclusive that the 67-kDa receptor is a transmembrane protein (Brown et al., 1983; Cody and Wicha, 1986; Wewer et al., 1986; Yow et al., 1988). Therefore, it is possible that the 67-kDa receptor does not span the membrane. In this case, our observation of vinculin and α-actinin involvement with 67-kDa cytoskeletal interactions would sug-
suest that one or more transmembrane proteins are required to complete the link between the receptor and cytoskeleton. Alternatively, the 67-kDa receptor could be a transmembrane protein that associates with vinculin, α-actinin, and possibly other proteins to form a connection with the cytoskeleton.

In summary, we report that the laminin-based adhesion signal, YIGSR, can promote cell spreading for a wide variety of cell types. However, it appears necessary for conformationally constrain or orient the YIGSR ligand so that receptor-ligand interactions are optimal for the promotion of cell spreading. The 32-, 45-, and 67-kDa laminin-binding proteins all play a role in cell spreading. The major receptor necessary for mediating cell spreading via YIGSR is the 67-kDa protein, since it appears to be solely involved in clustering and cytoskeletal association. These studies confirm the role of the 67-kDa receptor in recognition of YIGSR and mediation of cell adhesive interactions.

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