Cell Surface Proteoglycan Binds Mouse Mammary Epithelial Cells to Fibronectin and Behaves as a Receptor for Interstitial Matrix

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Abstract. The proteoglycan (PG) on the surface of NMuMG mouse mammary epithelial cells consists of at least two functional domains, a membrane-intercalated domain which anchors the PG to the plasma membrane, and a trypsin-releasable ectodomain which bears both heparan and chondroitin sulfate chains. The ectodomain binds cells to collagen types I, III, and V, but not IV, and has been proposed to be a matrix receptor. Because heparin binds to the adhesive glycoproteins fibronectin, an interstitial matrix component, and laminin, a basal lamina component, we asked whether the cell surface PG also binds these molecules. Cells harvested with either trypsin or EDTA bound to fibronectin; binding of trypsin-released cells was inhibited by the peptide GRGDS but not by heparin, whereas binding of EDTA-released cells was inhibited only by a combination of GRGDS and heparin, suggesting two distinct cell binding mechanisms. In the presence of GRGDS, the EDTA-released cells bound to fibronectin via the cell surface PG. Binding via the cell surface PG was to the COOH-terminal heparin binding domain of fibronectin. In contrast with the binding to fibronectin, EDTA-released cells did not bind to laminin under identical assay conditions. Liposomes containing the isolated intact cell surface PG mimic the binding of whole cells. These results indicate that the mammary epithelial cells have at least two distinct cell surface receptors for fibronectin: a trypsin-resistant molecule that binds cells to the sequence RGD and a trypsin-labile, heparan sulfate–rich PG that binds cells to the COOH-terminal heparin binding domain. Because the cell surface PG binds cells to the interstitial collagens (types I, III, and V) and to fibronectin, but not to basal lamina collagen (type IV) or laminin, we conclude that the cell surface PG is a receptor on epithelial cells specific for interstitial matrix components.

CELLULAR behavior appears to be regulated in part by interactions with the extracellular matrix. Binding of insoluble extracellular matrix proteins to cell surface receptors is transduced into specific cellular responses by mechanisms still unknown in detail. In many instances, these responses both appear to involve associations with the cytoskeleton and to occur in multiple stages. For example, fibroblast adhesion to fibronectin is a multistage process involving initial cell attachment followed by cell spreading and the appearance of stress fibers and focal adhesions (Heath and Dunn, 1978; Couchman and Rees, 1979; Thom et al., 1979; Badley et al., 1980). Initial fibroblast attachment to fibronectin has been suggested to involve recognition of a specific tripeptide sequence RGD (Arg-Gly-Asp) via a 140-kD cell surface receptor complex (Pierschbacher and Ruoslahti, 1984; Pytela et al., 1985). However, subsequent cell spreading, with the development of close contacts followed by focal adhesions and stress fiber formation, appears to involve interactions between a cell surface proteoglycan (PG)[1] and the heparin-binding domain of fibronectin (Laterra et al., 1983; Lark et al., 1985; Izzard et al., 1986; Woods et al., 1986). The evolution of the loss of close contacts and the appearance of focal adhesions correlates with the cessation of fibroblast cell movement (Couchman and Rees, 1979), suggesting a possible cell anchoring role for the cell surface PG of these cells.

These interactions between fibroblasts and the extracellular matrix differ markedly from those of epithelial cells. Simple epithelial cells exist in highly polarized sheets, bound together by junctional complexes, and contact the matrix solely at their basal surfaces. The cells in the sheets have sta-

1. *Abbreviations used in this paper*: Ecto, ectodomain; FN, fibronectin; GAG, glycosaminoglycan; GdnHCl, guanidine hydrochloride; GRGDS, glycine-arginine-glycine-aspartic acid-serine; HBD, heparin binding domain of fibronectin; Hep, heparin; LM, laminin; NMuMG cells, normal murine mammary gland epithelial cells; PG, proteoglycan; SDGRG, serine-aspartic acid-glycine-arginine-glycine.

ble shapes and do not spread or move with respect to their matrix. NMuMG mouse mammary epithelial cells demonstrate the properties of a simple epithelium, forming polarized sheets in culture (Owens et al., 1974) and ductal structures both in vitro (Bernfield et al., 1984) and in vivo (David et al., 1981). Therefore, we have utilized these cells to examine the receptors involved in the interaction of epithelial cells with the extracellular matrix.

Mouse mammary epithelial cells possess a cell surface PG with at least two functional domains; a lipophilic membrane-intercalated domain and a trypsin-releasable extracellular ectodomain (Rapraeger and Bernfield, 1985) that bears both heparan and chondroitin sulfate chains (Rapraeger et al., 1985). Immunolocalization of adult mouse tissues with the monoclonal antibody 281-2 (Jalkanen et al., 1985) and with a serum antibody (Jalkanen et al., 1988) (both directed against polypeptide determinants on the core protein) indicates that the antigen is on the surfaces of a wide variety of epithelial cells but is not on mesenchymal cells (Hayashi et al., 1987), and thus is a distinct gene product from the cell surface PG of fibroblasts. Because the mammary epithelial cell surface proteoglycan binds cells to fibrillar interstitial collagens (types I, III, and V) with high affinity and specificity (Koda et al., 1985), associates with the cytoskeleton when cross-linked in the plane of the plasma membrane (Rapraeger et al., 1986), and is polarized to the basolateral surface, we have proposed that it is a matrix receptor for interstitial collagens.

The known affinity of heparin and heparan sulfate for fibronectin (Laterra et al., 1980; Ruoslahti and Engvall, 1980; Yamada et al., 1980; Stamatoglou and Keller, 1982; Fujiwara et al., 1984) and for laminin (Sakashita et al., 1980; Woodly et al., 1983; Fujiwara et al., 1984) suggested that the cell surface PG might also be an epithelial cell receptor for these adhesive glycoproteins. Therefore, we have assessed its role in the binding of mammary epithelial cells to these glycoproteins. We find that trypsin-released cells bind to fibronectin but this binding is inhibited by the synthetic peptide Gly-Arg-Gly-Asp-Ser (GRGDS), suggesting the presence of a receptor similar to the 140-kD fibronectin receptor on fibroblasts. EDTA-harvested cells, however, bind to fibronectin via both this RGD receptor and the cell surface PG. In the presence of GRGDS, the binding of EDTA-harvested cells to fibronectin is sensitive to heparan sulfate lyase treatment of the cells and to competitive inhibition by both heparin and the purified ectodomain of the cell surface PG. This binding via the cell surface PG is to the COOH-terminal heparin binding domain of fibronectin. These binding properties are replicated by liposomes in which purified cell surface PG has been intercalated, confirming that the cell surface PG is responsible for the binding of the cells. Under identical conditions, neither EDTA-released cells nor liposomes containing intercalated cell surface PG bind to laminin. These data indicate that the cell surface PG is one of at least two fibronectin receptors on NMuMG cells and that this PG binds via its heparan sulfate chains to the COOH-terminal heparin-binding domain of fibronectin. The binding to interstitial collagens and fibronectin and the lack of binding to type IV collagen and laminin suggest that the cell surface PG is a specific receptor. Part of these data have been published in abstract form (Saunders et al., 1986).

Materials and Methods

Cell Culture

NMuMG mouse mammary epithelial cells (passages 13–22) were maintained in bicarbonate-buffered DME (Gibco, Grand Island, NY) as described previously (David and Bernfield, 1979). For cell binding assays, cells were plated on 35- or 60-mm dishes (Falcon Labware, Oxnard, CA) at approximately one-fifth confluent density, grown to confluency (3–4 d), and used within 2–3 d. For preparation of purified cell surface proteoglycan, cells were plated at approximately one-fifth confluent density on a 24.5 × 24.5-cm tissue culture plate (Falcon Labware), grown to confluency, and radiolabeled for 24 h in low sulfate medium (David and Bernfield, 1979) containing 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 100 μCi/ml $^{35}SO_4$ (ICN Biomedicals Inc., Irvine, CA).

Cell Binding Assay

This assay is based on a technique designed to test for the reaction of cell surface antigens with mAbs (Eisenbarth et al., 1980) and has been previously used to demonstrate the binding of NMuMG cells to various interstitial collagens (Koda et al., 1985). PVC multiwells (U-shaped bottom, Dynatech Corp., Alexandria, VA) were coated with proteins by incubating PBS (134 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) solutions of the desired protein (BSA, 10 mg/ml; fibronectin, 50 μg/ml; heparin binding domain, 15 μg/ml; laminin, 100 μg/ml) with the wells overnight at 4°C. Remaining binding sites were saturated before the binding assay by incubating the wells for 1 h at room temperature with 1 mg/ml BSA in PBS (buffer A). The media was removed from newly confluent monolayers of NMuMG cells and the cells were washed twice with cold (4°C) PBS plus 0.5 mM disodium EDTA (buffer B). Cells were harvested by either trypsinization or EDTA rounding and scraping.

For trypsinization cells on the washed monolayer were detached by 100 μg/ml trypsin (Gibco) in Tris-EDTA at room temperature. After detachment (5–10 min), the trypsinization was stopped by addition of 500 μg/ml soybean trypsin inhibitor (Calbiochem-Behring Corp., La Jolla, CA) and the cells transferred to a chilled centrifuge tube. After two washes by suspension and centrifugation (2 min, 240 *g*) in a clinical centrifuge (International Equipment Co., Needham Heights, MA), the cells were resuspended at ~3.5 × 10^5 cells/ml in cold (4°C) buffer A by pipetting until well dispersed.

For EDTA rounding-cell scraping, cells on the washed monolayer were rounded by a 10-min incubation with buffer B at room temperature. After carefully removing the buffer B from the rounded cells, the plate was placed on ice and the cells gently scraped into cold (4°C) buffer B using a rubber policeman. The scraped cells were gently washed at 4°C as just described, dispersed on ice by pipetting, and resuspended at ~3.5 × 10^5 cells/ml in buffer A. Suspension at cold temperatures prevents the shedding of the cell surface PG, as described elsewhere (Jalkanen et al., 1987).

Cell binding was assayed by pipetting 200 μl of the cell suspension into each well and incubating the cells with the wells for 30 min at room temperature. The microtiter plate was then centrifuged 15 min (Dynatech plate holder) at 800 rpm in a model UV centrifuge (International Equipment Co.). After centrifugation, 50 μl of 4% glutaraldehyde in PBS was added to each well and the plates fixed overnight at room temperature. The cells were then stained with trypan blue (Gibco) in PBS and photographed. If cells bind to the well, they remain as a uniform coating covering the bottom surface of the well; if the cells do not bind, they form a pellet at the bottom of the well.

Inhibitors of Cell Binding

To assay for the effect of heparin, we pretreated protein-coated wells with heparin before the addition of the cell suspension. After incubation with buffer A, the protein-coated wells were rinsed once with and subsequently incubated with 10 μM heparin (Upjohn Co., Kalamazoo, MI; beef lung 10,000 mol wt average, thrice precipitated with ethanol) in buffer A for 30 min at room temperature. The heparin solution was removed before addition of the cell suspension.

The ectodomain of the cell surface proteoglycan, prepared from NMuMG cell conditioned media by DEAE chromatography, $CsCl_2$ density centrifugation, and mAb affinity chromatography as described elsewhere (Jalkanen et al., 1987), was assayed as for heparin.

The effects of the synthetic peptides GRGDS and SDGRG (generously supplied by Dr. Russell Doolittle, Dept. of Chemistry, University of

California at San Diego) were assessed by addition of the peptide, at the concentrations indicated, to the cell suspension before its introduction into the protein-coated wells.

For heparitinase treatment, before harvesting the cells by EDTA rounding and scraping as just described, the washed monolayer was treated for 15 min at 37°C with 0.1 U/ml *Flavobacterium heparinum* heparitin sulfate lyase (heparitinase, Miles Laboratories Inc., Naperville, IL) in PBS containing 0.1% BSA and 1.25 mM $CaCl_2$. In addition, 0.1 U/ml of enzyme was included in the cell suspension during incubation of the cells with the wells.

Purification Of Human Plasma Fibronectin and Its Fragments

Fibronectin was prepared from human plasma by affinity chromatography on gelatin-Sepharose as described previously (Miekka et al., 1982). The purified fibronectin was concentrated, as necessary, by dialysis against 30% polyethylene glycol (Baker, 20,000 mol wt) in PBS.

The COOH-terminal heparin binding domain of fibronectin was prepared as described (Zardi et al., 1985). Briefly, purified fibronectin (ca. 30 mg at 1 mg/ml) in 25 mM Tris/HCl pH 7.6, containing 0.5 mM EDTA, 50 mM NaCl, and 2.5 mM $CaCl_2$ was digested with 5 μg/ml thermolysin (Sigma Chemical Co., St. Louis, MO) for 4 h at room temperature. After adding EDTA to a final concentration of 5 mM, the digestion products were dialyzed against 0.5 mM sodium phosphate containing 0.1% sodium azide, pH 6.8, and purified by hydroxyapatite (Bio-Rad Laboratories, Richmond, CA) chromatography (1.5 × 18-cm column, at 15 ml/h), using a 0.5–190 mM linear gradient of pH 6.8 sodium phosphate, 0.1% sodium azide. Fractions containing the COOH-terminal heparin binding domain were identified by polyacrylamide gel electrophoresis, pooled, dialyzed against PBS, and stored at −70°C.

Preparation Of Liposome Intercalated Cell Surface Proteoglycan

A radiolabeled 24.5 × 24.5-cm monolayer of NMuMG cells was washed three times in cold (4°C), Tris-buffered (Sigma Chemical Co., 10 mM, pH 7.4) isotonic saline, containing 1.25 mM $CaCl_2$ and 0.9 mM $MgSO_4$. The monolayer was then scraped and washed twice in cold (4°C) Ca^{2+}, Mg^{2+}-free Tris-buffered saline plus 0.5 mM disodium EDTA. The cells were suspended in 5 ml Tris-acetate buffered saline (TAS; 10 mM Tris, 50 mM sodium acetate, 150 mM sodium chloride, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM *N*-ethylmaleimide, 5 mM benzamidine) pH 5.0, containing 75 mM octyl glucoside (Calbiochem-Behring Corp.) for 10 min on ice. The detergent-extracted residue was centrifuged (100 *g*, 5 min) and resuspended in TAS containing 75 mM octyl glucoside and 0.5 M KCl for 10 min on ice.

The KCl extract, which contains the cell surface PG (Rapraeger et al., 1986), was diluted fivefold with 6 M urea (Schwarz-Mann, Spring Valley, NY), 50 mM sodium acetate pH 4.5 (Schwarz-Mann), 75 mM octyl glucoside, and subjected to DEAE chromatography at 4°C. A 1-ml DEAE-Sephacel column (Pharmacia Fine Chemicals, Piscataway, NJ) preequilibrated with 6 M urea, 0.4 M NaCl, 50 mM sodium acetate, pH 4.5, and 50 mM octyl glucoside (buffer D), was loaded at 8 ml/h and then washed sequentially with 6 ml of buffer D, followed by 6 ml buffer D without urea. The ^{35}S-labeled cell surface proteoglycan was eluted with 0.8 M NaCl, 50 mM sodium acetate, pH 4.5, and 50 mM octyl glucoside.

The cell surface PG was further purified on a mAb immunoaffinity column (1 mg/ml 281-2, linked to CNBr-Sepharose) (Jalkanen et al., 1985). The fraction from DEAE was diluted fivefold with 50 mM Tris pH 7.4, 50 mM octyl glucoside and loaded at 20 ml/h on a 2.5 × 3.5-cm immunoaffinity column preequilibrated with 50 mM Tris, pH 7.4, 50 mM octyl glucoside, and 0.15 M NaCl. The immunoaffinity column was washed with 15 ml of the equilibration buffer and then eluted with 50 mM triethylamine, pH 11.4, containing 50 mM octyl glucoside. The eluted ^{35}S-labeled cell surface PG was immediately neutralized with a one-tenth volume of 1 M Tris, pH 7.4. Purity was assessed by polyacrylamide gel electrophoresis (Koda et al., 1985).

Purified ^{35}S-labeled cell surface PG was intercalated into liposomes as described previously (Rapraeger and Bernfield, 1985). Briefly, the ^{35}S-labeled PG in 4 M GdnHCl (Schwarz-Mann), 50 mM Tris (pH 8.0), 0.1 M NaCl (buffer E) containing 75 mM octyl glucoside, was brought to 5 mg/ml with soybean phospholecithin (Sigma Chemical Co.) in buffer E containing 75 mM octyl glucoside. Liposomes were allowed to form by overnight dialysis against buffer E, and subsequently dialyzed against buffer A for use.

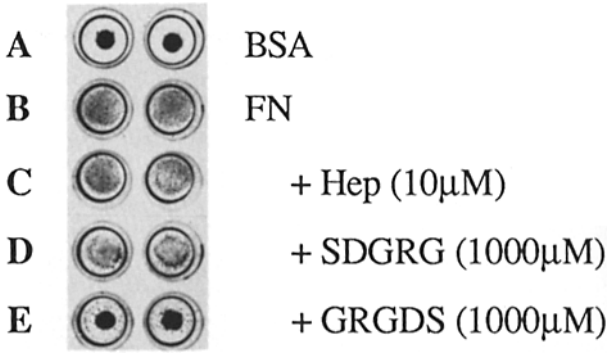

Figure 1. Trypsin-released cells bind to fibronectin via an RGD mechanism. NMuMG cells grown to confluency and released with trypsin as described in Materials and Methods do not bind to BSA (*A*), but bind to fibronectin (*FN*) (*B*). Heparin (*Hep*) (*C*) and the control peptide SDGRG (*D*) do not inhibit cell binding. However, binding to fibronectin is inhibited by the pentapeptide GRGDS containing the RGD binding sequence (*E*).

Solid Phase Binding Of Liposomes Containing Cell Surface Proteoglycan

Individual PVC wells (Dynatech) were coated and preincubated as described for the cell binding assay above. The preincubation solution (with or without heparin) was removed and 50 μl of liposomes containing ^{35}S-PG (10,000 cpm/well, Fig. 7, or 6,000 cpm/well Fig. 9) in buffer A added, and incubated for 2.5 h at room temperature. After incubation, the proteoglycan solution was removed and the wells washed five times with buffer A. The amount of ^{35}S-PG bound was quantitated by cutting out the wells, placing them in Aquasol scintillant and counting directly in a model LS-7500 scintillation counter (Beckman Instruments Inc., Fullerton, CA).

Results

NMuMG Epithelial Cells Possess More Than One Cell Surface Receptor for Fibronectin

The 140-kD cell surface complex that binds to a region of fibronectin containing the sequence Arg-Gly-Asp (RGD) is not removed from fibroblast cell surfaces by trypsin treatment (Tarone et al., 1982; Giancotti et al., 1985). To evaluate whether epithelia show similar binding properties, we assessed the binding of trypsinized NMuMG cells to fibronectin (Fig. 1). NMuMG cells harvested with 100 μg/ml trypsin in EDTA bind to fibronectin via the RGD sequence (Fig. 1 *B*). Binding to fibronectin was not inhibited by pretreatment of the wells with 10 μM heparin (Fig. 1 *C*). However, binding was inhibited by the synthetic peptide Gly-Arg-Gly-Asp-Ser (GRGDS) (Fig. 1 *E*), but not by the peptide Ser-Asp-Gly-Arg-Gly (SDGRG) (Fig. 1 *D*). Trypsin-released cells that were subsequently incubated for 45 min at 37°C in complete medium behaved identically (data not shown). Therefore, trypsinized NMuMG cells appear to bind similarly as the trypsinized fibroblastic cells, viz. via a receptor for an RGD sequence in fibronectin.

Trypsin treatment removes many integral and peripheral membrane proteins from cells, including fibronectin (Ruoslahti et al., 1982) and a cell surface PG (Rapraeger and Bernfield, 1985). To assess whether trypsin treatment modifies the binding of NMuMG cells to fibronectin, the cells were harvested following rounding with EDTA (Fig. 2). NMuMG

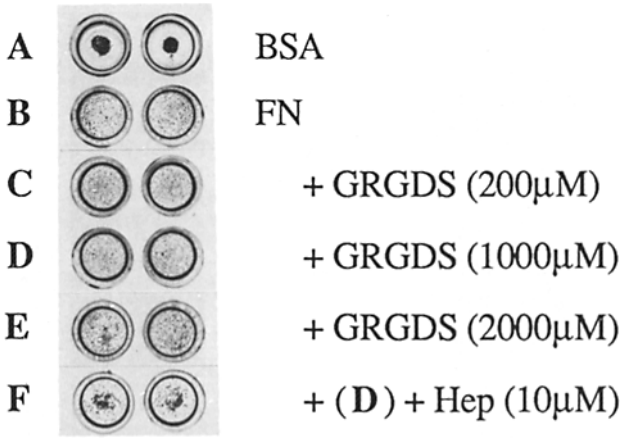

Figure 2. EDTA-released cells bind to fibronectin via both an RGD and a heparin-sensitive mechanism. NMuMG cells grown to confluency and released with EDTA, as described in Materials and Methods, do not bind to BSA (*A*) but bind to fibronectin (*FN*) (*B*). However, unlike trypsin-released cells, binding is not inhibited by GRGDS alone (*C*, *D*, and *E*), even at a concentration twice that which inhibits the binding of trypsin-released cells. After pretreatment of fibronectin-coated wells with heparin (*Hep*), cell binding is inhibited at 1,000 μM GRGDS (*F*).

cell monolayers were incubated for 10 min in EDTA at room temperature, placed at 4°C, then scraped and dispersed into a cell suspension in cold buffer. These cells bound to fibronectin (Fig. 2 *B*), but the binding was via a mechanism that is distinct from that of the trypsin-released cells. Unlike trypsin-released cells, the binding of EDTA-released cells was unaffected by the addition of GRGDS at concentrations up to 2,000 μM (Fig. 2, *C–E*), twice that which readily inhibits the binding of trypsin-released cells. However, GRGDS did inhibit binding when the fibronectin was pretreated with 10 μM heparin (Fig. 2 *F*). Thus, the EDTA-released cells apparently bind to fibronectin differently than the trypsin-released cells.

NMuMG Cells Bind to Fibronectin via the Cell Surface Proteoglycan

NMuMG cells possess a cell surface PG that is cleaved from the cells by mild proteolysis (Rapraeger and Bernfield, 1985) and which binds the cells to interstitial collagens (Koda et al., 1985). We investigated whether the cell surface PG also binds these epithelial cells to fibronectin. To test this possibility, EDTA-released cells were incubated with fibronectin. As expected, treatment with GRGDS did not inhibit cell binding (Fig. 3 *B*) except after pretreatment of the fibronectin with heparin (Fig. 3 *C*). This effect of heparin was duplicated by pretreatment of the fibronectin with the purified ectodomain of the cell surface PG at 0.4 μM (Fig. 3 *E*), suggesting that the heparan sulfate glycosaminoglycan (GAG) chains of the cell surface PG mediate the binding. Indeed treatment of NMuMG cells with heparan sulfate lyase (heparitinase), which preserves the integrity of the proteoglycan core protein while degrading the heparan sulfate GAG chains (Rapraeger et al., 1986), causes cell binding to become susceptible to GRGDS in a dose-dependent fashion (Fig. 4, *C–F*). Therefore, binding of NMuMG cells to fibronectin is mediated both by a trypsin-resistant cell surface molecule that recognizes the RGD sequence and by a trypsin-sensitive heparan sulfate PG.

NMuMG Cells Bind to the COOH-terminal Heparin Binding Domain of Fibronectin via the Cell Surface PG

Fibronectin has two sites that bind heparin, a lower affinity, divalent cation dependent site at the NH_2-terminus and a higher affinity site at the COOH terminus (Hayashi and Yamada, 1982). Binding of NMuMG cells via the cell surface PG occurs in the absence of divalent cations suggesting that the NH_2-terminal site is not involved and that this binding is to the high affinity site. To evaluate this possibility we purified the COOH-terminal heparin binding domain from thermolysin digests of fibronectin. EDTA-released NMuMG cells bound to the heparin binding domain (Fig. 5 *B*) but, in contrast with intact fibronectin, binding was inhibited by pretreatment with 10 μM heparin (Fig. 5 *C*) and was unaffected by GRGDS (Fig. 5 *D*). The COOH-terminal heparin binding domain from chymotryptic digestion of fibronectin yielded identical results (data not shown). These observations indicate that the binding of the NMuMG cells via the cell surface PG is mediated by the COOH-terminal heparin binding domain.

Liposomes Containing Intercalated Cell Surface Proteoglycan Mimic the Binding Properties of NMuMG Cells

The ability of the isolated ectodomain and intact cell surface PG to bind to fibronectin was investigated to assess whether either duplicated the binding properties of the cells. Although able to inhibit cell binding to fibronectin at high concentration, the isolated ectodomain of the cell surface PG bound poorly to fibronectin in a solid-phase assay (data not shown). Therefore, the binding of liposomes containing intercalated intact cell surface PG was examined. The liposomes bound to fibronectin (Fig. 6 *B*), but not to BSA (Fig. 6 *A*). This binding was inhibited by heparin pretreatment of the wells (Fig. 6 *C*), but was unaffected by GRGDS (Fig. 6 *D*). Furthermore, binding of liposomes to the COOH-terminal heparin binding domain (Fig. 6 *E*) was inhibited by pretreating the wells with heparin (Fig. 6 *F*). Therefore,

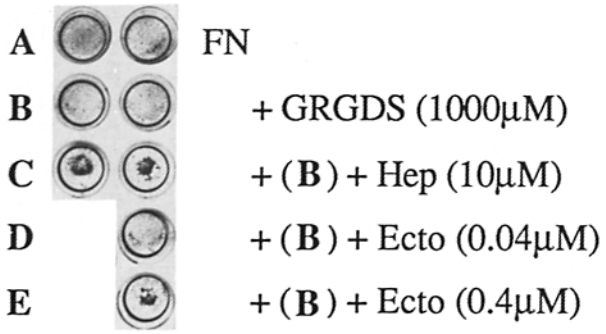

Figure 3. The extracellular domain (ectodomain) of the cell surface PG mimics heparin. EDTA-released cells, as in Fig. 2, bind to fibronectin (*FN*) (*A*) and this binding is not inhibited by GRGDS alone (*B*). When the fibronectin is pretreated with heparin (*Hep*), GRGDS treatment results in complete inhibition of binding (*C*). The purified ectodomain (*Ecto*) of the cell surface PG mimics this effect of heparin in a dose-dependent manner (*D*, *E*).

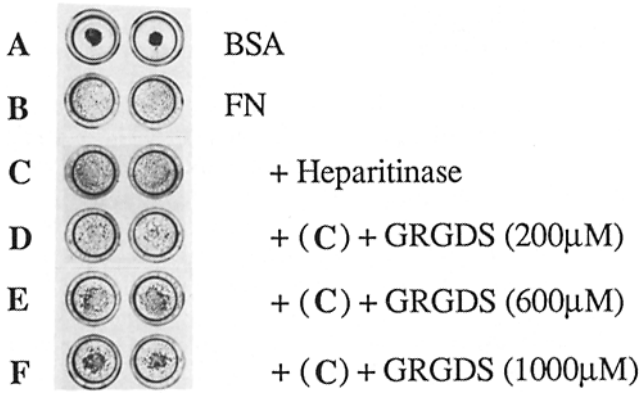

Figure 4. Removal of cell surface heparan sulfate prevents EDTA-released cells from binding via the cell surface proteoglycan. EDTA-released cells, as in Fig. 2, do not bind to BSA (*A*), but do bind to fibronectin (*FN*) both before (*B*) and after removal of cell surface heparan sulfate with heparan sulfate lyase (heparitinase) (*C*). After removal of cell surface heparan sulfate, binding of EDTA-released cells is inhibited by GRGDS in a dose-dependent manner (*D*, *E*, and *F*).

liposomes containing the isolated cell surface PG mimics the binding of EDTA-released cells.

Cell Surface Proteoglycan Does Not Bind NMuMG Cells to Laminin

Laminin is another adhesive glycoprotein reported to have heparin and heparan sulfate binding activities (Sakashita et al., 1980; Woodly et al., 1983; Fujiwara et al., 1984). Therefore, we investigated whether the cell surface PG of mouse mammary epithelial cells mediates cell binding to laminin. EDTA-released NMuMG cells bound to fibronectin (Fig. 7 *C*) and native type I collagen (Fig. 7 *B*) as reported previously (Koda et al., 1985), but failed to bind to laminin-coated wells (Fig. 7 *D*). This result was obtained with three distinct laminin preparations and at laminin concentrations sufficient to demonstrate the adhesion of a variety of other cell types (Goodman and Newgreen, 1985).

Liposomes containing intercalated cell surface PG duplicated the binding of cells; liposomes bound to fibronectin (Fig. 8 *B*) and to native type I collagen (Fig. 8 *C*), but not

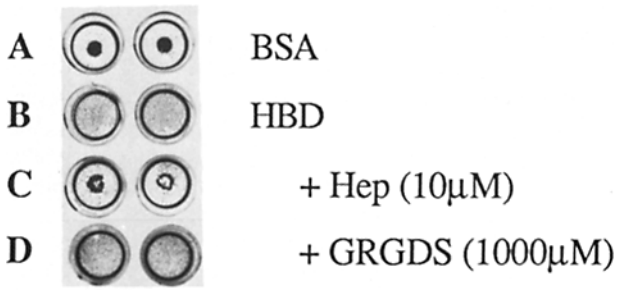

Figure 5. Cells bind to the COOH-terminal heparin binding domain of fibronectin via the cell surface proteoglycan. EDTA-released mammary epithelial cells, as in Fig. 2, do not bind to BSA (*A*), but bind to the purified COOH-terminal heparin binding domain (*HBD*) of fibronectin (*B*). This binding is inhibited by heparin (*Hep*) (*C*), but not by the RGD-containing peptide GRGDS (*D*).

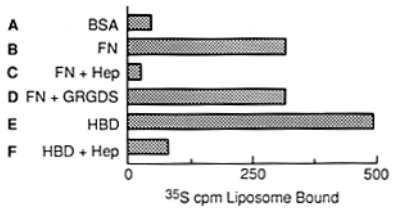

Figure 6. Liposomes containing the cell surface proteoglycan mimic the binding of cells to fibronectin. The cell surface proteoglycan of mouse mammary epithelial cells was metabolically labeled with $^{35}SO_4$. Proteoglycan was extracted with Triton X-100 containing 0.5 M KCl, purified by DEAE and immunoaffinity chromatography, and intercalated into liposomes. 10,000 cpm of ^{35}S-PG was added to each well and incubated for 2.5 h as described in Materials and Methods. The liposomes do not bind to BSA (*A*), but bind to fibronectin (*FN*) (*B*) and to the isolated COOH-terminal heparin binding domain (*HBD*) (*E*). This binding was inhibited by heparin (*Hep*) (*C* and *F*), but not by GRGDS (*D*).

to BSA (Fig. 8 *A*) or to laminin-coated wells (Fig. 8 *D*). Therefore we conclude that NMuMG cells do not bind to laminin via the cell surface PG.

Discussion

The binding of cells to extracellular matrix has been extensively evaluated but, in most instances, these studies have evaluated the behavior of fibroblastic cells. These cells migrate through the matrix in which they are embedded and have a different relationship with their extracellular matrix than epithelial cells which normally are sessile and interact with matrix components solely at their basal surfaces. We have evaluated the binding of NMuMG mouse mammary epithelial cells to the adhesive glycoproteins fibronectin, an interstitial matrix component, and laminin, a basal lamina component. We find that these cells possess at least two distinct cell surface receptors for binding to fibronectin: a trypsin-resistant receptor that binds cells to the sequence RGD and a trypsin-labile, heparan sulfate–rich PG that binds cells to the COOH-terminal heparin binding domain. Under the conditions where the cells bind to fibronectin via the cell surface PG, the cell surface PG does not mediate the binding of the cells to laminin. Because the cell surface PG binds cells to the interstitial collagens (types I, III, and V), and

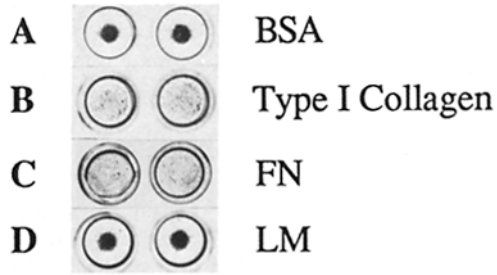

Figure 7. EDTA-released cells do not bind to laminin. EDTA-released cells, as in Fig. 2, do not bind to BSA (*A*), but bind to type I collagen (*B*) and fibronectin (*FN*) (*C*). However, under identical assay conditions, these cells do not bind to laminin (*LM*) coated wells (*D*).

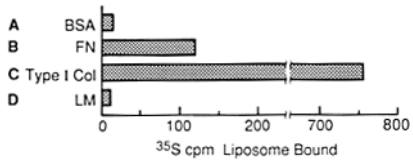

Figure 8. Liposomes containing the cell surface proteoglycan do not bind to laminin. Liposomes containing the cell surface proteoglycan were prepared as in Fig. 7. 6,000 cpm ^{35}S-PG was added to each well and incubated for 2.5 h as described in Materials and Methods. The liposomes bind to fibronectin (*FN*) (*B*) and to type I collagen (*C*) but not to BSA (*A*) or to laminin (*LM*) (*D*).

fibronectin, but not to basal lamina collagen (type IV) or laminin, we conclude that the cell surface PG binds these epithelial cells specifically to interstitial matrix components.

Cell Surface PG as a Fibronectin Receptor

The binding of NMuMG cells to fibronectin depends on the method of harvesting the cells from monolayer cultures. The binding of trypsin-released NMuMG cells to fibronectin is inhibited by a peptide containing the RGD sequence, identical with that of a variety of similarly harvested fibroblastic cells (Pierschbacher and Ruoslahti, 1984). Moreover, a serum antibody to the 140-kD complex (anti GP-140) stains the surfaces of these NMuMG cells and blocks their RGD-dependent binding (unpublished), suggesting that this binding is via the 140-kD complex. Because trypsin treatment removes the cell surface PG (Rapraeger and Bernfield, 1985) and the binding of trypsinized NMuMG cells to fibronectin is prevented by the RGD peptide, the 140-kD RGD receptor appears to be the sole functional fibronectin receptor on trypsinized cells under our assay conditions.

NMuMG cells harvested with EDTA bind to fibronectin by two receptors, the 140-kD RGD receptor and one that is prevented from binding by two types of pretreatment, pretreatment of the cells with heparitinase or pretreatment of the fibronectin with either heparin or the soluble ectodomain of the cell surface PG. Both EDTA-harvested NMuMG cells and liposomes containing the intact cell surface proteoglycan bind to the COOH-terminal heparin binding domain of fibronectin in a heparin-sensitive and GRGDS-insensitive manner. These data indicate that the cell surface PG acts as a fibronectin receptor on these cells.

Heparinlike polysaccharides are known to bind to fibronectin. The binding of heparin to fibronectin was among its earliest established properties (Stathakis and Mosesson, 1977; Laterra et al., 1980) and this binding occurs under physiological conditions to discrete proteolytic fragments (Yamada et al., 1980). Binding of heparin to fibronectin also appears to increase the affinity of the fibronectin for collagen (Johansson and Hook, 1980; Ruoslahti and Engvall, 1980), possibly the result of conformational changes in fibronectin (Osterlund et al., 1985).

Although several studies have described the interactions of PGs with fibronectin involving heparan sulfate chains (Hedman et al., 1982; Oldberg and Ruoslahti, 1982; Fujiwara et al., 1984), interactions of PGs involving chondroitin sulfate chains (Oldberg and Ruoslahti, 1982; Yamagata et al., 1986) and the core protein (Isemura et al., 1987; Schmidt et al., 1987) also have been observed. Most of these previous studies have involved PGs extracted from the extracellular and pericellular matrix.

The possibility that a cell surface–associated PG binds to fibronectin was suggested by the findings that heparan sulfate PG purified from rat liver microsomes binds to fibronectin in a column assay (Robinson et al., 1984) and that fibroblasts adhere to fibronectin via a heparinlike molecule (Laterra et al., 1983; Lark et al., 1985; Izzard et al., 1986; Woods et al., 1986). In the present study, cell binding experiments suggested the involvement of a cell surface PG and reconstitution of the purified cell surface PG into liposomes confirmed that this molecule mediates the binding of the cells.

Although the cell surface PG bound liposomes to fibronectin in a solid-phase assay, in this assay the isolated ectodomain of the cell surface PG bound poorly to fibronectin but was able to inhibit cell binding at high concentration. This behavior of the PG is consistent with the behavior of other isolated matrix receptors. For example, the isolated receptor anchorin CII binds with low affinity and poor selectivity to type II collagen. When intercalated into liposomes, however, anchorin CII binds with higher affinity and is selective for type II collagen (von der Mark et al., 1984). These results suggest that mobility of the PG in the plane of the membrane contributes to its affinity for fibronectin.

Cell Surface Proteoglycan Binds Cells to Interstitial Matrix Components

The cell surface PG polarizes solely to the basolateral surface of NMuMG cells in culture (Rapraeger et al., 1986). This distribution duplicates the localization of the cell surface proteoglycan on various simple epithelia in adult tissues in vivo (Hayashi et al., 1987) and is consistent with its proposed function as a matrix receptor. Despite the immediate proximity of the cell surface PG to the basal lamina, no binding of this molecule to the isolated basal lamina components laminin (this study) or type IV collagen (Koda et al., 1985) is observed, even when the cell surface PG is intercalated into liposomes, a procedure that is necessary to demonstrate binding to fibronectin. In contrast, the cell surface PG binds to components of the interstitial matrix such as fibronectin (this study), and types I, III, and V collagens (Koda et al., 1985). Therefore, at least under the conditions of our assay, we conclude that the cell surface proteoglycan is specific for components of the interstitial matrix.

We have proposed that the cell surface PG serves as an interstitial matrix anchor for epithelial cells. Interstitial matrix does exert behavioral influences on epithelial cells. For example, an interstitial matrix substratum (interstitial collagens and fibronectin) appears to stabilize epithelial sheets in vitro and may enhance the accumulation of specific differentiated gene products (Haeuptle et al., 1983; Hay, 1985). Development of stable epithelial morphology in vivo correlates with the deposition of interstitial matrix at the basal epithelial surface in mouse embryo salivary glands (Bernfield et al., 1984). This matrix anchoring hypothesis predicts that the cell surface PG extends through the epithelial basal lamina to interact with interstitial matrix, a prediction that is currently being tested.

Significance of Multiple Fibronectin Receptors

NMuMG cells contain at least two cell surface molecules that bind fibronectin, the 140-kD RGD receptor, and the cell surface PG. The presence of a single cell type bearing more

than one receptor for fibronectin may be common. For example, both the 140-kD complex and a 45-kD receptor for fibronectin (Aplin et al., 1981; Hughes et al., 1981; Oppenheimer-Marks and Grinnell, 1984) are present on Swiss 3T3 cells (Urushihara and Yamada, 1986). However, it is not known whether distinct matrix receptors that bind to the same ligand on a single cell type are independent and apparently redundant binding mechanisms or whether they cooperate or interact.

Fibronectin consists of a heterogeneous family of polypeptides resulting from the alternate splicing of its mRNA (Schwarzbauer et al., 1983; Kornblihtt et al., 1984; Schwarzbauer et al., 1985; Paul et al., 1986). Whereas the timing of expression and tissue localization of these various fibronectin forms have not been identified, they likely possess some unique biological attributes (Humphries et al., 1986). All of the alternate peptide inserts thus far identified fall into regions either between the RGD containing and COOH-terminal heparin binding domains, or immediately adjacent to these domains (Schwarzbauer et al., 1983; Paul et al., 1986). A single inserted peptide between these domains is found only in cellular fibronectin (Schwarzbauer et al., 1983; Kornblihtt et al., 1984). Thus, it is reasonable to speculate that cells containing both the 140-kD and cell surface PG receptors for fibronectin may respond distinctly to these distinct forms of fibronectin. The peptide linking the RGD-containing and the COOH-terminal heparin binding domains may allow cooperative interactions to occur between the fibronectin receptors. For example, Balb/c 3T3 cells, which are thought to contain both the 140-kD and a cell surface PG receptor, fail to form focal adhesions on substrata containing a mixture of the isolated RGD-containing and COOH-terminal heparin binding domains (Izzard et al., 1986).

Simple epithelial cells exist in vivo as single layers of cells in sheets or cords with the cells linked to one another by various types of cell junctions and cell adhesion molecules. In adult tissues, these cell sheets are relatively stable and the cells do not move or undergo changes in cell shape. The presence on epithelial cells of both the 140-kD and the cell surface PG fibronectin receptors may be understood by postulating distinct roles for such receptors in epithelial behavior. When the cell sheet is stable, we postulate that the cell surface PG is involved in stabilizing these linkages to the fibronectin-containing interstitial matrix. However, should an injury cause a defect or discontinuity in the sheet, the epithelial cells at the margins will spread and begin to migrate laterally to fill the defect (Kolega, 1986), a behavior that we postulate involves the 140-kD receptor. Consistent with these postulates, when NMuMg epithelial cells lose their attachment to the substratum in vitro they quantitatively shed the cell surface PG by cleavage of the GAG-bearing ectodomain from the membrane-associated domain (Jalkanen et al., 1987). In contrast, expression of the 140-kD receptor on these cells is unaffected by suspension. The loss of the cell surface PG in vivo would loosen cell anchorage to the interstitial matrix via the cell surface PG but allow spreading and subsequent migration of the epithelial cells on this matrix substratum.

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