Heparan Sulfate Proteoglycans from Mouse Mammary Epithelial Cells: Localization on the Cell Surface with a Monoclonal Antibody

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ABSTRACT Mouse mammary epithelial cells, of the normal murine mammary gland (NMuMG) cell line, bear a heparan sulfate–rich proteoglycan (HSPG) on their surfaces. A hybridoma (281-2) secreting a monoclonal antibody that recognizes this HSPG was produced by fusion of SP-2/0 myeloma cells with spleen cells from rats immunized with NMuMG cells. The 281-2 monoclonal antibody is directed against the core protein of the cell surface HSPG, as demonstrated by (a) recognition of the isolated proteoglycan but not its glycosaminoglycan chains, (b) co-localization of 281-2–specific antigen and radioactive cell surface HSPG on gradient polyacrylamide gel electrophoresis and on isopycnic centrifugation, and (c) abolition of immunofluorescent staining of the NMuMG cell surface by the intact, but not the protease-digested ectodomain of the cell surface HSPG. The antibody is specific for cell surface HSPG and does not recognize the HSPG that accumulates extracellularly beneath the basal cell surface. Therefore, the 281-2 antibody may be used to isolate the cell surface HSPG and to explore its distribution in tissues.

Interactions of cells with extracellular materials are likely mediated by cell surface matrix receptors (44). Among these are cell surface heparan sulfate proteoglycans (HSPGs) (25, 34). These molecules, consisting of a core protein bearing polyanionic heparan sulfate glycosaminoglycan (GAG) chains, are found at or near the surface of all adherent cell types (16). Cell surface HSPG may exist in two forms, either bound to the cell surface via the GAG chains (21) or intercalated into the plasma membrane via the core protein (22, 27, 34). There are also extracellular matrix forms of HSPG, found in parenchymal cell basement membranes (14) or in the pericellular matrices of fibroblasts (15).

We describe a monoclonal antibody directed against the core protein of an HSPG that is intercalated into the plasma membrane of mouse mammary epithelial cells of the normal murine mammary gland (NMuMG) cell line. The GAG composition, size, buoyant density, and/or resistance to reduction appear to distinguish this HSPG from some other cellular HSPGs such as those from rat hepatocytes (29), a hepatoma cell line (30), human fibroblasts (3, 5), bovine or rabbit aortic endothelial cells (2, 31), or human colon carcinoma cells (17), and also from those isolated from basement membranes, such as from the mouse EHS tumor (10, 14) and the rat kidney glomerulus (19, 39). Indeed, the chemical characteristics of each of these HSPGs appear to differ from the others in some respect. Despite these apparent differences, the various HSPGs that have been isolated have not been compared to the extent that their relationships are conclusively established. Based on immunological reactivity (14, 17, 30, 39, 43), some HSPGs may be similar, or perhaps the antibodies detect similar determinants on distinct HSPGs. To characterize a single type of HSPG immunologically, more specific immunoglobulins, such as monoclonal antibodies, may be required. A part of this study was presented in abstract form (1).

MATERIALS AND METHODS

Cell Culture and Labeling

NMuMG mouse mammary epithelial cells were obtained and cultured in bicarbonate-buffered Dulbecco's modified Eagle's medium (DME; Gibco Lab...
oratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Tissue Culture Biological, Tulare, CA) as described previously (7, 34). For sufficient for the cells to reach a steady state level of $^3$H sulfate incorporation (6).

**Preparation of HSPCs**

**TOTAL PROTEOGLYCANS:** Total cellular and basal extracellular proteoglycans (PGs) were isolated from cells cultured on collagen gels that were adsorbed to polylysine-polyacrylamide beads (Affi-Gel 731; Bio-Rad Laboratories, Richmond, CA). Type I collagen purified from rat tail tendons (4) and solubilized in 0.1 M acetic acid was adjusted to pH 5.7 with 1 M NaOH and incubated overnight with beads (~4 mg collagen per 1 g of beads). The beads were washed with 10 mM phosphate-buffered saline (PBS), pH 7.4 to remove non-bound collagen. Cells (~1.8-2.0 × 10$^5$) were harvested by settling. The beads were extracted by incubation (for 8 h at 4°C) with 500 g supernatant containing 0.5 M Tris, pH 8.0 and 0.2 M NaCl, and was purified on a DEAE-Sephacel column as described below.

The cell surface PG were prepared from confluent monolayers after they were scraped with a rubber policeman into cold TBS-EDTA (0.5 mM EDTA) containing 1% SDS, 100 mM NaCl, and 0.5% Triton X-100, followed by centrifugation at 200 g for 30 min at room temperature. The cells and pellets were plated into 175-cm$^2$ culture flasks (20 total) and incubated at 37°C without mixing. Medium was replaced with the same buffer, and eluted with a linear gradient of 0.2-0.8 M NaCl in 0.2 M sodium acetate (pH 4.5) containing 8 M urea and 0.2 M NaCl, washed twice with cold Tris-buffered saline (TBS; 10 mM Tris, pH 7.4, 150 mM NaCl, and 0.2 M MgSO$_4$). The monolayers were scraped with a rubber policeman into cold TBS-EDTA (0.5 M EDTA) containing the following proteins inhibitors: 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 brought to 1.4 g/ml with crystalline CsCl (38). Cetylpyridinium chloride-impregnated Whatman 3MM filter discs (Whatman, England Nuclear Co., Boston, MA) were dried for scintillation counting.

The 500 g supernatant was boiled and dialyzed against 8 M urea or 4 M GdnHCl, buffered as above, and boiled. The immunoglobulins in this medium were precipitated overnight at 4°C after addition of 2 M ammonium sulfate. The precipitate was collected by centrifugation at 20,000 g for 30 min, and the supernatant was dialyzed against TBS.

**Preparation of Monoclonal Antibodies**

**IMMUNIZATION AND PRODUCTION OF HYBRIDOMAS:** Confluent NMuMG cells were washed three times with cold PBS and the cultures harvested by scraping with a rubber policeman. An aliquot of this suspension (0.25 ml in PBS) containing ~4 × 10$^5$ cells was combined with an equal volume of Freund’s complete adjuvant and injected into two 12-week-old Fisher rats. The rats received an identical inoculum intraperitoneally twice at 10-d intervals. 10 d after the second intraperitoneal injection, blood samples were taken, and sera were tested for reactivity against PGs (see below). Rats with reactive serum received a third intraperitoneal injection 3 d before their spleens were harvested.

**Production and Purification of Monoclonal Antibodies:** Selected sublines of hybridoma 281-2, a hybrid producing an antibody specific for HSPG (see Results), were cultured in serum-free medium and as ascites in splenectomized nude mice. Hybridoma fluids (5 × 10$^6$ cells/ml) were plated onto 100-mm dishes (Falcon Labware) in HB101 medium (Hana Biologics, Berkeley, CA) without serum, and medium was harvested every 4 d. The immunoglobulins in this medium were precipitated overnight at 4°C after slowly adding ammonium sulfate to 50% saturation. The precipitate was collected at 10,000 g, as described by Hardy (13), dissolved in TBS, and dialyzed against TBS.

For the production of ascites fluid, athymic nude BALB/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were splenectomized, and 2 d later received a 0.5-ml i.p. injection of 2, 6, 10, 14-tetramethyl pentadecane (Aldrich Chemical Co.). 1 wk later, 10$^6$ hybridoma cells in 0.5 ml DME were injected intraperitoneally, and ascites fluids were harvested 7–10 d later. Immunoglobulins in clarified ascites fluids (25 ml) were isolated by dialuting 1:1 with TBS and precipitating as described above. The precipitate was dissolved in TBS and desalted over a Sephadex G-25 column (Pharmacia Inc.) in TBS.

**Immunotechniques**

**ASSAY FOR RAT SERUM ANTIBODIES:** Antibody with reactivity against PG was monitored by a sheep erythrocyte aggregation assay (12, 20). S. aureus (Calbiochem-Behring Corp.) was washed three times by suspension and centrifugation in 50 mM Tris-HCl, pH 8.3, 0.45 M NaCl, and 0.5% Nonidet-P40 (Calbiochem-Behring Corp.), resuspended in the same buffer containing 0.1% BSA and rabbit anti-rat IgG (final concentration 400 µg/ml; Dako Corp., Santa Barbara, CA), and incubated for 1 h at 4°C. After three more washes and resuspension, the S. aureus were incubated with rat serum (1:10 dilution) for 2 h. After removal of serum by similar washings, 6,000 cpm

**Sepharose CL-4B (Pharmacia Inc.) chromatography in 4 M GdnHCl buffered to pH 5.5 by 50 mM sodium acetate, or in 1% SDS, 100 mM NaCl buffered to pH 8.0 with 50 mM Tris-HCl. Radioactivities in solutions were assayed by scintillation counting using 6 vol of Aquasol (West Chemical Products, Inc., Nuevo, CA) for each sample. Samples containing 4 M GdnHCl were diluted 1:10 with water before mixing with Aquasol.
of purified $^{35}$SO$_4$-PG was added and incubated overnight in the same buffer. NAbound radioactivity was removed by washing, the pellet was suspended in 2% SDS, boiled, and the radioactivity released was measured by scintillation counting.

**Solid-Phase Radioimmunoassay** Individual wells of 96-well plates (Flow Laboratories, Inc., McLean, VA) containing 0.15 ml rabbit anti-rat IgG (final concentration 2 mg/ml in TBS; Dako Corp.) were incubated over night at 4°C. The IgG was replaced with TBS containing 0.5% BSA (BSA-TBS) for 1 h at room temperature, and the wells were filled (200 ml) with horseradish peroxidase solution and incubated overnight at 4°C. After washing the wells three times with BSA-TBS, 5,000–6,000 cpm of $^{35}$S-labeled PG in 0.15 ml BSA-TBS was added and incubated overnight at 4°C. After washing as in the previous step, the wells were removed by trimming with a hot wire, and each well was counted by liquid scintillation.

**Immunodot Assay on Deal-Paper** Similar sized pieces of DEAE-cellulose paper (Whatman DE 81) and Whatman 3 MM paper (Whatman Inc.) were wet with TBS and placed in a Microfold-dot apparatus (V & P Scientific, San Diego, CA) such that the 3 MM paper underlay the DEAE-paper. Antigen in 5 ml buffer was added to each well and incubated with rabbit IgG (final concentration 100 #g/ml in PBS; Cappel Laboratories, Cochranville, PA) in an ice bath. After the monolayers were washed five times with PBS, they were fixed in ice cold 30, 70, and 100% acetone each for 1 rain, rehydrated back in PBS; Dako Corp.) for 1 h at room temperature, and the wells were filled (200 ml) with horseradish peroxidase solution and incubated overnight at 4°C. After washing the wells three times with BSA-TBS, 5,000–6,000 cpm of $^{35}$S-labeled PG in 0.15 ml BSA-TBS was added and incubated overnight at 4°C. After washing as in the previous step, the wells were removed by trimming with a hot wire, and each well was counted by liquid scintillation.

**Immunostaining of Cells** NMuMG cell cultures contain HSPGs within the cells, at the cell surface, and extracellularly beneath the monolayer (6, 35). These PGs are apparently homogeneous in size, buoyant density, and lipophilicity but have the same charge density and types of GAG, suggesting that they may differ only in their core protein. To assess this possibility, we have attempted to prepare monoclonal antibodies specific for the different PG types, and here report on the preparation of an antibody directed against the core protein of the cell surface PG.

**Induction and Screening of Monoclonal Antibodies** Rats were immunized with a fraction containing both whole cells and the material accumulating beneath NMuMG monolayers. Spleen cells from these rats were fused with SP-2/0 myeloma cells. In one fusion, resulting in 188 wells that contained successful hybrids, media from 28 were positive, as assessed by a solid phase radioimmunoassay for total PGs (see Materials and Methods). The cultures of positive clones were expanded, then screened again, and four reactive clones (209, 230, 281, and 349) were selected (Fig. 1). The most positive (281) of these was evaluated further after cloning by limiting dilution. The subclones of 281 were assayed again with the total PG preparation as well as with the purified ectodomain of the cell surface PG. Using these assays, five subclones of 281 were selected that showed reactivity against both these preparations (data not shown). However, since antigenic reactivity may be lost because of subculturing, the selected clones were maintained in culture for 6 wk before recloning to ensure

**Results**

NMuMG cell cultures contain HSPGs within the cells, at the...
that they were stable. A single clone, 281-2, was selected for
further characterization.

Characterization of Clone 281-2

Analysis of PG-specific antibodies was facilitated by an
immunodot assay in which PGs were immobilized on DEAE-
cellulose paper by the binding of their GAG chains. Use of
DEAE-cellulose paper was necessary as the PG did not bind
well to either nitrocellulose or aminophenylthioether paper,
possibly due to its extensive glycosylation. Reactivity in the
assay, shown using medium from clone 281-2 cultures, was
dependent on both the antigen concentration and the dilution
of hybridoma medium (Fig. 2). The assay was sufficiently
sensitive to detect reactivity in a 1:1,000 dilution of culture
medium and with 10 ng of PG protein per dot (Fig. 2). Thus,
clone 281-2 is a satisfactory producer, and the PG can be
detected when bound to DEAE-paper.

Clone 281-2 was recloned by limiting dilution. Using the
immunodot assay, 35 out of 36 subclones were positive,
suggesting that 281-2 was monoclonal. Two of the subclones
(281-2-17 and 281-2-28) were selected for large scale produc-
tion of antibodies by culture in serum-free medium and for
the preparation of ascites fluid in nude mice. The 281-2 clone
and its subclones produced an IgG2a rat immunoglobulin as
shown by dot assay and indirect immunoassay on nitrocel-
lose paper (not shown), using class-specific rabbit anti-rat
antibodies (kindly provided by Dr. R. Coffman, DNAX, Palo
Alto, CA). The subclones produced antibodies that, after
purification (13), showed only a single major band on isoelec-
trofocusing by both protein stain and immunostain for rat
immunoglobulins (Fig. 3, lanes A and B, respectively). Thus,
the 281-2 antibody appears to be monoclonal (33). The iso-
electric point of 281-2 was 8.6, which is unusually high for
immunoglobulins, but typical for monoclonals (33). This high
isoelectric point facilitated the use of 281-2 in immunoassays
on DEAE-paper because its low binding to DEAE produced
low backgrounds.

Characterization of the Antigen

The antigenic determinant recognized by 281-2 may reside
in the PG core protein or its GAG chains. To assess whether
the GAG chains were reactive, total 35S-PG was treated with
either alkaline borohydride, to cleave the GAG chains from
the protein, or papain, to destroy the protein. These digest
were applied directly to DEAE-paper for immunoassay and
autoradiography. Alkaline borohydride, papain-digested, and
untreated materials showed identical radioactive patterns on
DEAE-paper (Fig. 4), indicating that the digestions did not
affect the binding of the GAG to the DEAE-paper. However,
when this sheet was immunostained with 281-2, only un-
treated samples gave a positive signal (Fig. 4), suggesting that
the 281-2 antibody does not recognize the GAG chains of the
PG.

If antibody 281-2 is directed against the PG core protein,
the immunoreactive material should co-distribute with the
the cells and extracellularly. In an attempt to localize the PG that reacts with antibody 281-2, cellular and basal extracellular $^{35}$S-PGs were fractionated according to their buoyant densities using isopycnic centrifugation. Extracts of cells showed a single major $^{35}$S peak with a density of 1.45–1.55 g/ml (Fig. 6A), corresponding in density to that of the cell surface PG (35). This peak and material of lower buoyant density were immunoreactive when dotted onto DEAE-paper and stained with 281-2. The basal extracellular fraction was separated into three $^{35}$S-labeled PG peaks, of buoyant densities > 1.55, 1.35–140, and 1.25–1.28 g/ml (Fig. 6B). Despite levels of radioactivity similar to that of the cellular extracts when these fractions were stained for 281-2, immunoreactivity was weak. The only stained material corresponded to the density of the immunoreactive material in the cellular fraction, presumably due to the presence of some residual cellular material. These data suggest that antibody 281-2 recognizes cellular PGs, including possible PG precursors, but does not recognize any of the basal extracellular PG fractions.

**Cellular Localization of the Antigen**

Radiosulfate is incorporated into macromolecules by NMuMG cells solely into PGs (7) that are found both with

![Image](https://example.com/image.png)
The isolated ectodomain of the cell surface PG (Fig. 5) and a cellular PG corresponding in buoyant density to the cell surface PG (Fig. 6) are recognized by antibody 281-2. To assess directly whether the antibody reacts with a cell surface antigen, NMuMG cells were stained by indirect immunofluorescence. Monolayers were incubated with 281-2 at 4°C, fixed, then washed and stained with a fluorescent second antibody. Antibody 281-2 stains the apical cell surface; staining was especially intense where cells abut one another (Fig. 7, A and B) and not seen with rat IgG (Fig. 7E). This staining was abolished by treating the monolayer with trypsin (20 µg/ml, 4°C, 10 min) (Fig. 7, C and D), suggesting that the antigen is at the cell surface.

Completion on experiments with the purified ectodomain were performed to ascertain whether the cell surface antigen is the cell surface PG. The ectodomain was incubated with 281-2, the mixture centrifuged, and the supernatant used to stain monolayers. The ectodomain prevented the staining of NMuMG cell cultures (Fig. 8A), indicating that the cell surface antigen is the cell surface PG. Sham-incubated ectodomain (Fig. 8B) and a mixture of core protein and GAG chains prepared by treating the ectodomain with alkaline borohydride (Fig. 8C) also prevented staining. However, papain-digested samples failed to compete (Fig. 8D), indicating that papain treatment destroys the antigenic site. These results suggest that antibody 281-2 recognizes a polypeptide antigen on the cell surface PG.

**DISCUSSION**

NMuMG mouse mammary epithelial cells contain a cell surface HSPG (34). We have isolated a monoclonal mouse-rat hybridoma clone 281-2 that produces antibodies against the core protein of this cell surface PG. The antigen detected by the antibody 281-2 is identical to the cell surface PG, as demonstrated by its size, buoyant density, and susceptibility to heparitinase and chondroitin sulfate ABC lyase, and by the specific staining of the surface of cultured NMuMG cells. The antibody binds to the PG core protein because removal of the GAG chains from the PG does not abolish binding, and PG devoid of its GAG chains is as effective as is untreated PG in competitively blocking the staining of NMuMG cells by the antibody. Extracellular PGs found beneath the NMuMG cell monolayer are distinct from the cell surface PG in buoyant density and are not recognized by the antibody 281-2. Thus, 281-2 is a specific probe for the core protein of the cell surface HSPG and can be used to study the metabolism of the core protein, the distribution of the PG in tissues, and the role of the PG in cell behavior.

**Antibodies Directed Against HSPGs**

HSPGs are found intracellularly, in the extracellular matrix, and at the cell surface. Where examined, considerable diversity of PG structure exists between and within these classes (16). Despite these apparent differences, polyclonal serum antibodies prepared against basement membrane and cell surface PGs have broad specificity and may show cross-reactivity. For example, an affinity purified antibody prepared against the HSPG isolated from a basement membrane-producing tumor recognizes the PG in the basement membranes of most, if not all, tissues studied (14), in the pericellular matrix of human fibroblasts (15), and on the surfaces of aortic endothelial (31) and intestinal epithelial cells (17). A serum antibody directed against the HSPG of the glomerular basement membrane also recognizes kidney and liver epithelial cell surfaces (39). Based on these immunological results, the HSPGs are either more similar than the size and compositional data would suggest or the antibodies recognize some common determinant(s) on apparently dissimilar molecules.

Because monoclonal antibodies detect only single determinants, we have pursued the monoclonal antibody approach in an attempt to obtain a specific immunoprobe for the HSPG on the surfaces of cultured epithelial cells. Our approach was to use intact NMuMG cells as the immunogen, circumventing the need to purify large amounts of this PG, and to use a radiochemically pure, soluble PG in a radioimmunoassay to screen for specific antibody production and to assist in characterizing the antigen at the cell surface. This PG is the 35S-ectodomain of the cell surface PG, readily prepared and purified from 35SO4-labeled cells by mild treatment with trypsin followed by DEAE-cellulose chromatography (35). Further analyses used DEAE-paper as a solid support for the PG in an immunodot assay and for immunological evaluation of electrophoretic transfers. DEAE-paper was used because the PG does not bind to either nitrocellulose or aminophenyl...
FIGURE 7 Trypsin removes the stain at the apical surface of NMuMG cells. Intact monolayers treated with or without trypsin (20 μg/ml, 4°C, 10 min) were incubated with purified ascites-derived 281-2 (60 μg/ml) followed by detection with an fluorescein isothiocyanate-conjugated rabbit anti-rat IgG (see text). (A) No trypsin treatment; (B) phase contrast of A; (C) trypsin treatment; (D) phase contrast of C; (E) no trypsin treatment, and a nonspecific rat IgG (100 μg/ml) was substituted for 281-2.

The Monoclonal Antibody 281-2 Is Specific for the Core Protein of Cell Surface HSPG

281-2 recognizes a polypeptide determinant on the cell surface PG. The 281-2 reactive determinant was identified as the core protein of the cell surface PG by observing the effects of treating the PG with alkali, protease, or selective GAG-degrading enzymes. Immobilization and immunodetection of protease- or alkali-treated 35S-ectodomain on DEAE-paper indicate that although the GAG chains remain bound, the 281-2 reaction site has been lost. When the 35S-ectodomain was fractionated by gradient SDS PAGE, either intact or after removal of GAG chains with heparitinase or chondroitin sulfate ABC lyase, then transferred onto DEAE-paper and stained for 281-2, identical profiles were revealed by both autoradiographic and immunological detection. These data suggest that the antibody-binding site does not reside in the GAG chains and that the antigen recognized by 281-2 and the 35S-ectodomain are identical regardless of whether either type of GAG chain is removed. These data also confirm the finding that the cell surface PG is a hybrid, containing both heparan and chondroitin sulfate chains on the same core protein (36).

The monoclonal antibody stains the apical surfaces of cultured subconfluent NMuMG cells. This stain is specific for the cell surface PG because the purified ectodomain competes with the cell surface staining. The site recognized by the monoclonal 281-2 on the cell surface is a polypeptide, because papain digestion destroys the ability of the ectodomain to compete. These data suggest that N-linked oligosaccharides, known to exist on heparan sulfate PGs (32, 45), are not part of the antigenic site recognized by 281-2.

The core protein determinant is not found on extracellular PGs. NMuMG cells produce a basal lamina-like structure rich in HSPG when grown on type I collagen gels (6, 7). These PGs are deposited beneath the monolayers when the cells are grown on plastic, contain a basement membrane PG (7), and can be harvested by scraping and centrifuging the cells, leaving the basal extracellular PGs in the supernatant (7, 23). This fraction contains three pools of PG that differ in buoyant density from each other and from the intact cell surface PG.
FIGURE 8 The ectodomain of the cell surface PG competes with NMuMG cells for antibody 281-2. Purified ascites-derived 281-2 (12 μg/ml) was incubated for 30 min at room temperature with purified ectodomain previously (A) untreated, (B) incubated in TBS, (C) treated with alkaline/borohydride, or (D) digested with papain, prepared as in the legend to Fig. 4. The mixtures were centrifuged (10,000 g), and the supernatants were used to stain intact monolayers as described in the text.

(6). Although the intact cell surface PG was detected by antibody 281-2 in this fraction, all three basal extracellular PG pools were negative. Thus, the 281-2 reactive determinant is either masked or not present in the basal extracellular PGs. The masking may be due to extracellular processing of HSPG or, despite the presence of 8 M urea and previous extraction with 4 M GdnHCl, interaction of the HSPG with other extracellular matrix molecules (10, 42). The HSPGs accumulating beneath the NMuMG cells may be products of a gene not coding for the 281-2 polypeptide determinant. In that case, the NMuMG cells can produce both a cell surface HSPG and a basement membrane HSPG, and antibody 281-2 distinguishes between these two types of PG.

The antibody 281-2 will be used to explore the function of this cell surface HSPG in cell anchoring, matrix organization, and the control of organ development, all of which involve cell–matrix interactions via cell surface matrix receptors.

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