The Cellular Interactions of Laminin Fragments

CELL ADHESION CORRELATES WITH TWO FRAGMENT-SPECIFIC HIGH AFFINITY BINDING SITES*

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The ability of the basement membrane protein laminin to promote the adhesion, spreading, proliferation, and differentiation of cells is related to its potential for cell binding (reviewed in Refs. 1 and 2). Laminin-binding proteins have been isolated from the plasma membranes of various tumor cells and muscle (3-5), cloned (6), and shown to have molecular mass of about 68 kDa. Such putative laminin receptors are thought to mediate the various biological activities expressed by cells when in contact with laminin. Other studies, however, have shown binding between laminin and sulfatides (7), gangliosides (8), a fibronectin-binding protein (9), and heparan sulfate (10), which may be alternative mediators of laminin-cell interactions.

In attempts to determine which domains of the laminin molecule are responsible for its cellular interactions, several proteolytic fragments of the protein have been shown to act as substrates for cell attachment (see Fig. 1). They include the disulfide-rich fragment 1, which consists of three rod-like segments originating from the center of the short arms of laminin (11, 12). This fragment was found to compete with the laminin-mediated cell attachment to collagen IV substrates (13, 14), to promote directly cell adhesion (15), and to bind to cells with an affinity comparable to that of laminin (16). Fragment 1 also decreased the metastatic potential of tumor cells (16,17) presumably due to blockade of the laminin receptors thought to be required for invasion through basement membranes. In addition, fragment 1 displayed metabolic effects similar to those of intact laminin in that it enhanced the proliferative response of adherent cells in culture (18) and stimulated the synthesis of a metalloproteinase which cleaves specifically basement membrane collagen (19).

Another cell-binding fragment of laminin, fragment 8, was originally identified in studies with neural cells. This fragment stimulated neurite outgrowth and potentiated the survival of cultured neurons (20) and was also implicated in the specific increase in levels of catecholamine synthetic enzymes seen when chromaffin cells are cultured on laminin substrates (21). Fragment 8 consists of a rod-like segment and a complex globular domain, representing the terminal half of the long arm of laminin (22, 23). It also contains the heparin-binding domain of laminin (fragment 3), although it could not be demonstrated that this domain was directly involved in the interaction of laminin with neurons (20). Other, small globular laminin fragments (fragments 5 and 6) were found to promote adhesion of hepatocytes (15) but have not yet been mapped within the laminin structure. Although antisera against these fragments could block cell attachment to these fragments themselves, no inhibition of cell binding to laminin was seen, indicating that fragments 5 and 6 do not correspond to the major cell-binding sites of the intact laminin molecule and may have arisen de novo due to the proteolysis.

A comparative analysis of the cell-binding potentials of the major laminin fragments 1 and 8 has so far not been undertaken. This study has become feasible after the development of a new method to isolate the laminin-nidogen/entactin complex (22) that allows the preparation of biologically active fragment 8 in a reproducible fashion. The data presented here demonstrate that many cell types can adhere to fragment 1 and/or fragment 8. The ability of these fragments to act as substrates for cell attachment correlates with the presence of two distinct laminin receptors, demonstrated by the high affinity binding of radiolabeled ligands to cells in suspension.

MATERIALS AND METHODS

Preparation of Laminin, Laminin Fragments, and the Laminin-Nidogen Complex—Laminin was purified from the mouse Engelbreth-Holm-Swarm tumor as previously described (24). Extraction and purification of the laminin-nidogen complex followed a more recent...
procedure (23). Dissociation of the complex for the preparation of intact complex was, however, used for the purification of laminin individual components was achieved with 2\(^{nd}\) ing previously published procedures sages over immunoadsorbents prepared from iaminin fragments fragment were prepared from conventionally purified laminin follow- says and electrophoresis demonstrated a high purity of fragments within the cruciform structure treated by ultrafiltration to 0.3-1.2 mg/ml, and their specificity was adsorbing proteins from solution in distilled water overnight at.

**Localization of cell-binding fragments 1 and 8**

within the cruciform structure of laminin. The cell-binding fragments are encircled by a dashed line. Additional laminin fragments used in the study are denoted by numbers 1–4, 3, and 25K (based on Refs. 12 and 22).

**Antisera and Immunoadsorption**—Rabbit antisera were raised against laminin (24) and the laminin fragments P1, E1-4, E3, and E4 and 25-kDa fragment were prepared from conventionally purified laminin following previously published procedures (11, 15, 22, 25). Radioimmunoassays and electrophoretic purity were determined by evaluating possessing antibodies. Antisera and purity of laminin fragments were also determined by radioimmunoinhibition assays.

**Cells and Cell Cultures**—A Chinese hamster ovary cell line (CHO), the human fibrosarcoma cell line HT 1080, and human rhabdomyosarcoma cell line RD were obtained from American Type Culture Collection (Rockville, MD). The human rhabdomyosarcoma cell line A 204 and astrocytoma cell line 251 MG were kindly provided by Dr. A. Vaberi (University of Helsinki). Rat schwannoma (RN 22) cells were obtained from Dr. S. Pfeiffer (University of Connecticut) and have been characterized previously (28). Human embryonic skin fibroblasts were those used in previous studies (29). Cells were grown to confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100–400 units/ml penicillin, 50–90 mg/ml streptomycin, 50 mg/ml ascorbate, and 300 mg/ml glutamine.

**Coating of Culture Dishes**—Tissue culture plastic dishes (24 multiwell plates with 2-cm\(^2\) surface area/well) from Costar, Cambridge, MA were coated by air-drying protein solutions overnight at 37 °C (29). Protein substrates were diluted in 1% BSA dissolved in distilled water prior to coating. In a second procedure, wells were coated by adsorbing proteins from solution in distilled water overnight at 4 °C, followed by blocking with 1% BSA for an additional 24-h absorption period. Wells coated with BSA alone served as negative controls.

**RESULTS**

**Comparison of Cell Adhesion to Substrates of Laminin, Lam- inin-Nidogen Complex, and Laminin Fragments**—The tumor cell lines and embryonic skin fibroblasts that were used to examine dose-response profiles of attachment on air-dried substrates had been previously shown to adhere to laminin substrates (28, 29). Many cell lines (including astrocytoma 251 MG, rhabdomyosarcoma RD, CHO, and fibroblasts, not

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1 The abbreviations used are: CHO, Chinese hamster ovary; BSA, bovine serum albumin; KRB, Krebs-Ringer-Henseleit.

2 V. Nurcombe and D. Edgar, unpublished results.
shown) behaved similarly to the fibrosarcoma HT 1080, displaying maximal attachment on 2-5 µg of laminin added per well (Fig. 2). Plateau values for attachment varied between 25 and 75% of total seeded cells, depending on the cell line. The rat schwannoma cell line RN 22 reached higher plateau values of 80-90% attachment when only 1-2 µg of laminin had been used to coat the wells, whereas the rhabdomyosarcoma cell line A 204 attached relatively poorly and did not reach plateau values even after using 20 µg of laminin/well in the same incubation period of 30 min (Fig. 2). Increasing the time of incubation did not change the plating efficiencies (not shown).

The cells were also compared in their ability to adhere to the laminin-nidogen complex. The complex showed generally higher activity than laminin at low concentrations, but there was no effect on the final plateau values of attachment obtained. The only exception was cell line A 204, which did not attach to the laminin-nidogen complex (Fig. 2). Components of the complex were separated from each other after dissociation in 2 M guanidine HCl (23) and were then examined in adhesion assays. Laminin obtained from the complex showed activity, whereas no adhesion-promoting activity could be detected with nidogen dissociated from the complex (data not shown).

Since previous studies with tumor cells (13, 15, 32) and neurons (20) have implicated fragments 1 and 8 of laminin (Fig. 1), respectively, as cell binding structures, we compared cell adhesion to both of these fragments (Fig. 2). Most of the cells (including 251 MG, CHO, RD, and fibroblasts, not shown) behaved like HT 1080 cells (Fig. 2) in displaying similar dose-response profiles for either fragment and laminin. Exceptions were the schwannoma A 204 cells which failed to attach to fragment 8 at all (Fig. 2). Further studies with laminin fragments 3 and 4 and 25-kDa fragment failed to demonstrate any adhesion of HT 1080 cells (not shown).

Cell adhesion was also examined with substrates prepared by adsorption from solution in order to determine if possible denaturation of molecules during air-drying had affected their ability to act as substrates. Adsorption resulted both in comparable amounts of substrate bound (see "Materials and Methods") and in similar attachment of HT 1080 cells to laminin and its fragments 1 and 8, showing that different coating procedures do not influence cellular recognition of laminin structures.

Antibody Inhibition of Cell Attachment—Eight different rabbit anti-laminin antisera of comparable radioimmunoassay titers were compared in attachment inhibition at a fixed dilution (1:25). Seven antisera showed significant inhibition of cell attachment which varied between 37-100% inhibition on an optimal laminin coat, depending on the particular antisera and cell line used. Antibodies of anti-laminin antisera were then fractionated by immunoadsorption on various laminin fragments in order to study the domain specificity of their inhibitory effect (Table I). Immunoadsorption of the antisera on fragments 1, 4, and 1-4, which are all structures of the short arms of laminin (25) (see Fig. 1), yielded antibodies which failed to inhibit HT 1080 cell attachment to laminin. Subsequent purification of the remaining antibodies by immunoadsorption on fragment 3 and laminin resulted in antibodies which reacted strongly with the long-arm fragments 3 and/or 8 of laminin (22) but failed to bind significantly short-arm fragments (Table I). Both of these antibody fractions inhibited attachment of HT 1080 cells on laminin (Table I).

Similar results were obtained when the inhibition of adhesion of a variety of cells (HT 1080, 251 MG, RD, CHO, and RN 22) was examined using rabbit antisera raised directly against fragments 1, 1-4, 3, 4, and 8. These antisera showed radioimmunoassay binding for the fragment used for immunization comparable to that for laminin but failed to react significantly with unrelated fragments (antigen-binding capacities usually 100-fold lower, not shown). Again, only antisera against fragments 3 and 8 showed strong inhibition of cell attachment on laminin. Antisera against 25-kDa fragment or fragments 1, 1-4, or 4 were either inactive or of lower potency (Table II). The low activity of the latter antisera indicates the presence of some antibodies recognizing epitopes near the active site of fragment P1. Similar antibodies are apparently missing in antisera against laminin (see Table I). The same set of antisera was, in addition, used to examine attachment of HT 1080 cells on the laminin-nidogen complex (Table II). Here again, only antisera to fragment 8 were inhibitory, whereas other fragment-specific antisera and antisera to nidogen were inactive.

Adhesion of HT 1080 cells to fragment 8 could be inhibited in an equivalent manner by antisera against fragments 8 and 3, whereas antisera against fragment 1-4 or 25-kDa fragment...
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TABLE II

Inhibition of attachment of various cell lines to laminin by antisera against laminin and laminin fragments

| Antiserum against laminin | Cell inhibition* | HT 1080 | 251 MG | RN 22 | CHO |
|--------------------------|------------------|---------|-------|-------|-----|
| HT 1080                  |                  | %       |       |       |     |
| I. Laminin               | 60               | 100     | 61    | 81    | 98  |
| Fragment E8              | 64               | 100     | 56    | 94    | 78  |
| Fragment E3              | 52               | 100     | 54    | 89    | D*  |
| Fragment E1–4            | 12               | 7       | 24    | 6     | ND  |
| Fragment P1              | 21               | 10      | 18    | -8    | -5  |
| Fragment E4              | 10               | 14      | 14    | -14   | ND  |
| Attached cell control numbers |           | %       |       |       |     |
| II. None                 | 41 ± 6           | 60 ± 5  | 85 ± 1| 29 ± 4| 40 ± 2|
| Normal IgG (0.1 mg/ml)   | 42 ± 0           | 67 ± 1  | 90 ± 3| 36 ± 1| 38 ± 3|

* Used at 1:25 dilution (antigen-binding capacities for laminin, 0.5–2 μg/ml).

† Compared to the control with normal IgG given in Part II, which lists the actual number of cells ± S.D. in the presence and absence of normal IgG.

‡ Coat with laminin-nidogen complex instead of laminin.

§ ND, not determined.

FIG. 3. Inhibition of adhesion of HT 1080 cells to laminin fragment 8 by antisera to various laminin fragments. Antisera were raised against fragments E8 (O), E3 (●), 25-kDa fragment (△), and E1–4 (□). Antigen-binding capacities were determined by radioimmunoassays with fragment E8 (antiserum against fragments E8 and E3, and 25-kDa fragment) or fragment E1–4 (antiserum against fragment E1–4) and were in the range 12–60 μg/ml antisera. Numbers of attached cells in the negative controls were similar to that in Table II.

were either inactive or of low inhibitory capacity (Fig. 3). No inhibition of cell adhesion on fragment 1 was seen with any of the antibodies used in this study; in particular, three different antisera raised against either fragment 1–4 or 1 directly were essentially noninhibitory (maximal 10% inhibition) even at the highest concentration used, which was 1 μg/ml antigen-binding capacity (Table II). Also, three different antisera against laminin which blocked HT 1080 cell adhesion on a laminin substrate failed to inhibit attachment of these cells on a fragment 1 substrate.

Cell-Binding of Radiolabeled Laminin and Laminin Fragments—The assays were carried out in cells in suspension using an assay method which has been useful in cell-binding studies with nerve growth factor (31, 33). A critical parameter of the assay was the use of freshly labeled ligands (within 4 days of labeling) which reduced background binding. Preliminary experiments showed maximal specific and nonspecific binding of radiolabeled laminin and its fragments after a 2-h incubation with the cells at 0 °C. This time point was therefore taken for the construction of equilibrium binding curves (Fig. 4). Such curves showed a sigmoidal shape extending above the inflexion point after plotting bound against free ligand in a semilogarithmic fashion (not shown). These data therefore appear suitable for analyzing affinity and receptor numbers involved in the cell binding of soluble ligands (34).

Apparent dissociation constants (K_d) and number of cell-binding sites for laminin and laminin fragments 1 and 8 were evaluated from Scatchard plots for which examples are shown in Fig. 4. The data best fit a straight line as determined with a linear regression program, consistent with the presence of a uniform class of high affinity receptors. The presence of low affinity binding sites (K_d > 10^-7 M) cannot be excluded in this experimental protocol. A summary of the data for HT 1080, RN 22, and A 204 cells (Table III) indicates receptor numbers between 3 and 12 × 10^6/cell and apparent K_d values in the range 1–10 nM for laminin binding. Similar values were obtained for the binding of fragment 1 to HT 1080 and A 204 cells and for the binding of fragment 8 to HT 1080 and RN 22 cells. It should be noted, however, that the RN 22 cells only displayed 10^6 binding sites/cell for fragment 1 with a comparatively low affinity (K_d = 11 nM). Furthermore, little, if any, specific binding of fragment 8 to A 204 cells could be detected, indicating the apparent lack of high affinity binding sites (Table III).

Specificity of fragment binding by HT 1080 cells was shown by competition assays (Fig. 5). Both laminin and fragment 1 competed in equivalent manner, with the binding of labeled fragment 1 resulting in 50% displacement at equimolar ratios. No displacement was seen with fragment 8 even at 125-fold molar excess. Conversely, laminin and fragment 8, but not fragment 1, were able to displace labeled fragment 8 from the cells. Thus, the cell-binding sites of the fragments are different in their ability to bind cells independently, consistent with the presence of two distinct cellular receptors.

**DISCUSSION**

This study shows that a variety of cells can adhere to fragment 1 or 8, or both, of laminin, reflecting the presence of distinct receptors for these fragments. The presence of such receptors was demonstrated directly by high affinity binding of radiolabeled ligands and the specific competition of this binding. These observations imply that the cell-binding sites of laminin on fragments 1 and 8 are structurally different.

The recognition by cells of one or both topologically separated domains of laminin seems to be a general phenomenon; cell interactions of fragment 1 have been reported previously for several metastatic tumor cell lines (13, 14, 16–18, 32) and hepatocytes (15), and cell interactions with fragment 8 have been reported with neurons (20) and chromaffin cells (21). Our present data show that fibroblasts and several cell lines (HT 1080, 251 MG, RD, and CHO) can adhere to both fragments 1 and 8 of laminin, showing that recognition of the latter is not a property restricted to neural cells. The dual recognition of laminin by cells does not appear, however, to be a uniform phenomenon; schwannoma RN 22 cells adhered more readily to fragment 8 substrate than to fragment 1, and similar observations have been made for myoblasts and several tumor cells lines. Conversely, the A 204 cell line behaved differently, adhering to laminin and fragment 1 substrates but not to fragment 8. Neurons may even differ slightly from this scheme since they respond to fragments 8 and 1–4 (20) but not to the fragment 1 substructure of fragment 1–4. Whether these differences are due to a third cell-binding

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S. Goodman, R. Deutmann, and K. von der Mark, personal communication.

D. Edgar, unpublished results.
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FIG. 4. Equilibrium binding and derived Scatchard analysis (insets) for the binding of 125I-labeled laminin (a), 125I-labeled fragment P1 (b), and 125I-labeled fragment E8 (c) to human fibrosarcoma HT 1080 cells as a function of free ligand concentration. The curves for total and specific binding are fit by eye, and standard deviations are shown for the specific binding curves. Specific binding (●) represents the difference between the binding in the absence (○) and presence of 100-fold excess unlabeled ligand. Incubations were performed at 0 °C for 90 min, at which time the binding had reached equilibrium (not shown). The Scatchard plots of the specific binding data are shown with the ratio bound/free $X_{10^{-1}}$ and the amount of bound ligand as nanograms/5 × 10⁶ cells. The regression coefficients for each experiment were $r = 0.97$ (a), 0.98 (b), and 0.88 (c).

TABLE III

| Cells      | Binding parameter | Radioligand | Laminin | Fragment 1 | Fragment 8 |
|------------|------------------|-------------|---------|------------|------------|
| HT 1080    | $K_D$ (nM)       |             | 3.8     | 3.2        | 9          |
|            | $R \times 10^{-3}$ |             | 98      | 98         | 19         |
| A 204      | $K_D$ (nM)       |             | 9.4     | 9.8        | >100       |
|            | $R \times 10^{-3}$ |             | 31      | 33         | ND*        |
| RN 22      | $K_D$ (nM)       |             | 2.4     | 11         | 1.2        |
|            | $R \times 10^{-3}$ |             | 117     | 10         | 103        |

*ND, not determined.

sequence only found on fragment 1-4 is not clear at present.

The laminin-nidogen complex where nidogen is bound to fragment 1 structures (23) shows a comparable or even better adhesion activity than laminin alone (Fig. 2). We attribute the increased adhesive activity of the complex to the gentle isolation procedure used which may keep fragment 8 structures more intact, rather than to a modulatory effect of nidogen in the complex. Nidogen, however, can block fragment 1 cell-binding sites as shown for A 204 cells which did not adhere to the laminin-nidogen complex, presumably because they lack alternative receptors for fragment 8 structures. The contribution of fragment 1 to the adhesion of other cells could not be further evaluated by antibody inhibition experiments; neither antibodies raised against fragment 1 directly nor anti-fragment 1 antibodies purified from blocking antisera raised against laminin could inhibit adhesion to fragment 1 or laminin. The reason why antibodies to fragment 8 inhibit the adhesion of cells to laminin substrates even though the cells apparently possess fragment 1-specific receptors remains to be elucidated.

Previous binding studies with the human breast carcinoma MCF-7 cell line (14, 32, 35), B16 mouse melanoma cells (3), and a mouse fibrosarcoma cell line (4) demonstrated receptors binding both laminin and fragment 1 with an apparent $K_D$ of about 2 nM and $10^4$-5 × 10⁴ receptor sites/cell. Binding data on the high affinity laminin-binding sites of the three cell lines examined here show apparent $K_D$ values in the range 1-
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10 nM with 10⁻⁶–10⁻⁷ receptor sites/cell and are in good agreement with the previous observations. In addition, however, it is shown here that fragment 8 can also bind specifically to cells with an affinity similar to that of laminin and with comparable numbers of receptor sites. The ability of laminin fragments to bind to cells in suspension closely paralleled their ability to mediate cell adhesion; the binding and adhesion to fragment 1 were low for RN 22 cells, and fragment 8 neither bound to A 204 cells nor stimulated their adhesion. There was also an inverse correlation between number of receptor sites for laminin or its fragments and the amount of laminin substrate required for optimal cell attachment (see Fig. 2 and Table III). This suggests that the rate of cell adhesion to matrix molecules is determined by the number of receptors expressed. Whether individual cells have differing numbers of receptors for each fragment, as suggested by Fig. 2, remains to be determined.

Several limitations should be considered in the interpretation of our binding data. The study was designed for the identification of high affinity binding sites; and any weaker interactions with laminin may therefore have escaped detection, although that is not to say that they are physiologically irrelevant. The accuracy of receptor number determinations may also be influenced by endogenous ligands which may have been incompletely removed during detachment of cells with EDTA. Oligomers of laminin which persist in the presence of EDTA (90) could also contribute to errors in the determination of \( \bar{K}_0 \) and receptor number. Very few and small oligomers are present in preparations of fragments 1 and 8 as shown by ultracentrifugation (11, 22). The error introduced by laminin aggregation therefore does not appear to be very substantial since apparent \( \bar{K}_0 \) and receptor numbers determined with laminin closely correspond to values determined for the laminin fragment binding most extensively to the cells.

The identification of two major cell adhesion sites on the laminin molecule that could be up to 80–100 nm distant from one another and different in structure predicts the existence of two distinct laminin receptors. This was clearly demonstrated by the lack of competition between fragments 1 and 8 in cell binding and because the numbers of each receptor vary independently between various cell types. It is not yet clear if the cells that possess the two receptors utilize them both for adhesion to laminin substrates. Different cell recognition sites have been identified on fibronectin (37), and it appears that at least two sites are necessary for the full interaction of fibroblasts with fibronectin substrates (38). A similar multiplicity of interactions might be required for cell adhesion to laminin.

Complexity of laminin receptors has also been indicated in previous studies which identified several cell-surface components as potential laminin-binding sites. A 68-kDa laminin-binding protein has been isolated from several sources (3–5). Cells possessing this receptor were demonstrated to react with laminin fragment 1 in competition assays (14) and showed similar \( \bar{K}_0 \) values for laminin and fragment 1 in radioligand binding (16). This receptor has also been shown to be eluted from affinity columns by a synthetic peptide corresponding to a structure present in fragment 1 (39). It appears likely therefore that this 68-kDa protein corresponds to the fragment 1 receptors identified in this study.

A laminin receptor with similarly high affinity should be expected to exist for fragment 8, but the nature of the molecule responsible for binding is unclear. Attempts to identify this receptor by affinity chromatography and ligand binding to blots have yielded equivocal results in our hands. Nevertheless, an avian fibronectin cell receptor (CSAT) has been claimed to be a bifunctional cell receptor in that antibodies to CSAT are also able to block cell adhesion and neurite outgrowth on laminin and also because isolated CSAT binds laminin (9, 40). The low affinity of this binding tends, however, to exclude CSAT as a binding protein for either fragment 1 or 8 structures of laminin. A more likely candidate for the second laminin receptor might be monogalactosyl sulfatides which, when purified from erythrocytes and brain (7), showed a high affinity for laminin (half-saturation of binding at 5 nM). Furthermore, these sulfatides do not bind laminin fragment 1. Cell membrane-bound forms of heparan sulfate proteoglycan (reviewed in Ref. 41) could also be involved in laminin binding since the major heparin-binding site of laminin has been localized to fragment 3, which is a globular domain of fragment 8 (22, 25). Whereas such proteoglycans might well be involved in cell interactions with fragment 8 (38), it is unlikely that these interactions alone are responsible for the high affinity since fragment 3 itself was found to be a poor adhesion substrate (see also Ref. 20). The cellular receptor for the long arm of laminin therefore remains to be identified.

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