Mouse Mammary Epithelial Cells Produce Basement Membrane and Cell Surface Heparan Sulfate Proteoglycans Containing Distinct Core Proteins

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Abstract. Cultured mouse mammary (NMuMG) cells produce heparan sulfate–rich proteoglycans that are found at the cell surface, in the culture medium, and beneath the monolayer. The cell surface proteoglycan consists of a lipophilic membrane-associated domain and an extracellular domain, or ectodomain, that contains both heparan and chondroitin sulfate chains. During culture, the cells release into the medium a soluble proteoglycan that is indistinguishable from the ectodomain released from the cells by trypsin treatment. This medium ectodomain was isolated, purified, and used as an antigen to prepare an affinity-purified serum antibody from rabbits. The antibody recognizes polypeptide determinants on the core protein of the ectodomain of the cell surface proteoglycan. The reactivity of this antibody was compared with that of a serum antibody (BM-1) directed against the low density basement membrane proteoglycan of the Englebarth-Holm-Swarm tumor (Hassell, J. R., W. C. Leyshon, S. R. Ledbetter, B. Tyree, S. Suzuki, M. Kato, K. Kimata, and H. Kleinman. 1985. *J. Biol. Chem.* 250:8098–8105). The BM-1 antibody recognized a large, low density heparan sulfate–rich proteoglycan in the cells and in the basal extracellular materials beneath the monolayer where it accumulated in patchy deposits. The affinity-purified anti–ectodomain antibody recognized the cell surface proteoglycan on the cells, where it is seen on apical cell surfaces in subconfluent cultures and in fine filamentous arrays at the basal cell surface in confluent cultures, but detected no proteoglycan in the basal extracellular materials beneath the monolayer. The amino acid composition of the purified medium ectodomain was substantially different from that reported for the basement membrane proteoglycan. Thus, NMuMG cells produce at least two heparan sulfate–rich proteoglycans that contain distinct core proteins, a cell surface proteoglycan, and a basement membrane proteoglycan. In newborn mouse skin, these proteoglycans localize to distinct sites; the basement membrane proteoglycan is seen solely at the dermal–epidermal boundary and the cell surface proteoglycan is seen solely at the surfaces of keratinocytes in the basal, spinous, and granular cell layers. These results suggest that although heparan sulfate–rich proteoglycans may have similar glycosaminoglycan chains, they are sorted by the epithelial cells to different sites on the basis of differences in their core proteins.

HEPARAN sulfate proteoglycans (PGs)[1] are found in several locations in animal cells: extracellularly, in the pericellular matrix of fibroblasts and in the basement membrane of various parenchymal cells, at the surface of mesenchymal, epithelial, and neural cells; and intracellularly, within secretory vesicles and the endoplasmic reticulum (see references in Gallagher et al., 1986). PGs with distinct molecular sizes or substituents may be at each location. For example, the cell surface PGs of hepatic (Kjellen et al., 1981) and mammary (Rapraeger and Bernfield, 1983) epithelial cells are apparently distinct in molecular mass, as are the basement membrane PGs of the glomerulus (Stow et al., 1983; Kobayashi et al., 1983) and the Englebarth-Holm-Swarm (EHS) tumor (Hassell et al., 1980) (see additional references in Hassell et al., 1986). Thus, although heparan sulfate PGs may contain virtually indistinguishable glycosaminoglycan (GAG) chains, they may differ in other characteristics, indicating that relationships between these PGs are best established by characterizing their core proteins (Hassell et al., 1986).

The cell surface PG of mouse mammary epithelial (NMuMG) cells consists of a membrane-associated lipophilic domain (Rapraeger and Bernfield, 1983; 1985) and a

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1. *Abbreviations used in this paper*: EHS, Englebarth-Holm Swarm; GAG, glycosaminoglycan; PG, proteoglycan; TBS, Tris-buffered saline.

GAG-bearing ectodomain that contains both heparan and chondroitin sulfate chains (Rapraeger et al., 1985). The PG behaves as an integral membrane protein, and the ectodomain can be shed intact into the extracellular space by cleavage from the membrane-associated domain (Jalkanen et al., 1987). NMuMG cells also synthesize a basement membrane PG (David and Bernfield, 1979, 1981; David et al., 1981) that contains the identical type and size of GAG chains as the cell surface PG (David and Van den Berghe, 1985) and is deposited beneath monolayers of cultured cells. The degradation of this PG is reduced when the cells are grown on a type I collagen gel (David and Bernfield, 1979).

Several lines of evidence suggest that other cell types also produce both cell surface and basement membrane PGs. But because these molecules may have similar GAG chains, differences in their size, buoyant density, and charge do not adequately distinguish them. Consequently, as has been recently emphasized, the molecules must be distinguished on the basis of their core proteins (Hassell et al., 1986). Here we have distinguished between the cell surface and basement membrane PGs produced by NMuMG cells by separating the molecules and identifying their core proteins with monospecific serum antibodies. Using these reagents, we show that NMuMG cells in vitro and mouse epidermal cells in vivo localize these PGs to distinct sites; the cell surface PG at the cell surface and the basement membrane PG beneath the cells. Because their GAG chains are similar, the accumulation of these PGs at distinct sites appears to result from differences in their core proteins. A part of these data has been published in abstract form (Jalkanen et al., 1985*b*).

Materials and Methods

Cell Culture

Early passages (12–20) of mouse mammary epithelial (NMuMG) cells were maintained in bicarbonate-buffered DME (Gibco, Grand Island, NY) containing 10% FBS (Tissue Culture Biological, Tulare, CA) as described previously (David and Bernfield, 1979). Cells were plated at one-quarter or less confluent density for routine use (100-mm dishes; Falcon Labware, Oxnard, CA), and fresh medium was replaced every 2–3 d. Medium conditioned by subconfluent cells was the source of a purified PG that is indistinguishable from the ectodomain of the cell surface PG, called here the medium ectodomain (Jalkanen et al., 1987). This PG was purified to homogeneity by sequential DEAE-cellulose chromatography, CsCl-density centrifugation, and monoclonal antibody (281-2) affinity chromatography. These procedures are described in detail elsewhere (Jalkanen et al., 1987).

Production of Affinity-purified Immunoglobulins Directed against the Medium Ectodomain

The purified medium ectodomain (25 μg protein) was mixed with Freund's complete adjuvant and injected into several intracutaneous sites on the back of a rabbit. After three to four boosters at 2–3-wk intervals in Freund's incomplete adjuvant, rabbit serum showed a positive reaction at dilutions up to 1:800 on DEAE-cellulose paper dot assay using the medium ectodomain as antigen. Affinity purification of a specific IgG fraction from the immune serum was performed at room temperature on columns of DEAE-cellulose containing the medium ectodomain. The affinity column was prepared by mixing the PG (50 μg) with a slurry of 1 ml of DEAE-cellulose (Pharmacia Fine Chemicals, Piscataway, NJ) in Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.4, 100 mM NaCl), which was poured into a column and rinsed with TBS. 5 ml of immune serum was passed through a similarly washed DEAE-cellulose column (1 × 10 cm) but not containing PG to remove materials that bind to DEAE-cellulose. This serum eluate was placed on the affinity column which was washed with 20 ml of TBS to remove unbound material. Bound material was released from the column using 8 M urea buffered to pH 7.4 (10 mM Tris-HCl, pH 7.4) and collected on ice. This cycle was repeated with the unbound material five to six times until the dot assay demonstrated that all immunoglobulins recognizing the medium ectodomain were removed. The immunoglobulins in urea were dialyzed against TBS, concentrated, when necessary, to 2.5 mg/ml by dialyzing against 30% polyethylene glycol 20,000 (J. T. Baker Chemical Co., Phillipsburg, NJ) in TBS, and aliquoted for −80°C storage.

Antibodies against the low density basement membrane heparan sulfate PG derived from the EHS tumor (Hassell et al., 1985) were kindly provided by Dr. J. Hassell (National Institute of Dental Research, National Institutes of Health) and used with dilutions indicated in the figure legends. mAb 281-2, the monoclonal antibody specific for the core protein of the cell surface PG, has been described previously (Jalkanen et al., 1985*a*) and was used in concentrations indicated in figure legends.

Dot Immunoassay for PGs

We have recently described the use of DEAE-cellulose paper as a solid phase for immunodetection of PGs (Jalkanen et al., 1985*a*). Briefly, wet DEAE-cellulose paper is placed into an immuno-dot apparatus (V&P Scientific, San Diego, CA), and samples in 8 M urea (buffered to pH 4.5 with 50 mM Na acetate) are loaded onto the paper using mild vacuum. Samples containing high salt concentrations (e.g., from CsCl gradients) were diluted with 8 M urea to a concentration of less than 0.2 M Cl^- ion to allow binding of the PG to the DEAE paper. After the loading, the paper was transferred to a solution of TBS supplemented with 1% FBS and washed several times to remove urea. The paper was exposed overnight at 4°C to the first antibody, washed five to six times with TBS, and then incubated with a peroxidase-conjugated second antibody in TBS containing 1% FBS for 30 min at room temperature. After five to six washes with TBS, the immobilized peroxidase conjugate was visualized using 0.05% (wt/vol) of 4-chloro-1-naphthol containing 0.03% (vol/vol) hydrogen peroxide in TBS (Esen et al., 1983).

Harvest of Radioactive PGs from NMuMG Cell Cultures

PGs deposited beneath the cell monolayer (basal extracellular PG), the intact cell surface PG, or its trypsin-released ectodomain were harvested from NMuMG monolayers labeled with radiosulfate (100 μCi/ml) for 24 h as described (David and Bernfield, 1981; Rapraeger and Bernfield, 1983). Monolayers were washed twice with cold TBS supplemented with 1.25 mM $CaCl_2$ and 0.9 mM $MgSO_4$. The monolayers were then scraped with a rubber policeman into cold TBS supplemented with 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 5 mM *N*-ethylmaleimide. The cells were centrifuged (200 *g*) and the supernatant, containing the basal extracellular PG, was mixed with an equal volume of 8 M GdnHCl buffered to pH 4.5 with 50 mM Na acetate and boiled. The cell pellet was washed once in the scraping solution and then extracted with the scraping solution containing 1% Triton X-100 and 0.5 M KCl. This suspension was centrifuged at 600 *g* to pellet nuclei and insoluble cytoskeletal materials, leaving the intact cell surface PG in the supernatant (Rapraeger et al., 1986). The superantant was mixed with an equal volume of 8 M GdnHCl and boiled.

For preparation of the ectodomain that is released from the cell surface by trypsin, cells that had been harvested by scraping and centrifuging were washed three times in 0.5 mM EDTA-PBS and incubated with cold trypsin (20 μg/ml) for 10 min on ice. After incubation, soybean trypsin inhibitor was added (100 μg/ml) and cells were pelleted again, leaving the cell surface ectodomain in the supernatant. The supernatant was mixed with an equal volume of 8 M guanidine HCl, boiled, and subjected to isopycnic centrifugation in CsCl.

Analysis of PGs

Isopycnic Centrifugation. Cellular, basal extracellular, and medium PGs were analyzed by isopycnic centrifugation in 50 mM Na acetate (pH 4.5) containing 4 M GdnHCl and 1% Triton X-100. Samples were brought to 1.4 g/ml with crystalline CsCl and centrifuged at 15°C for 72 h at 46,000 rpm in an SW 65 rotor (Beckman Instruments, Inc., Palo Alto, CA). Fractions were collected and analyzed for density by weighing. Total PG radioactivity was assessed by a modification of Stephens et al. (1978) procedure (Rapraeger et al., 1985). Briefly, cetylpyridinium chloride (CPC)-impregnated 3-mm filter discs (Whatman Inc., Clifton, NJ) were prepared by soaking in 2.5% CPC followed by drying. Aliquots were spotted on the dry discs which were washed five times in distilled water, soaked for 1 h in 25 mM Na sulfate, and rinsed several times in distilled water. Finally, the discs were

soaked for 30 min in 10% TCA followed by washes in distilled water and 95% ethanol and dried for scintillation counting.

Sizing Chromatography. The hydrodynamic volume of the PGs was assessed by Sepharose CL-4B chromatography in 4 M GdnHCl buffered to pH 5.5 by 50 mM Na acetate. Radioactivities in solutions were assayed by scintillation counting using 6 vol Aquamix for each sample volume. Samples containing 4 M GdnHCl were diluted 1:10 with water before mixing with Aquamix.

Western Blotting. For the measurement of the relative mass of the immunoreactive PG, selected fractions from CsCl gradients were dialyzed (against distilled water and then 0.1% SDS), then fractionated in a gradient (3.8–20%) polyacrylamide gel using PAGE buffer composed of 40 mM Tris, 60 mM boric acid (pH 8.0), 0.8 mM EDTA, 1 mM Na_2SO_4, and 0.1% SDS (Jalkanen et al., 1985*a*). The gel was run overnight to completion at 125 V and was transferred onto a pad of a model TE 52 transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA) and covered with DEAE-cellulose paper (Jalkanen et al., 1985*a*) or with Zeta-Probe (BioRad Laboratories, Richmond, CA), a cationic nylon membrane (Rapraeger et al., 1985). Transfer was performed according to Towbin et al. (1979) at 100 V for 6 h. The antigen was localized by indirect enzyme immunostaining using the affinity-purified immunoglobulins as described above for the dot assay, except that the nylon membrane was treated with 0.1% Tween-20-TBS for 1 h after the transfer to block the nonspecific binding of proteins to the membrane. The immobilized rabbit IgG was detected with affinity-purified goat anti-rabbit (alkaline phosphatase conjugated) and nitro blue tetrazaline/5-bromo-4-chloro-5 indoyl phosphate as substrate (ProtoBlot; Promega Biotec, Madison, WI).

Enzyme Digestions of PGs. Papain digestions (100 μg/ml; type II; Sigma Chemical Co., St. Louis, MO) were in 5 mM cysteine-EDTA pH 7.5 for 20 h at 60°C. For removal of GAG chains, PGs were digested with 0.1 U/ml *Flavobacterium heparinium* heparitin sulfate lyase (heparitinase; Miles Laboratories Inc., Naperville, IL) and with 0.05 U/ml chondroitin sulfate ABC lyase (chondroitinase ABC; Miles Laboratories) using conditions described in detail earlier (Rapraeger et al., 1985).

Amino Acid and Hexosamine Analyses. The composition of the isolated medium ectodomain (Jalkanen et al., 1987) was analyzed on Durrum D-500 and Beckman 6300 analyzers by the service operated by the University of California at Davis. For amino acid analysis, samples were hydrolyzed at 110°C in 6 M HCl for 24 and 72 h and for cysteine and methionine in 6 M performic acid for 24 h. The data presented are the means of values obtained by analysis of two separate ectodomain preparations, corrected for degradation, and incomplete hydrolysis using alanine as an internal standard.

Immunostaining of Cell Cultures and Tissue Sections

Competition with NMuMG Cell Surface Staining. Intact and enzyme-treated (papain or heparitinase plus chondroitinase ABC) medium ectodomain (1.25 μg; quantitated using a radioimmunoassay for the medium ectodomain (Jalkanen et al., 1987) were incubated with a 1:5 dilution of immune serum in PBS, then centrifuged at 10,000 rpm (Eppendorf microfuge) for 10 min, and the supernatants used to stain NMuMG cells. Subconfluent monolayers were rinsed three times with cold PBS and then incubated on ice for 30 min with a 1:10 dilution of these supernatants. After washing five times with cold PBS, the monolayers were fixed with 3.7% formaldehyde for 30 min followed by incubation in 50 mM ammonium chloride in PBS for 30 min on ice. The monolayers were rinsed with PBS and exposed to FITC-conjugated swine anti-rabbit, IgG (1:100 dilution in 1% FBS-PBS; Dako Corp., Santa Barbara, CA), washed five times with PBS, mounted in Eukitt (Calibrated Instruments, Ardsley, NJ), and viewed with epifluorescence

Triton-extracted Monolayers. NMuMG monolayers on coverslips were washed three times with cold TBS, incubated for 10 min with 1% Triton X-100 in TBS on ice, and then washed three times with TBS. Monolayers were then fixed in 3.7% formaldehyde for 30 min on ice followed by incubation in 50 mM NH_4Cl solution for 30 min on ice. Monolayers were treated sequentially with ice cold 50, 100, and 50% acetone for 5 min each. After a 15-min incubation in TBS, the monolayers were exposed to the first antibody for 30 min (when double staining, the first incubation was followed immediately by incubation with the other first antibody), then washed with TBS, and incubated for another 30 min with the second antibody (FITC- or TRITC-conjugated; in the case of double staining, incubation with both second antibodies was performed simultaneously). Finally, coverslips were washed five times in TBS and mounted in Aquamount for viewing with epifluorescence.

Mouse Skin. Fresh pieces of newborn mouse skin were mounted in Tissue Tek (American Scientific, Sunnydale, CA) on a dry ice/ethanol bath. Cryo-sections of 5–6 μm were cut, fixed in ice cold acetone for 10 min, and air-dried at room temperature. After rehydration in modified PBS (120 mM NaCl, 10 mM NaH_2PO_4, 4 mM K_2-HPO_4, and 0.05 mM thimerosal, pH 7.3), endogenous peroxidase was inhibited by incubating with 0.3% H_2O_2 in 99% methanol for 1 h at room temperature. All subsequent washes were done in modified PBS. After washing, the sections were incubated for 20 min with IgG (3 mg/ml) from the type of animal that produced the second antibody. The sections were washed again and incubated with the first antibody for 30 min at room temperature in a humidity chamber. The first antibody was removed by washing as above and sections were incubated, as in the previous step, with the second antibody. Before use, the antibody preparations and the horseradish peroxidase–conjugated IgG were incubated with 10% serum of the animal type of the first antibody for 30 min and then centrifuged for 5 min at 10,000 rpm. After at least five washings the sections were treated for 5 min with diaminobenzidine (0.5 mg/ml) in 0.03% H_2O_2 in modified PBS and the reaction stopped by rinsing with distilled water. The color intensity was enhanced by placing the sections in 0.5% copper sulfate for 5 min, after which the sections were dried in graded ethanol, transferred to xylene, and mounted in Permount.

Results

Production of an Affinity-Purified Serum Antibody against the Cell Surface PG

NMuMG cells shed their cell surface PG into the culture medium by cleaving the GAG-bearing ectodomain from the membrane-associated domain (Jalkanen et al., 1987). This shedding provides the ectodomain of the cell surface PG in the culture medium as a readily soluble PG. This "medium ectodomain," purified to homogenity from conditioned medium of NMuMG cell cultures and characterized as described (Jalkanen et al., 1987), was used to induce serum antibodies in rabbits. The medium ectodomain was a satisfactory immunogen because reactivity with the PG was obtained with the rabbit immune serum at dilutions as high as 1:800 in a DEAE-paper immunodot assay (not shown). The resultant antibody was characterized using NMuMG cells or the ectodomain released from the cells by trypsin.

To evaluate its specificity, the serum antibody was used to stain subconfluent NMuMG cell monolayers. Preimmune serum showed no fluorescent stain (Fig. 1 *B*), but the immune serum showed staining of the apical cell surfaces, especially at the lateral cell boundaries and occasionally in a punctate distribution (Fig. 1 *C*). This staining is identical with that shown by monoclonal antibody 281-2 (Hayashi et al., 1987), which is known to react with the core protein of the cell surface PG (Jalkanen et al., 1985*a*). Adding the intact medium ectodomain or its core protein, prepared by enzymatic removal of the GAG chains, completely prevented staining (Fig. 1, *D* and *E*). This inhibition was abolished when the medium ectodomain was pretreated with papain (Fig. 1 *F*). Thus, most, if not all epitopes recognized by the immune serum, are polypeptide determinants on the core protein of the cell surface PG.

Monospecific antibodies directed against the medium ectodomain were purified by antigen-based affinity chromatography. The affinity column contained the purified medium ectodomain bound to DEAE-cellulose, taking advantage of both the high affinity of the GAG chains and the low affinity of immunoglobulins for this resin at neutral pH and physiological salt concentration. Immune serum was initially passed through a DEAE column and the flow-through was slowly loaded onto a DEAE column containing bound medium ectodomain. After thorough washing, the retained antibody was eluted with 8 M urea. This procedure was repeated three to five cycles (Fig. 2). Comparison by immunodot assay of

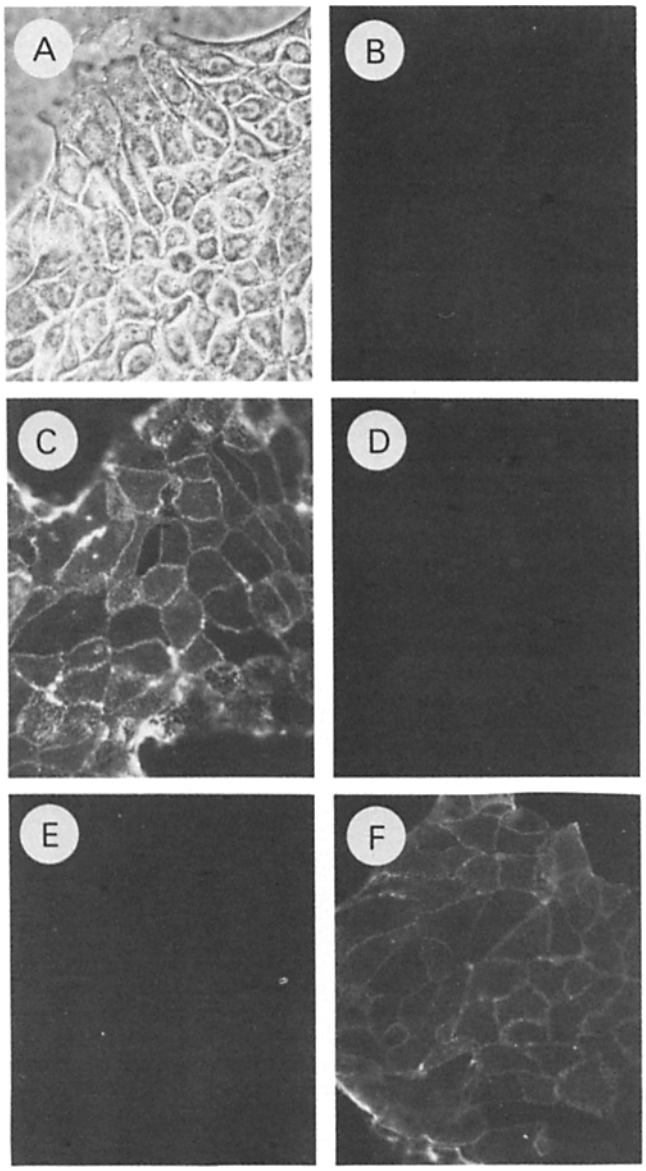

Figure 1. Immune serum against the medium ectodomain detects the core protein of the cell surface PG. Subconfluent NMuMG monolayers (*A*; phase contrast) were stained with preimmune serum (*B*) and the medium ectodomain immune serum (*C*) at 1:50 dilution. For the experiments in *D–F*, the immune serum was incubated for 30 min at room temperature with purified medium ectodomain (1.2 μg/40 μl of immune serum) either previously untreated (*D*), digested with heparan sulfate lyase (heparitinase) and chondroitin sulfate ABC lyase (chondroitinase ABC) (*E*), or digested with papain (*F*), as decribed in the text. These mixtures were centrifuged (10,000 *g*), diluted to 2 ml with TBS (1:50 final dilution of serum), and used to stain cells as in *B* and *C*.

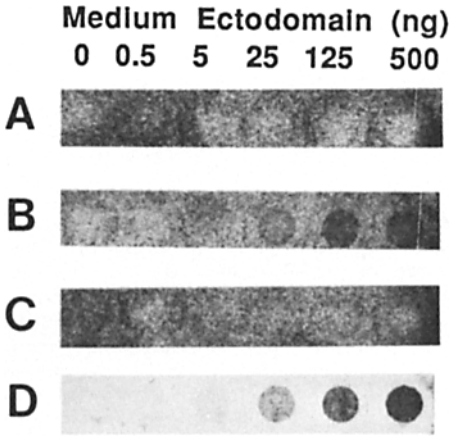

Figure 2. Immunoaffinity isolation of antibodies against the medium ectodomain. Varying amounts of purified medium ectodomain were applied to DEAE-paper strips (*round dots*). The paper strips were incubated with preimmune serum (*A*), immune serum (*B*), the flow-through from passing immune serum through a DEAE-cellulose column containing the medium ectodomain (*C*) (see text) and the urea eluate from the same column (*D*) (see text). *A–C* were used at 1:100 dilution and *D* at 10 μg/ml. The presence of immobilized rabbit IgG was detected with swine anti-rabbit peroxidase conjugate as described in the text.

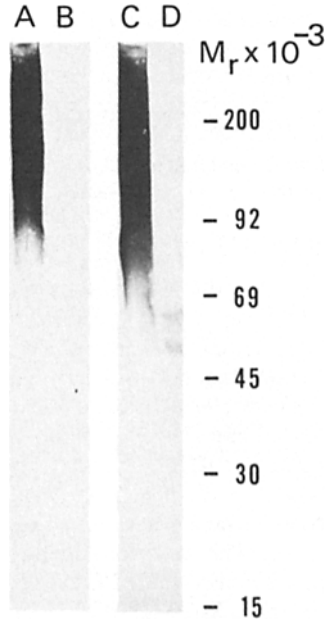

Figure 3. Affinity-purified antibody detects the trypsin-released ectodomain and its core protein in Western blots. Trypsin-released ectodomain (0.1 μg/6,500 cpm) (lanes *A* and *C*) and its heparitinase/chondroitinase ABC digest (0.3 μg/20,000 cpm) (lanes *B* and *D*) were subjected to electrophoresis in a 3.8–20% polyacrylamide gel and transferred to a cationic nylon membrane for antibody probing as described in the text. Lanes *A* and *B* are after 1-d autoradiographic exposure and lanes *C* and *D* are after immunostaining. The affinity-purified antibody was used at 5 μg/ml and goat anti-rabbit alkaline phosphatase conjugate at 1:7,500 dilution. Indicated molecular mass markers were ^{14}C-labeled myosin (200 kD), phosphorylase *b* (92.5 kD), BSA (69 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD), and lysozyme (14.3 kD).

preimmune and immune serum with the flow-through and eluates from the affinity column showed that this procedure yields an antibody preparation that retains the PG reactivity of the original serum while markedly reducing other components that produce background staining (Fig. 2). The material eluated by urea showed a single band detectable by anti-rabbit IgG (alkaline phosphatase–conjugated) after SDS-PAGE and transfer to nitrocellulose (not shown).

The affinity-purified antibody was shown to react with the core protein of the cell surface PG. The ectodomain released by trypsin from cells labeled with $^{35}SO_4$ was partially purified by isopycnic centrifugation. Autoradiography after gradient SDS-PAGE with and without prior enzyme treatment to remove GAG chains showed that the trypsin-released ectodomain migrates as a smear (lane *A*) and that the enzyme

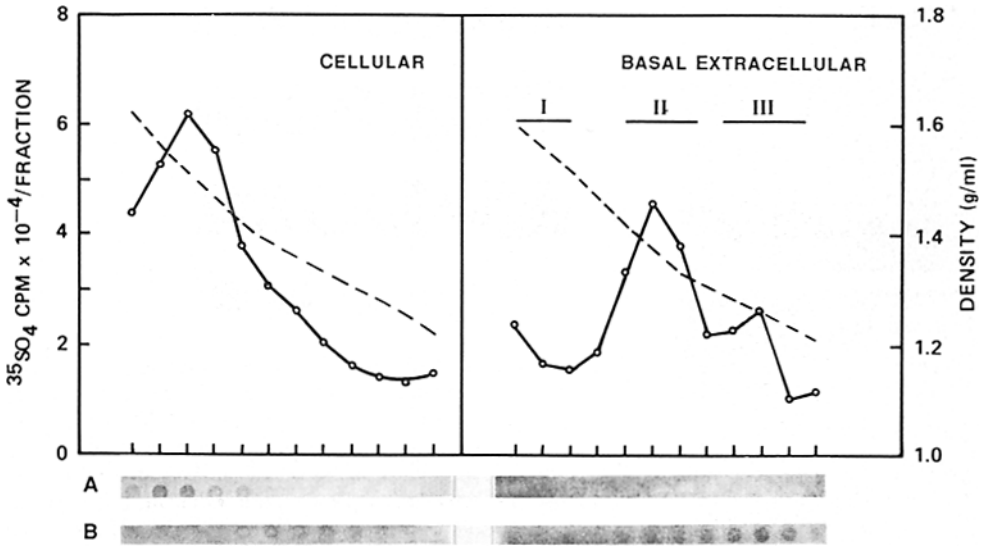

Figure 4. Separation of $^{35}SO_4$-PGs produced by NMuMG cells. Cellular (*left*) and basal extracellular proteoglycans (*right*) were harvested and centrifuged in a CsCl density gradient as described in the text. Fractions from the gradients were analyzed for density (*dotted line*) and for PG-bound radiosulfate (*open circles*). Aliquots (20 μl out of 250 μl) were diluted 100-fold with 8 M urea buffered to pH 4.5 with 50 mM Na acetate and loaded onto DEAE-paper as described in the text. These were then incubated with (*A*) affinity-purified antibody (20 μg/ml) and (*B*) with the BM-1 antibody (1:50 serum dilution). Antibodies were stained using 1:200 diluted peroxidase-conjugated second antibodies as described in the text.

treatments remove all the GAG (lane *B*) (Fig. 3). The affinity-purified antibody recognizes both the ectodomain (lane *C*) and a ~53-kD protein, representing the enzyme-digested core protein (lane *D*), as previously shown for mAb 281-2 (Rapraeger et al., 1985). By antibody detection, the core protein region (lane *D*) appears faint and as a doublet. Although the core protein may be heterogeneous, it is more likely that the cationic nylon membrane is blocked at sites where contaminating proteins, added as carrier or present in the enzyme preparation, are transferred, resulting in non-uniform retention of the core protein in these regions (Rapraeger et al., 1985). These data indicate that an affinity-purified antibody directed against the medium ectodomain reacts with the core protein of the cell surface PG.

Analysis of PGs Produced by NMuMG Cells

Cultured NMuMG cells produce PGs that are shed into the culture medium, deposited beneath the cells, and retained by the cells. Their distinct location in the cultures and response to the culture substratum suggest that they may differ in their core proteins. To assess this possibility, the cellular and basal extracellular PGs were fractionated and evaluated immunologically using antibodies specific for the cell surface and basement membrane heparan sulfate–rich PGs. Newly confluent monolayers were labeled to the steady state with $^{35}SO_4$ and harvested by scraping on ice. The materials released from beneath the monolayer, the basal extracellular PG, and the cellular materials extracted in 1% Triton X-100 0.5 M KCl were subjected to isopycnic centrifugation in CsCl (Fig. 4). The cells showed a single major PG peak at a buoyant density of ~1.55 g/ml, the value previously reported for the cell surface PG (Rapraeger and Bernfield, 1985). This peak was recognized by the affinity-purified antibody (Fig. 4 *A*), confirming it as the cell surface PG. With the BM-1 antibody (Fig. 4 *B*), some stain at lower buoyant densities was seen, presumably representing intracellular basement membrane PG or its precursors (Ledbetter et al., 1985).

The basal extracellular materials showed three fractions with peaks at ~ >1.60, 1.38, and 1.25 g/ml. None of these was recognized by the affinity-purified antibody and only fraction III, density of 1.2–1.3 g/ml, was recognized by the BM-1 antibody (Fig. 4). The hydrodynamic volume of this latter fraction was compared with that of the trypsin-released ectodomain by chromatography on Sepharose 4B and gradient PAGE. On Sepharose 4B, the basal extracellular PG reacting with BM-1 antibody and the trypsin-released ectodomain eluted at K_{av} of 0.12 and 0.30, respectively (not shown). On gradient SDS-PAGE, the basal extracellular PG reacting with the BM-1 antibody had an appreciably larger molecular mass than the ectodomain (Fig. 5, *E* and *F*). Transfer of the PGs to cationic nylon membranes and reactivity with the BM-1 and the affinity-purified antibodies confirmed that the BM-1 reactive PG is appreciably larger than the ectodomain (Fig. 5). Antibody reactivity on the transfers confirmed that these PGs do not contain shared epitopes recognized by these antibody preparations (Fig. 5, *A–D*).

The amino acid and amino sugar composition of the isolated medium ectodomain differs substantially from that reported for the low density basement membrane PG of the EHS tumor (Table I), which was used as the antigen to induce the BM-1 antibodies (Hassel et al., 1985). These data, together with those previously obtained, indicate that the core proteins for the cell surface and the basement membrane PGs are encoded by different genes.

Distinct Cellular and Tissue Location of the Cell Surface and Basement Membrane PGs

mAb 281-2, the affinity-purified antibody against the cell surface PG, and the BM-1 antibody were used to localize PGs in NMuMG cell cultures and in sections of newborn mouse skin. To study the distribution of antigens in the NMuMG cultures, newly confluent monolayers were first treated with Triton X-100 to allow penetration of the antibodies, then in-

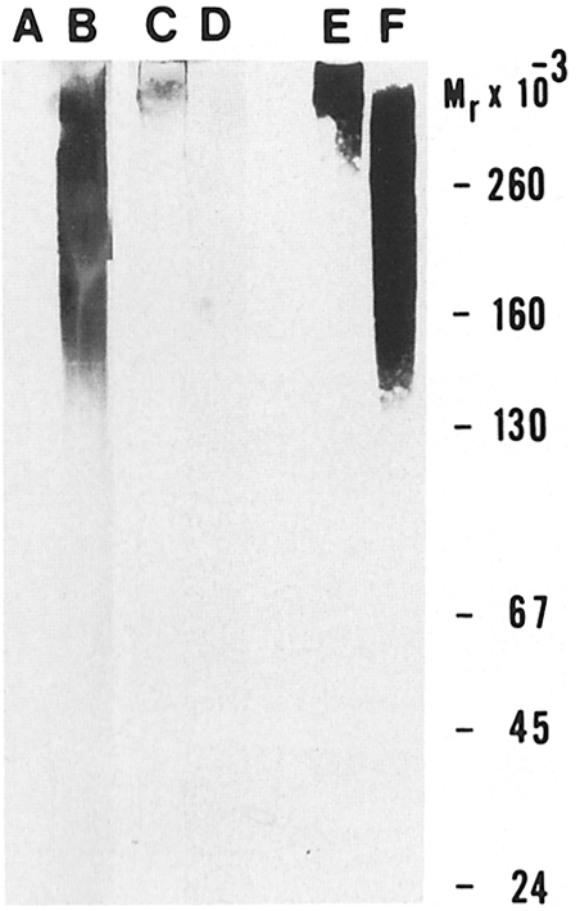

Figure 5. Comparison of the trypsin-released ectodomain and basement membrane PGs on Western blots. Radiosulfate-labeled trypsin-released ectodomain (lanes *B*, *D*, and *F*) and BM-1 positive PG (lanes *A*, *C*, and *E*) were isolated from CsCl gradients, subjected to electrophoresis in 3.8–20% PAGE, and transferred onto cationic nylon membranes. The PGs were detected by immunostaining (*A–D*) or by autoradiography (*E*, *F*). Lanes *A* and *B* were stained with the affinity-purified antibody (20 μg/ml), and lanes *C* and *D* with BM-1 antiserum (1:50 dilution), as described in the text. Molecular mass markers (collagen type I beta-chains, 260 kD; mouse IgG, 160 kD; collagen type I alpha chains, 130 kD; BSA, 67 kD; ovalbumin, 45 kD; and chymotrypsinogen, 24 kD) are indicated on the right.

cubated with the antibodies and finally processed for double immunostaining (Fig. 6). Observations were made throughout the cell, but the photomicrographs show only a focal plane at the basal cell surface. Neither nonimmune pooled rat (or rabbit) IgG (Fig. 6 *A*) showed specific staining. Both mAb 281-2 (Fig. 6 *C*) and the affinity-purified antibody (Fig. 6 *D*) stained a fine filamentous network near the basal cell surface and surrounding the nucleus. These antibodies showed extensive co-localization (Fig. 6, *C* and *D*). In contrast, mAb 281-2 and the BM-1 antibody showed quite distinct staining patterns (Fig. 6, *E* and *F*); the BM-1 antibody showed patchy deposits that were especially prominent where cells were closely packed.

The affinity purified and BM-1 antibodies also showed distinct localizations in sections of newborn mouse epidermis. Using peroxidase-conjugated second antibody, neither nonimmune pooled rat (Fig. 7 *A*) or rabbit (Fig. 7 *B*) IgG showed

Table I. Amino Acid Composition of Proteoglycans

Residue	Medium Ectodomain residues/1,000	Basement membrane low density PG*
Asx	82	81
Thr	116	64
Ser	74	102
Glx	148	127
Pro	102	78
Gly	102	111
Ala	86	73
Cys	0	9
Val	76	76
Met	4	10
Ileu	12	31
Leu	46	88
Tyr	36	25
Phe	30	25
Lys	24	16
His	26	75
Arg	36	36

* Data from the paper by Hassell et al. (1985).

specific stain. The mAb 281-2 (Fig. 7 *C*) and the affinity-purified antibody (Fig. 7 *D*) stained the epidermis similarly; stain was intense and surrounded the keratinocytes in the stratum germinatirum and spinosum. Stain was reduced in the stratum granulosum and absent from the stratum corneum. Neither antibody stained the dermis or the dermal–epidermal border. Interestingly, the surface of the cells in the stratum basale that abuts on the dermis showed more consistent staining with the affinity-purified antibody than with the monoclonal antibody, suggesting that the binding site for the monoclonal antibody on the PG may be partially masked at the basal cell surfaces. In contrast with these stainings, the BM-1 antibody did not stain cell surfaces and showed a continuous layer of stain at the dermal–epidermal border (Fig. 7 *E*), presumably representing the basement membrane, as previously reported (Hassell et al., 1980).

Discussion

The heparan sulfate PGs are grouped together by their similar GAG chains, but these molecules can differ in cellular location, tissue distribution, and presumed function. Antibodies directed against the core proteins of these PGs, however, can distinguish among them. Here we describe an affinity-purified serum antibody against the core protein of the cell surface PG of NMuMG mouse mammary epithelial cells. Using this reagent, an antibody against basement membrane PG and chemical separation procedures, we show that NMuMG cells produce at least two distinct heparan sulfate–rich PGs: a cell surface PG and a basement membrane PG. These PGs are at distinct sites in NMuMG cell cultures and in newborn mouse skin. In culture, the cell surface PG is present at the cell surface and is shed into the culture medium, whereas the basement membrane PG accumulates beneath the cells. In the skin, the cell surface PG is at keratinocyte cell surfaces, but is absent from the cells in the more superficial layers. The basement membrane PG is at the dermal–epidermal junction, the site of the basement membrane. Although the cell surface PG is readily shed in cultures, it does not appear to accumulate extracellularly in

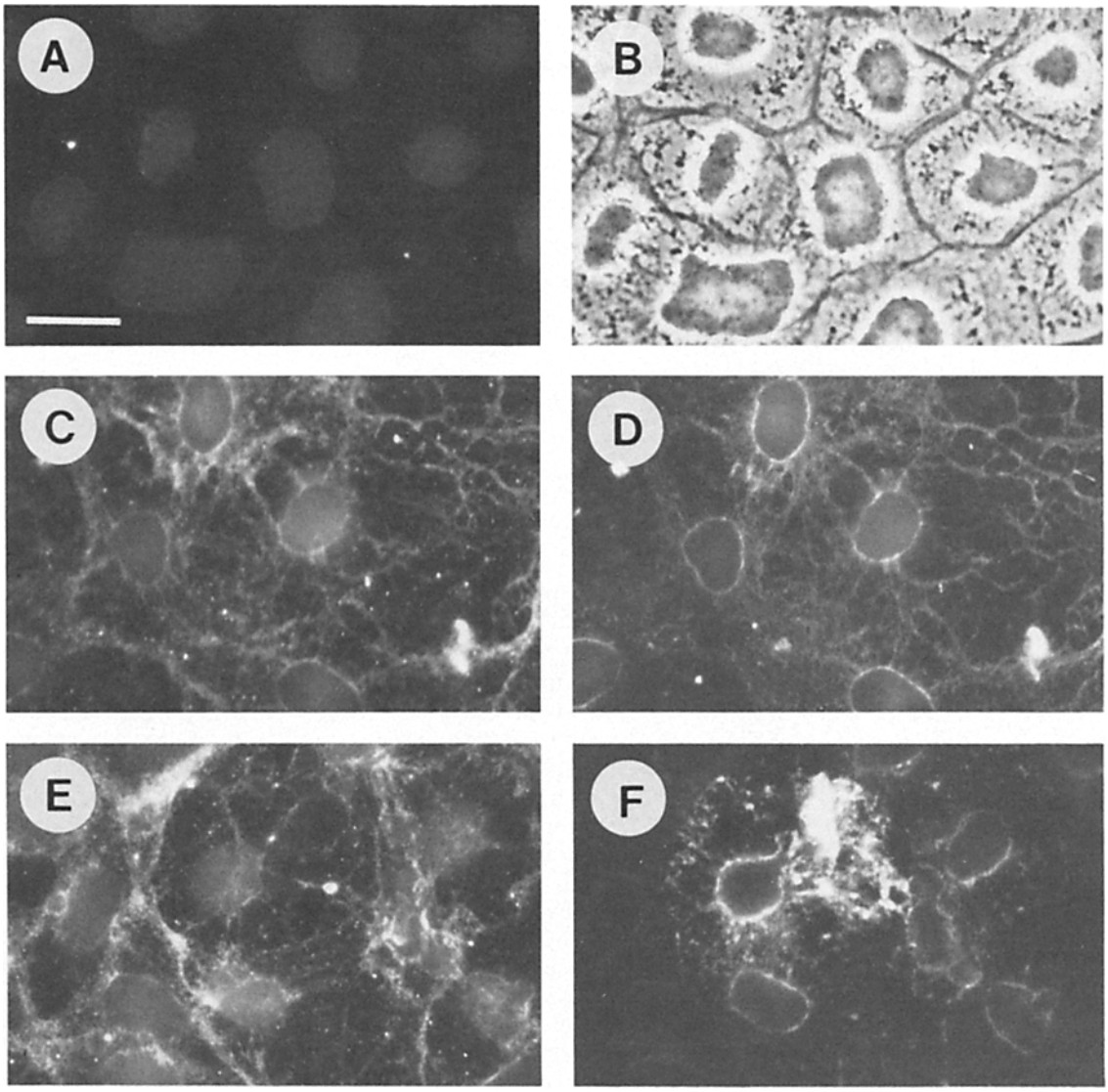

Figure 6. Immunolocalization of cell surface and basement membrane PGs in NMuMG cell cultures. Newly confluent NMuMG cells on coverslips were extracted with Triton X-100 and analyzed by double-immunostaining as described in the text. (*A*) Nonimmune pooled rat IgG (200 μg/ml), (*B*) phase micrograph of the same field as in *A*. (*C* and *E*) mAb 281-2 (20 μg/ml), (*D*) affinity-purified antibody (20 μg/ml) against the cell surface PG, (*F*) affinity-purified BM-1 antibody (1:5 dilution). TRITC-conjugated second antibody was used for mAb 281-2 and FITC-conjugated second antibody for the rabbit antibodies. Photos were taken at the focal plane of the basal cell surface. Nonimmune IgG stained nuclei slightly (*A*, *B*). mAb 281-2 (*C*) and the affinity-purified antibody (*D*) against the cell surface PG stained filamentous materials at the basal cell surface, showing extensive co-localization. However, the filamentous staining with mAb 281-2 (*E*) does not co-localize with the patchy deposits detected by the BM-1 antibody (*F*). Bar, 10 μm.

the skin. These results indicate that the core proteins of the cell surface and basement membrane heparan sulfate-rich proteoglycans are distinct gene products and suggest that their different cell and tissue localization results from their sorting by the cell on the basis of these distinct proteins.

NMuMG Cells Produce at Least Two Distinct Heparan Sulfate-rich Proteoglycans

The conditioned medium of NMuMG cell cultures contains a PG, the "medium ectodomain," that is indistinguishable by several criteria from the trypsin-released ectodomain of the cell surface PG (Jalkanen et al., 1987). We have taken advantage of this source of the ectodomain to prepare a serum antibody. This serum antibody was affinity-purified on columns containing the medium ectodomain, providing a monospecific reagent that recognizes a considerably larger number of epitopes than mAb 281-2, a monoclonal antibody that recognizes a polypeptide epitope on the core protein of the cell surface PG. The affinity-purified serum antibody also recognizes polypeptide determinants on the ectodomain core protein and thus can be used to define the core protein. The reactivity of this serum antibody confirms that the ectodomain of the cell surface PG is shed into the medium by cleavage from its membrane-associated domain (Jalkanen et al., 1987).

Serum antibodies against the cell surface PG and against the low density basement membrane PG (BM-1; Hassell et al., 1985) were used to identify the PGs associated with the NMuMG cells and deposited beneath the monolayer into the

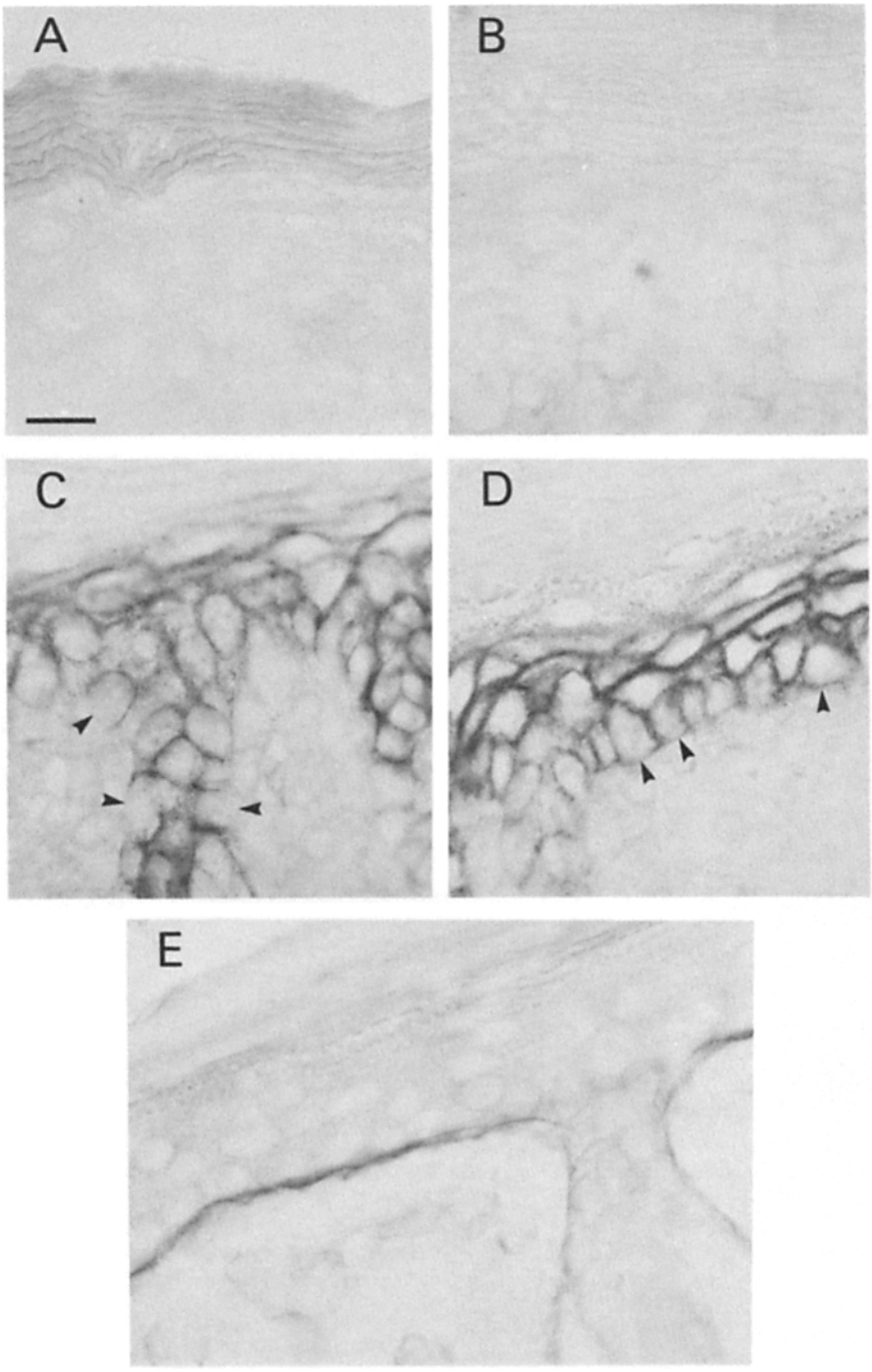

Figure 7. Immunolocalization of cell surface and basement membrane PGs in mouse skin. Frozen sections (5 μm) of newborn mouse skin were incubated with (*A*) nonimmune pooled rat IgG (25 μg/ml), (*B*) nonimmune pooled rabbit IgG (25 μg/ml), (*C*) mAb 281-2 (20 μg/ml), (*D*) affinity-purified antibody against the cell surface PG (20 μg/ml), and (*E*) affinity-purified BM-1 antibody (1:5 dilution). The bound antibodies were detected with peroxidase-conjugated second antibodies (1:50 dilution) as described in the text. The nonimmune IgG showed little stain (*A*, *B*). mAb 281-2 stains the surfaces of the cells in the stratum germinatirum and spinosum intensely; cells in the stratum granulosum stain less well and there is no stain in the stratum corneum. (*C*). There is poor or no staining of the basement membrane (*arrows* in *C*). The affinity-purified antibody against the cell surface PG stains nearly identically as mAb 281-2 except that the serum antibody stains the basal surface of the cells abutting the basement membrane (*arrows* in *D*). The BM-1 antibody stains only the basement membrane (*E*). Bar, 20 μm.

basal extracellular space. Cells labeled with radiosulfate to the steady state showed predominantly cell surface PG on isopycnic centrifugation and this PG had a buoyant density of ~1.55 g/ml. It was at the apical surfaces of subconfluent cells and, in confluent monolayers extracted with detergent to allow access to antibody, was in filamentous arrays at basal cell surfaces. This latter localization is presumably in association with actin filaments, as previously described (Rapraeger et al., 1986). The cells also contained basement membrane PG in a broad range of buoyant densities, from ~1.25 to 1.45 g/ml. This wide distribution could represent the conversion of the low density PGs into higher density PGs, as described in the EHS tumor (Hassel et al., 1985; Ledbetter et al., 1985).

The basal extracellular PGs showed three major labeled fractions. The least dense, at ~1.25 g/ml, was the basement membrane PG, confirming previous work (Hassell et al., 1980, 1985; Fujiwara et al., 1985). The other fractions, which constituted the bulk of the labeled GAG, were not identifiable immunologically, suggesting that these cells may produce additional PGs. In NMuMG cell cultures, the basement membrane PG was seen in patchy deposits beneath the monolayer in a distinct distribution from that of the cell surface PG. The extent of these patchy deposits increased with time in culture.

The cell surface and basement membrane PGs described here are rich in heparan sulfate and may contain variable amounts of chondroitin sulfate. For example, although the cell surface PG can contain both types of GAG, a substantial

proportion of this PG contains solely heparan sulfate chains (Rapraeger et al., 1985). The $^{35}SO_4$ label on the isolated basement membrane PG was susceptible to heparan sulfate lyase (unpublished), however, a PG thought to be a basement membrane PG produced by NMuMG cells is clearly a hybrid, bearing both heparan and chondroitin sulfate chains (David and Van den Berghe, 1985). Thus, the PGs produced by NMuMG cells appear to contain identical classes of GAG chains.

The serum antibody directed against the membrane-associated heparan sulfate PG from NMuMG cells did not react with the basement membrane PG. Other work, however, suggests that membrane-associated PGs may share antigenic determinants with other heparan sulfate PGs. The serum antibody directed against the rat hepatic cell surface PG recognizes a PG that is released from cell surfaces with heparin as well as a PG that is apparently intercalated into the hepatic plasma membrane (Stow and Farquhar, 1987). The heparan sulfate PG at the surface of human colon carcinoma cells is apparently intercalated into the plasma membrane (Iozzo et al., 1986), but reacts immunologically with the BM-1 antibody (Iozzo, 1984). The serum antibody described here should be useful in exploring immunological relationships among PGs.

The Cell Surface and Basement Membrane PGs Accumulate Independently

Although both the cell surface and basement membrane PG were detected on or near the basal cell surfaces of NMuMG cells, we were not able to detect the ectodomain of the cell surface PG in the basal extracellular space (Fig. 4). We also could not detect basement membrane PG in conditioned culture medium (unpublished). These results could be due to the insensitivity of our methods or could reflect distinct handling of the PGs by these cells. Therefore, we investigated the distribution of these PGs in mouse skin. Here, as previously noted (Hassell et al., 1980), the basement membrane PG was seen solely at the epidermal–dermal junction. The cell surface PG, detected by either the affinity-purified or monoclonal antibody, was solely at cell surfaces, and although the most superficial cells did not stain, the entire surface of the cells in the more basal layers stained. Interestingly, the cell surfaces facing the basement membrane stained with the affinity-purified serum antibody, but only poorly with the monoclonal antibody, suggesting that the epitope recognized by mAb 281-2 is masked at this site. No cell surface PG was detected in the interstitial spaces or on the cells in the dermis, consistent with the epithelial localization of this PG (Hayashi et al., 1987). These results suggest that the cell surface and basement membrane PGs are handled distinctly by the epidermal cells.

The function of the cell surface PG on the surface of the epidermal keratinocytes is unclear. Its presence on the basal cell surfaces is consistent with its postulated role as a matrix receptor, but its location over the entire surfaces of these cells suggests a potential role in cell–cell adhesion, possibly by self-association of the heparan sulfate chains (Fransson et al., 1983) or interactions with other cell surface proteins (Cole et al., 1986). Its loss from the most superficial cells is consistent with its known absence from terminally differentiated cells (Hayashi et al., 1987).

Multiplicity of Heparan Sulfate PGs

Most cells likely produce distinct PG core proteins containing similar GAG chains. Indeed, analogous results with cultured liver, kidney, and Chinese hamster ovary cells have recently been described by Stow and Farquahar (1987). These findings indicate that cells must distinguish between these PG molecules, in terms of synthesis, secretion, and possibly other parameters, by means of the structure of their core proteins. Unique core protein sequences undoubtedly direct the glycosylation of specific types of GAG chains (Bourdon et al., 1987). There are also PG core proteins that bear distinct types of GAG chains depending on the cell type (Tantravahi et al., 1986). Thus, the nature of core protein and the cell of origin rather than the presence of a specific type (or types) of GAG chain should be used to designate these molecules.

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