Attachment of Cells to Basement Membrane

Collagen Type IV

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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.

Basement membranes are extracellular protein matrices that are involved in cell–matrix interactions due to their anatomical location and wide-spread occurrence in tissues. Laminin, a cross-shaped 1,000-kD protein (16, 50) has been identified as one of the major cell-binding components of basement membranes (21, 43) and in this respect appears to be functionally similar to fibronectin (54). Binding sites for hepatocytes and tumor cells were identified in structures of the short arms of laminin (7, 38, 42, 46) while a neuron-binding site was found in the long arm of laminin (15). Some of these binding sites interact specifically with receptors which have been identified as 68-kD integral plasma membrane proteins in muscle and tumor cells (26, 27, 37). Further possibilities for molecular interactions include the binding of laminin to collagen IV (11, 53), to proteoglycans (19, 53), and to nidogen (14). Whether these other basement membrane proteins also directly interact with receptors of cell membranes has so far not been established. It is thought that major cell–matrix interactions occur via laminin which anchors the cells to basement membranes by forming bridges to collagen IV (21, 43).

Collagen IV, which forms large networks within basement membranes (51, 55), has so far been considered to provide mechanical stability to these matrices while less is known about other potential biological properties (reviewed in reference 45). Several studies, however, indicated that some cells may attach and respond to collagen IV substrates including keratinocytes (31), hepatocytes (40), endothelial cells (35), myocytes (9), and tumor cells (13, 17, 34, 52). The molecular basis of this interaction and the possible requirement for mediator proteins has not yet been adequately analyzed. A cell surface–associated protein which binds native collagen IV has been identified for mouse embryonic parietal endoderm cells although it is not known whether this protein participates in cell attachment and spreading (23).

In the present study, we have identified several cell lines that interact directly with collagen IV and have characterized this potential with regard to known interaction patterns for laminin and fibronectin.

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, M. 140,000) and large fragments of this region—P1 (COOH-terminal peptide fragment of all[IV], 50 kD), P2 (COOH-terminal peptide 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 α[I]ICB7 was prepared from rat collagen I (80).

Antisera and Immunoblotting

Rabbit antisera against laminin were prepared as described previously (50). Similar immunization protocols were used for the production of antisera against laminin fragment E 1-4 which contains the three short arms of laminin (33). Antisera 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 fragments P1, P2, and P3, was performed as described previously (51).
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 × 10^5 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 chondrosarcoma 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. Yuspa, National Cancer Institute, Bethesda. Human breast carcinoma cells (MCF-7) 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).

Cells were grown to confluency in Dulbecco's modified Eagle's medium (DME) supplemented with glutamine (300 μg/ml), sodium ascorbate (50 μg/ml), penicillin (400 U/ml), streptomycin (50 μg/ml), and 10% fetal calf serum. For attachment assays, cell monolayers were incubated for 30-60 min with serum-free medium containing cycloheximide (25 μg/ml). Cells were then detached by 0.05% trypsin, 0.02% EDTA in phosphate-buffered saline (PBS) at 37°C. The results obtained with both coating procedures did not differ by more than 8%. All data reported under Results were obtained with the air-drying procedure. Control dishes were coated with serum albumin alone and showed only 0-10% attachment of the various cells studied. These background values were subtracted from the number of cells attached to various other proteins.

**Attachment and Spreading Assays**

Coating of tissue culture plastic dishes (24 multi-well plates or 35-mm Falcon dishes) was performed by air drying protein solutions overnight at 37°C. Coating with collagen IV was also done without air drying (incubation overnight at 4°C). The results obtained with both coating procedures did not differ by more than 8%. All data reported under Results were obtained with the air-drying procedure. Proteins were diluted to a standard concentration of 40 μg/ml in distilled water containing bovine serum albumin (10 mg/ml) and used in volumes of 125 μl per cm² well surface. Control dishes were coated with serum albumin alone and showed only 0-10% attachment of the various cells studied. These background values were subtracted from the number of cells attached to various other proteins.

Aliquots of the cell suspensions (see above) were plated onto coated dishes at a density of 5 × 10^4 cells/cm² and cells were allowed to attach for 30-60 min at 37°C in a humidified incubator. At the end of the attachment period, the medium was removed and the attached cells were once washed with NaC1/Pi and photographed to record cell spreading. The number of attached cells was then determined after resuspension in 0.05% trypsin, 0.02% EDTA in NaC1/Pi in a Coulter counter or by using a flurometric assay for DNA (24). The original cell suspension was used for calibrating the latter assay.

Some assays were carried out in the presence of soluble proteins or in the presence of specific antisera against basement membrane proteins. Coated dishes were incubated with serum-free medium containing laminin or fibronectin (concentrations between 100 and 400 μg/ml) and/or antisera (diluted 1:25) for 60 min at 37°C before the addition of cells. The attachment assay was then performed in the presence of these proteins and/or antibodies.

**Protein Binding Assays**

For solid-phase binding assays, following the principle of enzyme immunoassays (4), ELISA plates were coated with collagen IV (10 μg/ml; 100 μl/well) and nonspecific binding sites were subsequently masked by incubation (4°C, overnight) with bovine serum albumin in NaC1/Pi (10 mg/ml). Wells were then incubated with various dilutions of fibronectin (maximal concentration, 20 μg/ml), with or without NaC1/Pi, and incubated with appropriate dilutions of antisera (1:250) against fibronectin. Bound antibodies were then detected by applying peroxidase-conjugated second antibody (49).

Protein binding was also determined by a variant of radioimmunoassays (49). Collagen IV was labeled with 125I by the chloramine T method and used at concentrations of 2 ng/0.1 ml. Binding to fibronectin was analyzed by precipitating the complexes with antiserum to fibronectin followed by goat antiserum to rabbit IgG (24). In the inhibition assay, an appropriate amount of fibronectin giving 50% maximal binding was preincubated with various diluents of the inhibitors overnight at 4°C before the binding assay (49).

**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 variable 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 embry 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
Table I. Attachment of Different Cell Lines to Extracellular Matrix Proteins

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

Multiwell plates were coated by air drying the proteins (40 µg/ml of 1% BSA in water, 250 µl/well). Cells (0.5-1 × 10⁶ 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.

Reactions were, however, observed for similar extracts prepared from PYS-2 and PAM 212 cells.

**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 antilaminin 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**

| Cell line | % cells attached to | Collagen IV | Collagen IV plus laminin | Fibronectin |
|-----------|---------------------|-------------|--------------------------|-------------|
|           | Laminin             | Collagen IV | Collagen IV plus laminin | Fibronectin |
| **A** HT 1080 | 39 ± 2              | 43 ± 2      | 29 ± 1                   |             |
|           | 23 ± 2              | 50 ± 1      | 60 ± 6                   | 7 ± 1       |
| A 204     | 0 ± 0               | 0 ± 0       | 2 ± 1                    |             |
|           | 3 ± 0               | 5 ± 4       | 3 ± 1                    | 1 ± 1       |
| 251 MG    | 58 ± 1              | 25 ± 1      | 17 ± 2                   | 0 ± 0       |
|           | 37 ± 3              | 8 ± 1       | 1 ± 1                    |             |
| EC        | 11 ± 2              | 3 ± 1       | 84 ± 5                   | 20 ± 1      |
|           | 5 ± 1               | 5 ± 1       | 58 ± 4                   | 4 ± 1       |

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.
Localization of the bryo skin fibroblasts. A binding equivalent, or slightly better not shown). These antisera had particularly strong antibody titers against the terminal domains of collagen IV (39, 51).

Since collagen IV is a multidomain 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 pepsin 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 two-fold better than that, of denatured collagen IV was found for pepsin 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(1) CB7 (reviewed

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

| Antibodies against | Laminin | Collagen IV | Collagen IV plus laminin | 251 MG on a coat of laminin |
|-------------------|---------|------------|--------------------------|-----------------------------|
| None              | 68 ± 5  | 45 ± 1     | 46 ± 3                   | 31 ± 3                      |
| Laminin           | 18 ± 2  | 48 ± 3     | 52 ± 2                   | 15 ± 4                      |
| Fragment E 1–4    | 72 ± 4  | 49 ± 3     | 44 ± 3                   | 39 ± 4                      |

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

| Coat               | Absent | Present |
|--------------------|--------|---------|
| Collagen IV        | 56 ± 5 | 67 ± 6  |
| Collagen IV, denatured* | 18 ± 5 | 11 ± 3  |
| Major triple helix domain | 53 ± 5 | 63 ± 6  |
| Major triple helix domain, denatured‡ | 0 ± 0  | 14 ± 1  |
| 7S collagen domain | 0 ± 0  | 5 ± 3   |
| Globular domain NC1 | 6 ± 2  | 6 ± 2   |

* Multiwell plates were coated by air drying the different peptides (40 µg/ml of 1% BSA in water, 250 µl/well). Cells (0.5–1 x 10⁶ 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 antisera (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.

‡ Chain constituents of 140 kD (P large) isolated after reduction by chromatography under denaturing conditions (44).

| Coat*               | Absent | Present |
|---------------------|--------|---------|
| Collagen IV, native | 56 ± 9 | 48 ± 1  |
| Collagen IV, denatured | 6 ± 2  | 26 ± 5  |

* The substrates were reduced and alkylated under non-denaturing conditions in order to prevent refolding of the triple helix after heat denaturation (20 min, 56°C).
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 NCI (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 α2(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 microscopical 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 α1 CB7 of collagen I also strongly competed with the binding of collagen IV. The binding site in α1 CB7 has been mapped to a segment containing not more than 34 amino acid residues (22). Other data, however, demonstrated a low homology between α1(I) and α1(IV) chains (8). Collagens I and IV may, however, share a short consensus sequence responsible for fibronectin binding which may be identified upon further sequence analysis.

The study was supported by the Deutsche Forschungsgemeinschaft and by the Fritz Thyssen Stiftung.

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

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