Attachment of Cells to Basement Membrane
Collagen Type IV

Monique Aumailley and Rupert Timpl
Max-Planck-Institut für Biochemie, Abt. Bindegewebsforschung, D-8033 Martinsried, Federal Republic of Germany

Abstract. Of ten different cell lines examined, three showed distinct attachment and spreading on collagen IV substrates, and neither attachment nor spreading was enhanced by adding soluble laminin or fibronectin. This reaction was not inhibited by cycloheximide or antibodies to laminin, indicating a direct attachment to collagen IV without the need of mediator proteins. Cell-binding sites were localized to the major triple-helical domain of collagen IV and required an intact triple helical conformation for activity. Fibronectin showed preferential binding to denatured collagen IV necessary to mediate cell binding to the substrate. Fibronectin binding sites of collagen IV were mapped to unfolded structures of the major triple-helical domain and show a similar specificity as fibronectin-binding sites of collagen I. The data extend previous observations on biologically potential binding sites located in the triple helix of basement membrane collagen IV.

Materials and Methods

Preparation of Proteins and Protein Fragments
Collagen IV was solubilized from the mouse Engelbreth-Holm-Swarm (EHS) tumor by limited digestion with pepsin at 4°C or with bacterial collagenase, and was then purified on DEAE cellulose (51). The same tumor was also used to prepare acid-extracted collagen IV (48). These preparations gave identical results in cell attachment and are collectively referred to as collagen IV. The major triple helix of collagen IV (fragment P large, Mr 140,000) and large fragments of this region—P1 (COOH-terminal peptide of all[IV], 50 kD), P2 (COOH-terminal peptic fragment of α2[IV], 50 kD), and P3 (disulfide-linked NH2-terminal portion of the major triple helix, 70 kD)—were obtained by further digestion with pepsin and purified in either triple-helical or denatured state (44). These fragments have been previously localized within collagen IV molecules as shown in Fig. 1 (47). Reduction and alkylation of collagen IV under non-denaturing conditions was done as described previously (51). Laminin was purified from the EHS tumor according to a standard protocol (50). Fibronectin was purified from human plasma using affinity chromatography on gelatin and heparin and was kindly supplied by Dr. H. Richter, Martinsried. Cyanogen bromide peptide αIIIC7 was prepared from rat collagen I (10).

Antiseras and Immunoblotting
Rabbit antisera against laminin were prepared as described previously (50). Similar immunization protocols were used for the production of antiserum against acid-extracted collagen IV and its purified 7S and NC1 domain were those used in previous studies (28, 39, 48).

Immunoblotting with anti-laminin antisera, which allows the detection
of laminin A and B chains (36), was used to analyze the presence of these chains in cultured cells. Cell layers were extracted overnight with 3 M guanidine–HCl and aliquots corresponding to 2–6 × 10⁶ cells were, after reduction, used for electrophoresis and transferred to nitrocellulose.

Cell Culture

Human embryonic skin fibroblasts (HEF) were those used in previous studies (6). A Chinese hamster ovary cell line (CHO) and a human fibrosarcoma cell line (HT-1080) were obtained from American Type Culture Collection (Rockville, MD) and characterized previously (5). The mouse neuroblastoma cell line C-1300 (3), human rhabdomyosarcoma cell line A204 (4), and human astrocytoma cell line 251 MG (2) were kindly provided by Dr. A. Vaheri, University of Helsinki. Murine endothelial cell line EC was a gift of Dr. G. Grotendorst, National Institute of Dental Research, Bethesda, and mouse epidermal cell line PAM 212 (43) was a gift of Dr. S. Yupsa, National Cancer Institute, Bethesda. Human breast carcinoma cells (MCF7) were from the Michigan Cancer Foundation. Mouse partial yolk sac (PYS-2) cell line was a gift from Dr. J.M. Lehman, University of Colorado, Denver, and previously characterized (32).

Results

Patterns of Cell Attachment to Collagen IV, Laminin, and Fibronectin

Nine different cell lines and embryonic fibroblasts were tested for their ability to attach and spread on the basement membrane substrates collagen IV and laminin, or on a combination of both proteins, using collagen IV as substrate and laminin as soluble mediator. According to the attachment patterns, cells could be classified into four groups (A to D, Table I). HT-1080 fibrosarcoma, CHO cells, and fibroblasts (group A) showed good attachment to both substrates but lack of substantial promotion by exogenous laminin when adhering to collagen IV. Three other cell lines preferred the combination collagen IV/laminin for attachment but did not adhere to either protein alone (group B). These cells included the epidermal cell line PAM 212 for which this particular attachment pattern has been originally described (43). The other cells showed either exclusive adherence to laminin but a poor spreading (group D) or no substantial attachment to any of these substrates (group C). A comparison with attachment patterns to fibronectin showed distinct binding only for group A cells and the teratocarcinoma cell line PYS-2 (group C) but not for any other cell (Table I).

Attachment values for a particular substrate were rather invariable within a single test. An interassay comparison showed occasionally larger variations (Table II) but without changing the general patterns described in Table I.

Attachment reached plateau values within the standard assay period (30–60 min) and no further increase was observed upon four- to eightfold longer incubations (not shown). Substrate concentrations of 5 µg/cm² appeared to be optimal as shown for HT-1080 cell attachment to collagen IV (Fig. 2). Cells that attached well to a particular substrate showed complete spreading within 40–60 min. Cells with a poor attachment rate did not spread on the substrates.

All assays were performed in serum-free medium and in the presence of cycloheximide (25 µg/ml) which completely abolished endogenous protein synthesis as judged from metabolic labeling in human embryo fibroblast cultures. This indicates that cell attachment to collagen IV and/or laminin is independent of serum components and of the production of soluble mediator for proteins by the cells. The possible presence of preformed laminin on the cells was examined by immunoblotting and failed to detect laminin chains in extracts of HT 1080 cell layer. Strongly positive

1. Abbreviation used in this paper: DME, Dulbecco's modified Eagle's medium.
Table I. Attachment of Different Cell Lines to Extracellular Matrix Proteins

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Laminin</th>
<th>Collagen IV</th>
<th>Collagen IV plus laminin</th>
<th>Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong> HT 1080 (human fibrosarcoma)</td>
<td>45 ± 2</td>
<td>50 ± 1</td>
<td>60 ± 6</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>CHO (Chinese hamster ovary)</td>
<td>22 ± 1</td>
<td>26 ± 2</td>
<td>27 ± 1</td>
<td>21 ± 0</td>
</tr>
<tr>
<td>HEF (human embryo fibroblast)</td>
<td>13 ± 1</td>
<td>41 ± 4</td>
<td>50 ± 3</td>
<td>31 ± 1</td>
</tr>
<tr>
<td><strong>B</strong> PAM 212 (mouse epidermal)*</td>
<td>0 ± 1</td>
<td>2 ± 1</td>
<td>36 ± 3</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>EC (murine capillary endothelium)</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
<td>58 ± 4</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>MCF-7 (human breast carcinoma)</td>
<td>4 ± 2</td>
<td>16 ± 2</td>
<td>23 ± 2</td>
<td>5 ± 1</td>
</tr>
<tr>
<td><strong>C</strong> A204 (human rhabdomyosarcoma)</td>
<td>3 ± 0</td>
<td>5 ± 4</td>
<td>3 ± 1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>PYS-2 (mouse teratocarcinoma)</td>
<td>5 ± 1</td>
<td>0 ± 1</td>
<td>0 ± 1</td>
<td>80 ± 9</td>
</tr>
<tr>
<td><strong>D</strong> C-1300 (mouse neuroblastoma)</td>
<td>15 ± 1</td>
<td>2 ± 1</td>
<td>1 ± 1</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>251 MG (human astrocytoma)</td>
<td>37 ± 3</td>
<td>8 ± 1</td>
<td>1 ± 1</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

Multiwell plates were coated by air drying the proteins (40 µg/ml of 1% BSA in water, 250 µl/well). Cells (0.5–1 × 10⁴ cells/ml, 250 µl/well) were seeded on the coats in DME containing cycloheximide (25 µg/ml) without or with addition of soluble laminin (collagen IV plus laminin) at a concentration of 200 µg/ml (50 µg/well). After 30 min of incubation, the unattached cells were removed by gentle washing with NaCl/Pi. The firmly attached cells were resuspended using trypsin and counted with a Coulter counter. Results are given as mean ± SD of quadruplicate wells in a single experiment.

* Incubation of 2 h for maximal attachment.

Antibody Inhibition Studies

Antisera against various proteins were used to study the substrate specificity of attachment by inhibition assay. The binding of HT 1080 and 251 MG cells to laminin coats could be clearly inhibited by several but not all antisera against laminin; one example of inhibition is given in Table III. Other antisera directed against a restricted segment of laminin (fragment E 1–4) were found to be inactive (Table III) even though they had radioimmunoassay titers comparable to that of anti-laminin sera. The inhibitory anti-laminin antisera failed, however, to block the attachment of HT 1080 cells to collagen IV coats carried out in the absence or presence of laminin (Table III).

Table II. Variability of Attachment to Extracellular Matrix Proteins between Two Experiments

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% cells attached</th>
<th>Collagen IV</th>
<th>Collagen IV plus laminin</th>
<th>Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT 1080 1</td>
<td>39 ± 3</td>
<td>33 ± 2</td>
<td>43 ± 2</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>23 ± 2</td>
<td>50 ± 1</td>
<td>60 ± 6</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>A 204 1</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>3 ± 0</td>
<td>5 ± 4</td>
<td>3 ± 1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>251 MG 1</td>
<td>58 ± 1</td>
<td>25 ± 1</td>
<td>17 ± 2</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>2</td>
<td>37 ± 3</td>
<td>8 ± 1</td>
<td>1 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>EC 1</td>
<td>11 ± 2</td>
<td>3 ± 1</td>
<td>84 ± 5</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
<td>58 ± 4</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

Coating of wells was carried out as described Table I; the cells were seeded in the same conditions and incubated 30 min. Values represent mean of quadruplicate tests ± SD in two separate experiments (1 and 2).

Figure 2. Concentration dependence of the attachment of HT-1080 cells to collagen IV (○) and its major triple-helical domain (●) used as substrates. The incubation time was 30 min.
Published October 1, 1986

Table III. Inhibition of Cell Attachment to Various Substrates by Antibodies against Laminin and Laminin Fragment E 1-4

<table>
<thead>
<tr>
<th>Antibodies against</th>
<th>Laminin</th>
<th>Collagen IV</th>
<th>Collagen IV plus laminin</th>
<th>251 MG on a coat of laminin</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>68 ± 5</td>
<td>45 ± 1</td>
<td>46 ± 3</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>Laminin</td>
<td>18 ± 2</td>
<td>48 ± 3</td>
<td>52 ± 2</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>Fragment E 1-4</td>
<td>72 ± 4</td>
<td>49 ± 3</td>
<td>43 ± 3</td>
<td>39 ± 4</td>
</tr>
</tbody>
</table>

Multiwell plates were coated by air drying the laminin or collagen IV (40 µg/ml; 250 µl/well) in the presence of BSA. Cells (0.5-× 10^6 cells/ml, 250 µl/well) were seeded onto the coated wells in DME containing cycloheximide (25 µg/ml) without or with addition of soluble laminin (collagen IV plus laminin) at a final concentration of 200 µg/ml and without or with addition of specific antibodies (diluted 1:25) against laminin or against a laminin fragment (E 1-4). After 30 min of incubation, the unattached cells were removed by gentle washing with NaCl/Pi and the firmly attached cells were counted as described in Table I. Values represent mean of quadruplicate tests ± SD in a single experiment.

Several other antisera raised against collagen IV or some of its fragments lacked inhibiting capacity in attachment assays of HT 1080 cells on collagen IV or laminin coats (data not shown). These antisera had particularly strong antibody titers against the terminal domains of collagen IV (39, 51). Lack of sufficient antibodies against the central triple helix may therefore explain their failure to prevent attachment to collagen IV (see below).

Localization of the Cell Attachment Region on Collagen IV

Since collagen IV is a multidendritic protein (51), further studies were aimed at the identification of cell binding sites using attachment assays with HT-1080 cells and human embryo skin fibroblasts. A binding equivalent, or slightly better than that, of intact collagen IV was found for its major triple-helical domain which can be released from the protein by moderate peptic treatment (Fig. 2). Other segments of collagen IV, including the triple helical 7S domain and the globular domain NC1, were relatively ineffective in attachment (Table IV). Low binding to these domains as well as the strong binding to the triple helix was not substantially improved by adding soluble laminin as a mediator (Table IV).

Reduction of disulfide bonds under conditions which do not destroy the triple-helical conformation of collagen IV (48, 51) did not impair its cell-binding activity (Table V). A subsequent heat denaturation which unfolds the triple helix resulted, however, in an 80-90% decrease in cell binding (Tables IV and V) and lack of cell spreading. This was confirmed by showing that unfolded chain constituents of the major triple helix were also inactive in cell binding (Table IV).

Interaction between Collagen IV and Fibronectin, and Its Effect on Cell Attachment

Like laminin, fibronectin was unable to modulate HT-1080 cell binding to native collagen IV substrates when added in soluble form (Table V). Since fibronectin usually interacts poorly with collagens in native form (54), we examined its interaction with native and denatured collagen IV by an enzyme immunoassay reaction (Fig. 3) and by radioligand binding (data not shown). Both assays demonstrated strong binding to the denatured but not to the native protein. This type of interaction is apparently responsible for a fibronectin-mediated fourfold increase of cell binding to denatured collagen IV which is otherwise a poor substrate for HT-1080 cells (Table V). The fibronectin-binding site on denatured collagen IV was identified by an inhibition assay using various collagen IV fragments (Fig. 4). An inhibitory activity equivalent, or twofold better than that, of denatured collagen IV was found for peptic fragments P3 and P2, which originate from the NH₂-terminal half and from the a2(IV) chain COOH-terminal segment of the major triple helix, respectively (47, 48). Other collagenous fragments such as P1 (a1[IV] chain COOH-terminal segment of the major triple helix) or the denatured 7S domain were 50- to 500-fold less active, indicating that this weak activity is due to small contaminations (0.2-2%) by other, more active fragments. No activity was found for the noncollagenous domains, NC1 and NC2. A major fibronectin-binding segment of collagen I has been previously identified as CNBr peptide a1(I) CB7 (reviewed

Table IV. Attachment of HT1080 Cells to Various Domains of Collagen IV

<table>
<thead>
<tr>
<th>Coat</th>
<th>Absent</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen IV</td>
<td>56 ± 5</td>
<td>67 ± 6</td>
</tr>
<tr>
<td>Collagen IV, denatured*</td>
<td>18 ± 5</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Major triple helix domain</td>
<td>53 ± 5</td>
<td>63 ± 6</td>
</tr>
<tr>
<td>Major triple helix domain,</td>
<td>0 ± 0</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>denatured†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7S collagen domain</td>
<td>0 ± 0</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>Globular domain NC1</td>
<td>6 ± 2</td>
<td>6 ± 2</td>
</tr>
</tbody>
</table>

Multiwell plates were coated by air drying the different peptides (40 µg/ml of 1% BSA in water, 250 µl/well). Cells (0.5-× 10^6 cells/ml, 250 µl/well) were seeded in DME containing cycloheximide (25 µg/ml) with or without addition of laminin to the wells (400 µg/ml, 100 µg/well). After 30 min of incubation, the unattached cells were removed by gentle washing with NaCl/Pi and the firmly attached cells were counted as described in Table I. Values represent mean of quadruplicate tests ± SD in a single experiment.

* Reduced and alkylated under nondenaturing conditions before heat denaturation (20 min, 56°C).
† Chain constituents of 140 kD (P large) isolated after reduction by chromatography under denaturing conditions (44).

Table V. Effect of Conformation and Presence of Fibronectin on Attachment of HT1080 Cells to Collagen IV

<table>
<thead>
<tr>
<th>Coat*</th>
<th>Absent</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen IV, native</td>
<td>56 ± 9</td>
<td>48 ± 1</td>
</tr>
<tr>
<td>Collagen IV, denatured</td>
<td>6 ± 2</td>
<td>26 ± 5</td>
</tr>
</tbody>
</table>

Multiwell plates were coated by air drying native and denatured collagen IV (40 µg/ml of 1% BSA in water, 250 µl/well). Cells (0.5-× 10^6 cells/ml; 250 µl/well) were seeded in DME containing cycloheximide (25 µg/ml) with or without addition of soluble fibronectin (400 µg/ml; 100 µg/well). After 30 min of incubation, the results were quantitated as described in Table I. The substrates were reduced and alkylated under nondenaturing conditions in order to prevent refolding of the triple helix after heat denaturation (20 min, 56°C).
in reference 54). A comparison of α1(I) CB7 in the fibronectin–collagen IV assay demonstrated an inhibitory activity almost identical to that of the most active fragment P2 (Fig. 4). This indicates an interesting structural relationship between both collagenous proteins.

**Discussion**

The present study identifies two cell lines (HT-1080 and CHO) and embryonic fibroblasts that showed distinct attachment and spreading on collagen IV substrates equal to or exceeding that on laminin and fibronectin. This attachment occurred in a reproducible fashion and was independent of serum proteins, fibronectin, laminin, and endogenous protein synthesis. Furthermore, attachment to laminin could be blocked by addition of specific antibodies that did not prevent attachment to collagen IV. The data indicate direct interaction with collagen IV structures presumably due to specific cell receptors that have not yet been identified. Still another interaction pattern is based on collagen IV as secondary substrate and laminin as mediator protein as shown previously for epidermal cells (43), some tumor cells (27, 42), and muscle cells (26), and in the present study for an endothelial cell line.

The distinct interaction of HT-1080 cells with collagen IV correlates with the production of collagen IV as a major collagenous component (5). It should be noted, however, that these cells produce very little laminin. Some collagen IV production is also observed by immunofluorescence analysis of embryonic fibroblasts (our unpublished data). It indicates that these cells in culture may use collagen IV as an endogenous substrate. The production of collagen IV, however, must not necessarily correlate with the expression of corresponding receptors as indicated for neuroblastoma C-1300 cells (3) and PYS-2 teratocarcinoma cells (32) which produce collagen IV but attach only poorly to this substrate.

Binding of HT-1080 cells to collagen IV is due to its major triple helix, which has a length of ~330 nm (51) and is dependent on an intact triple-helical conformation. The restricted localization of the cell-binding site indicates the presence of a specific three-dimensional binding structure which is not found on the other triple helical 7S domain and the globular domain of collagen IV. This interaction therefore differs from that of hepatocytes which bind to various types of collagens including collagen IV, unfolded collagen α chains, and even small synthetic peptides with repeating collagen-like sequences (40). A similarly broad specificity has also been found for bacterial collagen receptors (41) and may exist for platelet receptors which bind collagen α chains (12). Chondrocytes, however, possess a 33-kD receptor (anchorin) which binds preferentially to triple-helical collagen II (29, 30). These cell-binding sites are sensitive to proteases, indicating that anchorin is different from the collagen IV receptor. Binding of collagen I to fibroblasts has also been attributed to a high affinity receptor (20) but its specificity has so far not been examined. Together, the data indicate that cell-binding to collagenous substrates is a frequently observed phenomenon but may vary substantially in its molecular features.

The multidomain structure of collagen IV (51) is apparently reflected in a diverse distribution of various biological activities. The terminal segments comprising the 7S domain and globular NC1 domain are the most important sites for self assembly, and thus are crucial structures for macromolecular organization and stability (51). The major triple
helix which connects these terminal domains has been identified as possessing binding sites for laminin and fibronectin (II, 25, 53) and for heparan sulfate proteoglycan which are also present on domain NC1 (19, 25). Electron microscopy has indicated that laminin and fibronectin binding sites involve different segments of the major triple helix. Since neither laminin nor fibronectin interferes with attachment of cells to collagen IV it is likely that the cell-binding sites are located on a different segment. A possible colocalization with heparan sulfate–binding sites remains to be studied.

Our binding analyses also confirmed previous data that fibronectin interacts preferentially with denatured collagen IV when compared to native collagen IV (17, 18, 53). It thus appears unlikely that this kind of interaction contributes to cell binding in basement membranes. The fibronectin-binding sites could be localized to pepsin fragments P2 (50 kD) from the a2(IV) chain and P3 (70 kD) originating from the major triple helix of collagen IV (44). A single site with a rather broad distribution was attributed to fibronectin binding of collagen IV by electron microscopic studies (25).

These data are not necessarily incompatible since fragments P2 and P3 are located adjacent to each other but do not overlap (44, 45, 47). Most interesting was the observation that the fibronectin-binding peptide aI CB7 of collagen I also strongly competed with the binding of collagen IV. The binding site in aI CB7 has been mapped to a segment containing not more than 34 amino acid residues (22). Other data, however, demonstrated a low homology between aI(I) and aI(IV) chains (8).

Collagens I and IV may have, however, share a short consensus sequence responsible for fibronectin binding which may be identified upon further sequence analysis. The study was supported by grants of the Deutsche Forschungsgemeinschaft and by the Fritz Thyssen Stiftung.

Received for publication 14 February 1986, and in revised form 20 May 1986.

References


The Journal of Cell Biology, Volume 103, 1986


