

# Cell Surface Proteoglycan of Mouse Mammary Epithelial Cells Is Shed by Cleavage of Its Matrix-binding Ectodomain from Its Membrane-associated Domain

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**Abstract.** The cell surface proteoglycan on normal murine mammary gland (NMuMG) epithelial cells consists of a lipophilic domain, presumably intercalated into the plasma membrane, and an ectodomain that binds via its glycosaminoglycan chains to matrix components, is released intact by proteases and is detected by monoclonal antibody 281-2. The antibody 281-2 also detects a proteoglycan in the culture medium conditioned by NMuMG cells. This immunoreactive proteoglycan was purified to homogeneity using DEAE-cellulose chromatography, isopycnic centrifugation, and 281-2 affinity chromatography. Comparison of the immunoreactive medium proteoglycan with the trypsin-released ectodomain revealed that these proteoglycans are indistinguishable by several criteria as both: (a) contain heparan sulfate and chondroitin

sulfate chains; and (b) are similar in hydrodynamic size and buoyant density; (c) have the same size core protein ( $M_r$ , ~53 kD); (d) are nonlipophilic as studied by liposomal intercalation and transfer to silicone-treated paper. Kinetic studies of the release of proteoglycan from the surface of suspended NMuMG cells are interpreted to indicate that the immunoreactive medium proteoglycan is derived directly from the cell surface proteoglycan. Suspension of the cells both augments the release and inhibits the replacement of cell surface proteoglycan. These results indicate that the cell surface proteoglycan of NMuMG cells can be shed by cleavage of its matrix-binding ectodomain from its membrane-associated domain, providing a mechanism by which the epithelial cells can loosen their proteoglycan-mediated attachment to the matrix.

**P**ROTEOGLYCANS are found on the surfaces of most, if not all, nucleated cell types (Höök et al., 1984). Of the various types of cell surface proteoglycans (PGs),<sup>1</sup> heparan sulfate-rich PGs are the most prevalent and these can be peripheral membrane components, bound by their heparan sulfate chains, or may be integral, apparently intercalated into the plasma membrane by a lipophilic core protein (Höök et al., 1984). All of the cell surface proteoglycan on normal murine mammary gland (NMuMG) mouse mammary epithelial cells contain a lipophilic domain on its core protein, which associates with the plasma membrane (Rapraeger and Bernfield, 1985), and an extracellular domain, called the ectodomain, which contains both heparan and chondroitin sulfate chains on a protein core (Rapraeger et al., 1985). Because the ectodomain of the mammary epithelial cell surface PG binds with high affinity and specificity

to extracellular matrix components (Rapraeger et al., 1987) and because cross-linking of the ectodomain causes the PG to associate with the cytoskeleton (Rapraeger et al., 1986), we have proposed that this molecule is a matrix receptor. In this role, the PG would link the cytoskeleton of epithelial cells to the interstitial matrix, thus stabilizing cell and tissue shapes.

The metabolism of cell surface PGs has been studied with a variety of cultured cell types. These PGs are actively metabolized. Some cell types predominantly secrete the PG while others degrade the bulk of the PG intracellularly (Hassell et al., 1986). During the course of investigating the metabolism of the cell surface PG in NMuMG cells we found that these cells both degraded the PG and released a substantial proportion into the medium.

To assess whether release of the PG into the medium is a physiologically significant process we characterized the material released into the medium, examined factors accelerating its release, and evaluated its source. Using a monoclonal antibody directed against the ectodomain of the cell surface PG (Jalkanen et al., 1985), we have purified to homogeneity the immunoreactive PG that accumulates in medium conditioned by NMuMG cells. This PG is nonlipophilic and indistinguishable from the ectodomain produced by mild protease

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1. *Abbreviations used in this paper:* CPC, cetylpyridinium chloride; GAG, glycosaminoglycan, GdnHCl, guanidine hydrochloride; NMuMG cells, normal murine mammary gland epithelial cells; PG, proteoglycan.

treatment of the cells. Suspension of the cells with EDTA augments the release of this PG and kinetic studies of the release indicate that the immunoreactive medium PG is derived directly from the cell surface PG. Moreover, suspension of the cells prevents further accumulation of PG at the cell surface. Thus, the cell surface PG is shed from NMuMG cells by cleavage of its ectodomain from its membrane-associated domain. This cleavage, which is presumably via proteolysis, is enhanced by rounding of the cells. These results indicate that when the cells change their shape and/or their association with the substratum, the matrix-binding ectodomain of the cell surface PG is released and is not readily replaced, providing a mechanism by which the cells can loosen their attachment to the matrix.

## Materials and Methods

### Reagents

**Materials Were Obtained as Follows.** Carrier-free  $H_2^{35}SO_4$  was obtained from New England Nuclear (Cambridge, MA), DEAE-Sephacel, Sepharose CL-4B, and CNBr-activated Sepharose CL-4B from Pharmacia (Piscataway, NJ), DEAE-cellulose (DE-81) and silicone-treated (IPS) papers from Whatman Inc. (Clifton, NJ), Zeta-probe cationic nylon membrane from Bio-Rad Laboratories (Richmond, CA), ultra pure urea, CsCl, and guanidine hydrochloride (GdnHCl) from Schwartz-Mann, Spring Valley, NY, 4-chloro-1-naphthol from Sigma Chemical Co. (St. Louis, MO), heparan sulfate lyase and chondroitin ABC lyase from Miles Laboratories, Inc. (Naperville, IL), and Stains All dye (1-ethyl-2-(3-(1-ethylnaphthol(1,2d)-thiazolin-2-ylidene)-2-methyl-propenyl)naphthol(1,2d)thiazolium bromide) from Eastman Kodak Co. (Rochester, NY). All other reagents and chemicals were of the highest purity available.

**Immunochemicals.** We have recently described a mAb 281-2 against the cell surface proteoglycan of NMuMG cells (Jalkanen et al., 1985). This monoclonal recognizes the polypeptide core protein of the PG and is a rat IgG<sub>2a</sub> immunoglobulin. In this paper, the mAb 281-2 was purified from hybridoma-induced ascites fluid as described (Jalkanen et al., 1985). The concentrations used are indicated in the figure legends. Nonspecific rat IgG and peroxidase-conjugated rabbit anti-rat IgG were purchased from DAKO (Accurate Chemicals, Westbury, NY).

### Preparation and Purification of Proteoglycans

**Harvesting of NMuMG Cell-conditioned Medium.** Early passages (12–20) of mouse mammary epithelial cells (NMuMG) were maintained in bicarbonate-buffered DME (Gibco, Grand Island, NY) containing 10% FBS (Tissue Culture Biologicals, Tulare, CA) as described previously (David and Bernfield, 1979; Rapraeger and Bernfield, 1983). These cells were routinely plated at one-quarter or less confluent density (100-mm dishes; Falcon Labware, Oxnard, CA) and fresh medium was replaced every 2–3 d. The conditioned medium harvested during cell growth, before confluence, was used as the starting material to purify the immunoreactive (281-2) medium PG.

**Purification of Immunoreactive PG from Culture Medium.** Conditioned medium (2–10 liters), mixed with tracer amounts of  $^{35}SO_4$ -labeled conditioned medium (see next section), was brought to 2 M in urea, 50 mM in Na-acetate, pH 4.5. The mixture was loaded at 4°C onto a DEAE-Sephacel column (2.6 × 8 cm) previously equilibrated with this loading buffer. The column was washed with four-column vol of loading buffer supplemented with 0.2 M NaCl, and then was eluted with a linear 0.2–0.8 M NaCl gradient in the same buffer at 16 ml/h, collecting 4-ml fractions. The elution was followed by analyzing aliquots (50  $\mu$ l) from each fraction by immunodot assay for 281-2-positive material, as described below. Positive fractions were pooled (see Fig. 1) and precipitated with 3 vol of ethanol at –20°C. The pellet (20,000 g) was dried and then dissolved in three 4-ml aliquots of 4 M guanidine hydrochloride (GdnHCl), buffered to pH 4.5 with 50 mM Na-acetate. Solid CsCl was added to bring the density to 1.4 g/ml and this mixture was centrifuged at 15°C in rotor model SW 65 (Beckman Instruments, Inc., Palo Alto, CA) at 46,000 rpm for 72 h. The gradients were harvested into 250- $\mu$ l aliquots, 10  $\mu$ l of which was analyzed by immunodot assay. Positive fractions were pooled, boiled for 15 min, and dialyzed at 4°C

against three changes of Tris-buffered (10 mM, pH 7.4) saline (150 mM NaCl) (TBS).

The mAb 281-2, isolated by DEAE-chromatography from hybridoma-induced ascites fluid (Jalkanen et al., 1985), was bound to CNBr-activated Sepharose 4B-CL (2 mg protein/ml wet Sepharose) as in the manufacturer's instructions. The 281-2-Sepharose 4B-CL column (2.6 × 10 cm) was equilibrated at 4°C with TBS and the dialyzed PG sample was loaded at 20 ml/h. The column was washed with 5 bed vol of TBS and then eluted with 20 ml of 50 mM triethylamine (pH 11.5) at the same rate to release the bound material (Parham, 1979). Eluents were rapidly neutralized with 1:10 vol of 1 M Tris, pH 7.4, and aliquots (50  $\mu$ l) were analyzed by immunodot assay. Positive material was precipitated with ethanol as before, dried, dissolved in water, rehyophilized, and stored dry at –20°C. This treatment yielded a PG preparation that was readily water soluble. The purification procedure was followed by dialyzing aliquots at each step against 0.1% SDS, lyophilizing and analyzing the material by SDS-PAGE and Western blotting (Fig. 3), as described below.

**Harvesting of Radioactive PGs from NMuMG Cell Cultures.** The intact cell surface PG, its trypsin-released ectodomain, and medium PGs were harvested from NMuMG monolayers labeled with radiolabeled (10  $\mu$ Ci/ml) for 24 h (Rapraeger and Bernfield, 1983; David and Bernfield, 1982). Medium was first removed from the monolayers and submitted to the purification described above for nonlabeled medium. Monolayers were washed twice with ice-cold PBS (10 mM phosphate buffered, pH 7.4, saline) supplemented with 1.25 mM  $CaCl_2$  and 0.9 mM  $MgSO_4$ , and then scraped with a rubber policeman into cold Ca- and Mg-free TBS supplemented with 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF; Calbiochem-Behring Corp., La Jolla, CA), 5 mM benzamide (Aldrich Chemical Co., Milwaukee, WI), and 5 mM *N*-ethylmaleimide (Calbiochem-Behring Corp.) The cells were centrifuged (200 g), the supernatants discarded, and the cell pellet washed again in the cold scraping solution. The cells were then extracted at 4°C with 1% Triton X-100 and 0.5 M KCl in the scraping solution. This suspension was centrifuged again at 600 g to pellet nuclei and insoluble cytoskeletal materials, but leaving the intact cell surface heparan sulfate PG in the supernatant (Rapraeger et al., 1986). The supernatant was then mixed with an equal volume of 8 M GdnHCl buffered to pH 4.5 with 50 mM Na-acetate and boiled. For the total cellular extract, the pelleted cells were directly solubilized into 4 M GdnHCl without any prior extraction.

For preparation of the trypsin-released ectodomain, cells pelleted after scraping were washed three times in 0.5 mM EDTA-TBS and exposed to trypsin (20  $\mu$ g/ml) for 10 min on an ice bath. After incubation, soybean trypsin inhibitor was added (100  $\mu$ g/ml) and cells were centrifuged again leaving the ectodomain in the supernatant (Rapraeger and Bernfield, 1985), which was made 4 M in GdnHCl and boiled as for the intact cell surface PG.

The cell surface PG and the trypsin-released ectodomain were further fractionated by isopycnic centrifugation as described above for medium PG, except that 1% Triton X-100 was included in the buffers. Labeled PG in the gradients was assayed by aliquoting onto cetylpyridinium chloride (CPC)-impregnated 3 MM filters (Whatman, Inc.), followed by washing in 10% TCA, and rinsing with water and 95% ethanol before scintillation counting (Stephens et al., 1978; Rapraeger and Bernfield, 1985; Jalkanen et al., 1985).

**Kinetics of Ectodomain Release from Suspended NMuMG Cells.** Newly confluent NMuMG monolayers were washed thrice with ice-cold EDTA-PBS, scraped from the dish, and washed once by centrifugation in the same buffer on ice. The pelleted cells were suspended in 10 ml of this buffer and two 1-ml aliquots were removed for the zero time controls. These were submitted to trypsin treatment to release the ectodomain as described above. The remaining cells were centrifuged, resuspended in 10 ml of DME containing 10% FBS at 37°C, and aliquoted into 1-ml aliquots in polycarbonate tubes. After varying times at 37°C, during which the cells were regularly resuspended, duplicate tubes were removed and the cells were centrifuged at 4°C. The media were collected and the cells were trypsinized to release the ectodomain.

### Qualitative Assay of Proteoglycans

**Dot Immunoassay for PG.** We used the recently described solid phase assay for immunodetection of PGs (Jalkanen et al., 1985). Briefly, wet DEAE-paper was placed into an immunodot apparatus (V&P Scientific, San Diego, CA) and samples in 8 M urea (buffered to pH 4.5 with 50 mM Na-acetate) were loaded onto the paper using mild vacuum. When samples contained high salt concentrations (i.e., CsCl), they were diluted to <0.2 M Cl concentration with 8 M urea to allow the binding of the PG to the DEAE-paper.

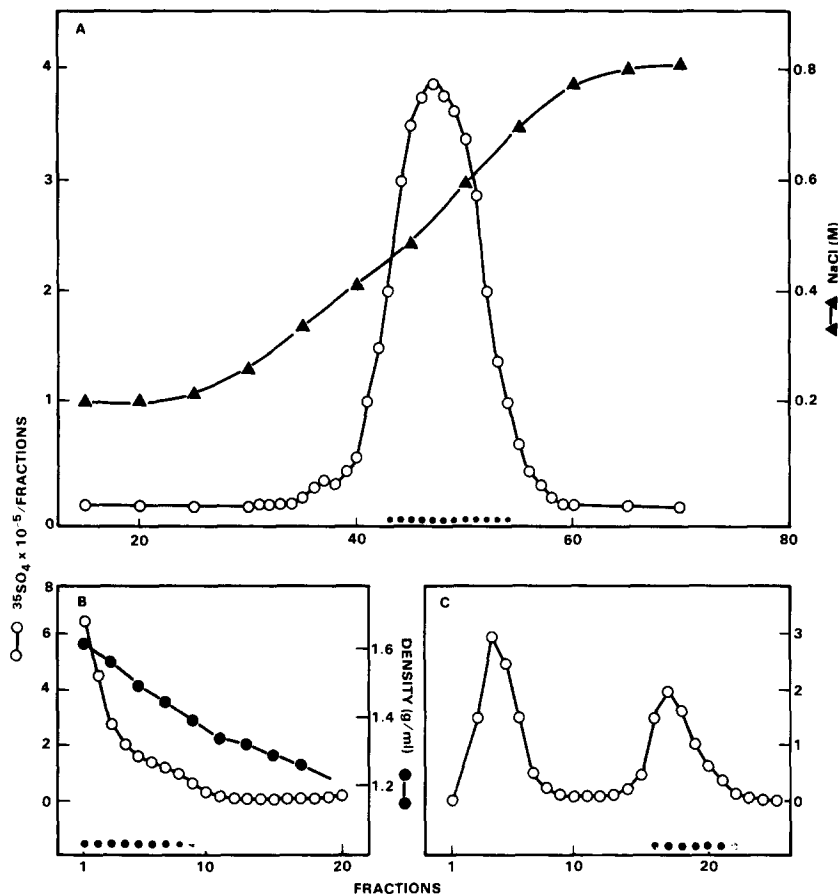


Figure 1. Isolation of immunoreactive proteoglycan from culture medium conditioned by NMuMG cells. Medium conditioned by NMuMG cells was subjected to DEAE-cellulose chromatography (A), isopycnic centrifugation (B), and 281-2 immunoaffinity chromatography (C) as described in the text. (A) One major  $^{35}\text{SO}_4$ -labeled peak (open circles) eluted from the DEAE-column at 0.45–0.65 M NaCl (solid triangles). This peak also contained immunoreactive PG as probed with the mAb 281-2 on DEAE-immunodot assay (dots at the bottom). (B) In CsCl gradient both the radioactive (open circles) and the immunoreactive (dots at the bottom) materials were found at buoyant densities (solid circles) of 1.4 g/ml or higher. (C) The immunoaffinity column separated two  $^{35}\text{SO}_4$ -labeled peaks; only the material that bound to the column reacted positively with 281-2 (dots at the bottom).

After loading, the paper was transferred to a dish containing TBS supplemented with 1% FBS and washed several times to remove the urea. The paper was then incubated overnight at 4°C with mAb 281-2 (10–50 µg/ml). After 5–6 washes with TBS, the DEAE-paper was incubated with the peroxidase-conjugated second antibody (rabbit anti-rat; 1:200 dilution) in TBS containing 1% FBS for 30 min at room temperature, and washed again five to six times with TBS. The immobilized peroxidase conjugate was visualized using 0.5% (wt/vol) of 4-chloro-1-naphthol containing 0.03% (vol/vol) hydrogen peroxide in TBS (Esen et al., 1983). Subsequent work has shown that cationic nylon membranes can replace the DEAE-paper in this assay.

**Sizing Chromatography.** The relative mass of the PGs was assessed by Sepharose Cl-4B chromatography in 4 M GdnHCl buffered to pH 5.5 by 50 mM Na-acetate. Radioactivity in solutions was assayed by scintillation counting using 6 vol Aquamix (Westchem, San Diego, CA) for each sample volume. Samples containing 4 M GdnHCl were diluted 1:10 with water before mixing with Aquamix.

**Enzyme Digestions of Glycosaminoglycan (GAG).** The nature of the GAG chains was established by various enzyme treatments before electrophoresis and Western blotting. Proteoglycans were treated with (a) heparan sulfate lyase (0.1 U/ml) in 50 mM Tris, pH 7.0, and 5 mM calcium acetate for 3 h at 37°C to digest heparan sulfate, or (b) with chondroitin sulfate ABC lyase (0.5 U/ml) in Tris, pH 8.0, and 30 mM sodium acetate to digest chondroitin and dermatan sulfates. Both Tris buffers also contained 0.1 mg/ml of BSA and 1 mM PMSF.

**Western Blots.** To measure the relative mass of immunoreactive PG or its enzyme digestion products, they were 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  $\text{Na}_2\text{SO}_4$ , and 0.1% SDS (Jalkanen et al., 1985). The gel was run overnight to completion at 125 V and was transferred onto a pad of a transfer apparatus (model TE52; Hoefer Scientific Instruments, San Francisco, CA) and covered with DEAE-cellulose paper (Jalkanen et al., 1985), with a nitrocellulose sheet (0.45 µm), or with Zeta-Probe (Bio-Rad 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 sheets were analyzed by autoradiography by exposing to X-Omat film and the antigen was localized by in-

direct enzyme immunostaining as described above for the dot immunoassay, except that the nitrocellulose sheet or the nylon membrane was treated after the transfer with 0.1% Tween-20-TBS for 1 h to block nonspecific binding of proteins to the membrane. Occasionally, a portion of the gel was developed for silver staining (Oakley et al., 1980) by fixing the proteins first in 10% TCA-50% methanol, washing in methanol/acetic acid/water (5:7:88) for 3 h, 50% methanol overnight, followed by silver staining. Proteoglycans in the gel were identified with the carbocyanine dye, Stains-All (Eastman Kodak Co.), by fixing the gel in 25% isopropanol for 30–65 min at 65°C, and then staining as described by Green et al. (1973).

**Hydrophobic Western Blots.** Electrophoretic transfers from the SDS polyacrylamide gels were also performed with a hydrophobic support, silicon-treated paper (IPS paper, Whatman, Inc.) A sheet of this paper was wet overnight in 75% isopropanol and transferred 24 h at 75 V in a transfer apparatus (model TE52; Hoefer Scientific Instruments). After the transfer, the paper was washed for 2 h in 3 M NaCl, 50 mM Tris-HCl (pH 7.4), and for 1 h in 50 mM Tris at the same pH, dried, and exposed to X-Omat film. Parallel samples were transferred onto cationic nylon membranes which bind PGs regardless of their lipophilicity.

### Quantitative Assay of Proteoglycans

**Lipophilicity.** A new method was developed to assay membrane proteins based on their ability to bind to hydrophobic paper. Silicone-treated paper disks (IPS paper; Whatman, Inc. were soaked overnight in 75% (vol/vol) isopropanol and then stored several days in 3 M NaCl buffered to 7.4 with 50 mM Tris-HCl. Complete prewetting and high salt concentration facilitates binding of lipophilic materials to the paper. For assay, disks (26 mm) were placed on a parafilm sheet and aqueous samples in 4 M GdnHCl, buffered to 7.5 with 100 mM Tris, were applied. Optimal volume for this size disk varies from 150 to 300 µl. The filters were then incubated at room temperature for 20–24 h by covering them with a lid to which a wet 3 MM sheet (Whatman, Inc.) was held by capillarity (prevents evaporation of samples). After incubation, the filters were washed sequentially under mild vacuum suction (model FH-225V; Hoefer Scientific Instruments) with 3 M

NaCl (pH 7.4) and distilled water (40 ml of each), dried, and scintillation counted in Liquifluor-toluene (New England Nuclear).

Liposome intercalation, as described by Rapraeger and Bernfield (1983, 1985), was used to compare with the results of IPS paper assay. Proteoglycans in 4 M GdnHCl (pH 7.4) were brought to 75 mM octylglucoside (Calbiochem-Berhing Corp.) and 5 mg/ml soybean phospholipase (Sigma Chemical Co.) and dialyzed against 4M GdnHCl in which the Tris was replaced with 50 mM sodium acetate, pH 5.8, to form liposomes. The association of the PG with liposomes was assessed by flotation of the liposomes in sucrose solutions formed by combining 90  $\mu$ l of the PG/liposome mixture with an equal volume of 40% sucrose in above mentioned buffer. These were centrifuged in an Airfuge (Beckman Instruments, Inc.) at 28 psi for 2 h and the gradients formed were frozen immediately in a dry ice/ethanol bath. The frozen tubes were cut in half and PG was measured on CPC-impregnated discs as described above.

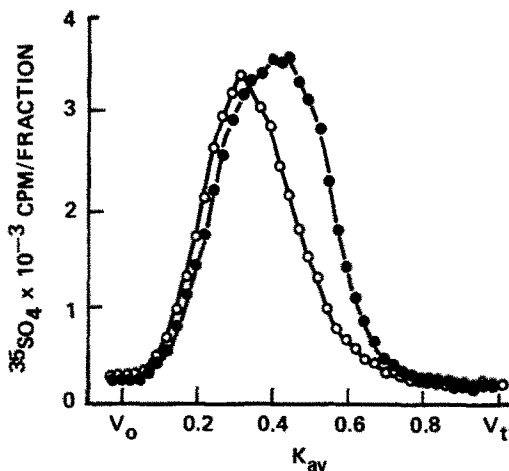
**Quantitation of Medium 281-2-positive PG.** A radioimmunoassay, based on a modification of the PG immunodot assay (Jalkanen et al., 1985), was used to quantitate the amounts of 281-2-positive PG in medium samples from NMuMG cell cultures. For the assay, mAb 281-2 was radioiodinated by the Chloramine T oxidation procedure (Stähli et al., 1983) to a specific activity of  $2.5 \times 10^5$  cpm/ $\mu$ g protein.

For PG analysis, medium samples were brought to 50 mM sodium acetate (pH 4.5). Known standard amounts of purified medium PG (quantitation based on amino acid analysis) and medium samples for assay were loaded onto DEAE-cellulose paper as described above for the immunodot assay. The DEAE-paper was then exposed to  $^{125}$ I-labeled mAb 281-2 (10,000 cpm/ml) overnight at 4°C. After removing the nonbound 281-2 by washing with TBS five to six times, the DEAE-paper was dried, the dots were cut out, and counted in a gamma counter (model 1195; Searle Analytic, Des Plaines, IL). The radioactivities from the standard curve of purified medium PG allowed the estimation of the amounts of 281-2-positive PG in the experimental samples. This assay was suitable for samples containing from 5 to 500 ng PG.

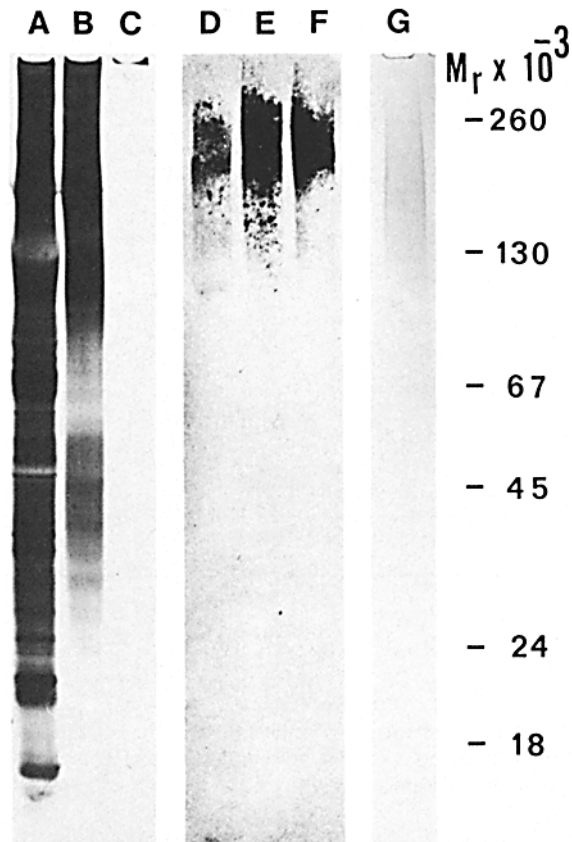
## Results

### Isolation and Purity of Immunoreactive Medium Proteoglycan

Conditioned medium from NMuMG cell cultures was subjected to DEAE-cellulose chromatography to remove serum proteins and to reduce the volume of the sample. A single  $^{35}$ SO<sub>4</sub> peak eluted from the column at 0.5–0.65 M NaCl (Fig. 1 A), identical to the elution of the ectodomain of the



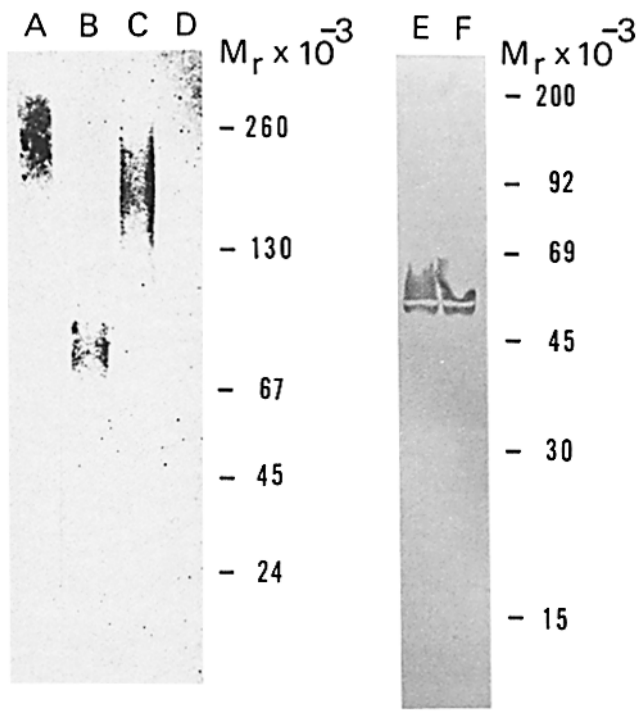
**Figure 2.** Sepharose 4B chromatography of proteoglycans separated on a 281-2 immunoaffinity column. An equal amount of radioactivity, 25,000 cpm, from materials that bound (open circles) and failed to bind (solid circles) to a mAb 281-2 affinity column (see Fig. 1 C), were fractionated on a Sepharose CL 4B column as described in the text.



**Figure 3.** Purity of the immunoreactive medium proteoglycan. Aliquots after each purification step containing identical amounts of 281-2 reactive material were loaded onto SDS-PAGE. (Lanes A and D) After DEAE-cellulose chromatography; (lanes B and E) after CsCl-gradient centrifugation; (lanes C and F) after 281-2 affinity chromatography (see the text also). The reactive material represented 0.8  $\mu$ g of purified protein which was placed in lanes C, F, and G. Proteins were visualized by silver staining (lanes A–C) and immunoreactive PG by Western blotting against the mAb 281-2 (10  $\mu$ g/ml) on DEAE paper (lanes D–F). Lane G was stained with Stains All dye (Eastman Kodak Co.). Molecular mass estimates are based on the mobilities of the silver-stained beta and alpha chains of type I collagen (Freytag et al., 1979), BSA, ovalbumin, chymotrypsinogen A, and myoglobin.

cell surface PG (Koda et al., 1985). This labeled peak contained immunoreactive PG, as shown by immunodot assay with antibody 281-2 (Fig. 1 A). The antibody-positive fractions were pooled and fractionated by isopycnic centrifugation; the bulk of radioactivity was found at buoyant densities  $>1.4$  g/ml (Fig. 1 B). The antibody-positive fractions (bottom nine tubes) were pooled and placed on a 281-2 immunoaffinity column. This column separated the radioactivity into two pools, one nonimmunoreactive that failed to bind to the column, and an immunoreactive pool that bound to the column and eluted as a single peak (Fig. 1 C). The nonreactive peak was reloaded onto the 281-2 column, but again did not bind. The immunoreactive material behaved homogeneously on Sepharose 4B chromatography (Fig. 2) with a peak at  $K_{av}$  0.32, which the nonreactive material was more heterogeneous and smaller in size (Fig. 2) and was not studied further.

Samples containing equal amounts of immunoreactive



**Figure 4.** Western blot analysis of immunoreactive medium PG and trypsin-released ectodomain. The immunoreactive medium PG (1  $\mu\text{g}$ ) was subjected to selective GAG removal before Western analysis on DEAE paper (lanes A–D) or on cationic nylon membrane (lanes E and F). The intact PG (lane A), after treatment with heparan sulfate lyase (lane B), chondroitin sulfate ABC lyase (lane C), or with both enzymes (lane D) was fractionated in 3.8–15% PAGE and Western blotted as described in the text. DEAE paper does not retain the core protein (Rapraeger et al., 1985; Jalkanen et al., 1985). Lanes E and F present Western blots on 3.8–20% PAGE for medium PG (lane E) and trypsin-released ectodomain (lane F) after heparan sulfate lyase and chondroitin sulfate ABC lyase digestions. The limited capacity of the nylon membrane allows it to become blocked at sites where proteins added as carrier or present in the enzyme preparations are transferred, resulting in nonuniform retention of the core protein in these regions and the spurious appearance of multiple bands. Relative molecular mass is estimated from the front of the bands. See Fig. 3 for molecular mass markers for lanes A–D and Fig. 6 for lanes E and F.

materials from each purification step were analyzed by gradient PAGE followed by silver staining to detect protein (Fig. 3, lanes A–C), Western blot analysis to detect the immunoreactive components (Fig. 3, lanes D–F) and Stains All–carbocyanine dye staining to detect glycosaminoglycan chains (Fig. 3, lane G). The  $^{35}\text{SO}_4$ -labeled peak from DEAE-cellulose contained several polypeptides (Fig. 3, lane A) and a smear of high relative molecular mass immunoreactive material (Fig. 3, lane D). This smear corresponds to the PAGE appearance of the cell surface PG and its ectodomain and is due to the variable extent of glycosylation of the core protein (Rapraeger et al., 1985; Jalkanen et al., 1985). Several polypeptides were removed by isopycnic centrifugation in 4 M GdnHCl, but the immunoreactive smear remained (Fig. 3, lanes B and E). The material that bound to the 281-2 column no longer showed any proteins by silver staining, although the immunoreactive smear was again seen (Fig. 3, lanes C and F). The smear of immunoreactive material stained with

a carbocyanine cationic dye (Stains All) which stains polyanions, including glycosaminoglycans (GAG) (Bader et al., 1972) (Fig. 3, lane G). The lack of silver staining, the homogeneous behavior on Sepharose 4B, the single smear of immunoreactivity, and the GAG staining indicate that the procedure yielded a medium PG free of detectable contaminants. This protocol results in a  $>10^5$ -fold purification from medium conditioned by subconfluent NMuMG cells, producing 10–15  $\mu\text{g}$  of purified PG (as protein) per liter, a yield of 60–90% based on radioimmunoassay measurement.

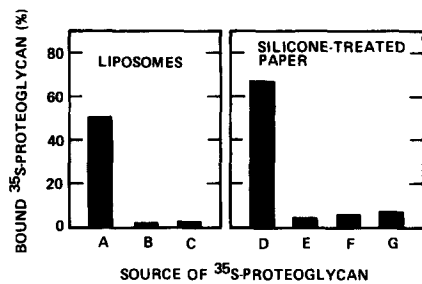
#### **Characterization of the Immunoreactive Medium Proteoglycan**

The 281-2 monoclonal antibody is directed against the cell surface PG of NMuMG cells, but these data indicate that it also recognizes a PG in the medium. Although these PGs could contain dissimilar proteins with a shared epitope, this result suggests that the immunoreactive medium PG is derived from the PG at the cell surface. Therefore, we characterized the medium PG to assess its possible relationship to the cell surface PG.

**Immunoreactive Medium PG Is Chemically Related to the Trypsin-released Ectodomain of the Cell Surface PG.** The cell surface PG contains both heparan sulfate and chondroitin sulfate chains, shown using the ectodomain of the cell surface PG that is released from the cells by trypsin treatment (Rapraeger et al., 1985; Jalkanen et al., 1985). The immunoreactive medium PG is also a hybrid PG, as demonstrated by heparan sulfate lyase and/or chondroitin ABC lyase digestion before Western blot analysis (Fig. 4). After enzymatic removal of the GAG chains, the core proteins of the trypsin-released ectodomain and the medium PG migrate at nearly the same  $M_r$ ,  $\sim 53$  kD.

**Medium PG Recognizes Matrix Molecules.** The cell surface PG binds to types I, III, and V collagens (Koda et al., 1985) and to the COOH-terminal heparin-binding domain of fibronectin (Saunders and Bernfield, 1988). The immunoreactive medium PG has similar binding properties: all the medium PG applied to a fibrillar collagen column bound to the column and eluted in a  $\text{Na}_2\text{SO}_4$  gradient, beginning at 80 mM and completed at 200 mM (not shown), mimicking results obtained with the ectodomain (Koda et al., 1985). Liposomes containing the intact cell surface PG bind to the heparin-binding domain of fibronectin immobilized on polyvinylchloride wells. This interaction is abolished by pretreatment of the wells with the immunoreactive medium PG (Saunders and Bernfield, 1988), indicating that it binds to the same site as the intact cell surface PG.

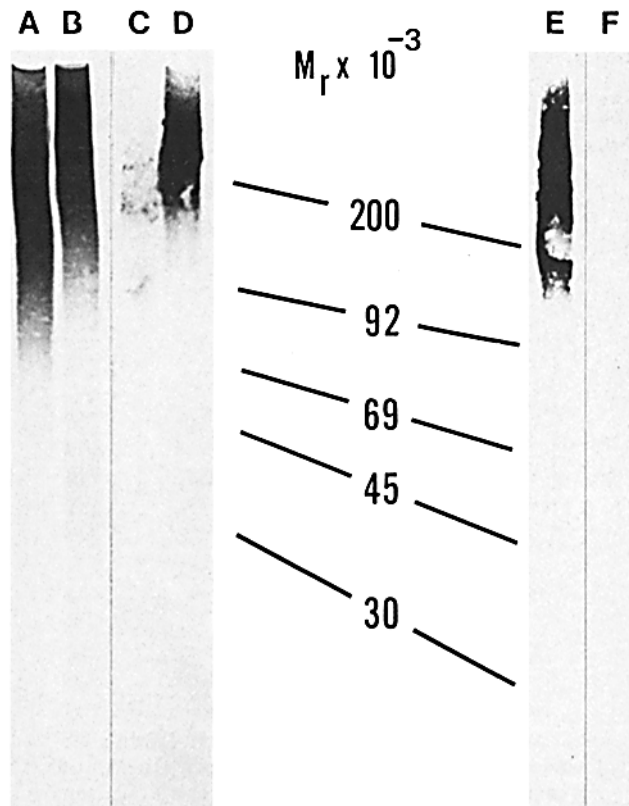
**Immunoreactive Medium PG Is Nonlipophilic.** The cell surface PG has a lipophilic domain and it can be quantitatively and vectorially intercalated into liposomes, whereas the trypsin-released ectodomain does not contain a lipophilic domain (Rapraeger and Bernfield, 1985). To evaluate the lipophilicity of the medium PG, we have compared its ability to intercalate into liposomes and to bind to hydrophobic silicone-treated paper with the PG from whole cells and PG released from cells by trypsin treatment. Initial experiments compared liposome intercalation and binding to the hydrophobic paper. An extract of radiolabeled cells, labeled materials released from the cells by brief treatment with ice-cold trypsin and labeled conditioned medium were



**Figure 5.** Analysis of PG lipophilicity by liposomal intercalation and binding to silicone-treated paper. <sup>35</sup>SO<sub>4</sub>-labeled fractions from NMuMG cell cultures were prepared in 4 M GdnHCl and used in assays as described in the text. (A and D) Total cell extract; (B and F) trypsin-released cell surface material; (C and G) NMuMG-conditioned medium; (E) as D, but supplemented with 1% Triton X-100. The total radioactivity in PG for each fraction was measured using the CPC-filter assay and compared with the radioactivity bound to the silicone-treated paper and intercalated into liposomes. The percent PG bound for each sample group represents a mean of 4 for silicone-treated paper and 2 for liposomal intercalation.

made 4 M in GdnHCl and intercalated into liposomes or applied to hydrophobic paper as described in Materials and Methods (Fig. 5). 48% of the cellular label could be intercalated into liposomes compared with <2% of the trypsin-released label, consistent with previous work showing that cellular PG is lipophilic but that the trypsin-released ectodomain is not (Rapraeger and Bernfield, 1985). Less than 2% of the label in the medium could be intercalated into liposomes, suggesting that medium PG is also not lipophilic. Qualitatively identical results were obtained by binding to silicone-treated paper; 65% of the cellular label bound to silicone-treated paper, and Triton X-100 reduced this binding to 4%, suggesting that binding to silicone-treated paper is due to hydrophobic interactions (Fig. 5). Only a small proportion of trypsin-released label (4.5%) and of the label in the medium (6.5%) bound to silicone-treated paper, confirming the evidence provided by the liposome intercalation studies. The lower proportion of lipophilic PG shown by liposomal intercalation is likely due to lower recoveries of lipophilic PG than nonlipophilic PG during the several steps involved in the intercalation procedure.

Purified proteoglycans and the silicone treated paper, as a hydrophobic transfer matrix, were used to assess differences in the lipophilicity of the cell surface PG, the ectodomain and the medium PG. Radiosulfate-labeled cell surface PG, ectodomain, and medium PG purified on DEAE-cellulose and by isopycnic centrifugation were subjected to PAGE in duplicate; one set was transferred to a cationic nylon membrane and the other to silicone-treated paper. The ectodomain and the cell surface PG transferred to and bound nearly equally well to the cationic membrane (Fig. 6, lanes A and B). On silicone-treated paper, only trace amounts of the ectodomain bound (Fig. 6, lane C) while the cell surface PG bound well (lane D), indicating that the hydrophobic paper preferentially accepts lipophilic PGs for transfer. The purified immunoreactive medium PG transferred and bound to the cationic membrane, but not to silicone-treated paper (Fig. 6, lanes E and F), showing its lack of lipophilicity.



**Figure 6.** Hydrophobic blot of immunoreactive medium proteoglycan. <sup>35</sup>SO<sub>4</sub>-labeled PGs (10,000 cpm/lane) were separated in gradient SDS polyacrylamide gels (left, 3.8–20%; right, 3.5–15%) and then electrophoretically transferred onto cationic nylon membranes (Zeta Probe; Bio-Rad Laboratories; lanes A, B, and F) or to prewetted silicone-treated paper (lanes C, D, and F), which were then autoradiographed as described in text. The ectodomain (lanes A and C) and the cell surface PG (lanes B and D) bound to the cationic membrane, but only the cell surface PG bound appreciably to silicone-treated paper. Immunoreactive medium PG (lanes E and F) bound to the cationic membrane (lane E), but not to silicone-treated paper (lane F). The indicated molecular mass markers (<sup>14</sup>C-labeled myosin, phosphorylase b, BSA, ovalbumin, and carbonic anhydrase [Amersham Corp., Arlington Heights, IL]) were transferred onto cationic membranes with each run.

#### **Immunoreactive Medium Proteoglycan Is Shed from the Cell Surface**

Both the PG purified from the conditioned medium and the ectodomain released from the cell surface PG by trypsin react with antibody 281-2, are nonlipophilic, contain heparan and chondroitin sulfate, have a core protein of ~53 kD, and bind interstitial matrix materials (see Table I), suggesting that the medium PG is derived from the cell surface PG. If so, then loss of PG from the cell surface should occur concomitantly with appearance of the PG in the medium. Because the cell surface PG is anchored to the cells, at least in part by a linkage with the actin cytoskeleton (Rapraeger et al., 1986), removing the cells from their substratum by causing the cells to round up might be expected to enhance the rate of PG loss. Although the mechanism is unclear, pilot studies demonstrated that detachment of the cells did accelerate the rate of PG loss.

**Table I. Comparison of the Ectodomain and Medium Proteoglycan from NMuMG Cultures**

Parameter	Trypsin-released ectodomain*	Medium proteoglycan
Lipophilic	No	No
281-2 positive	Yes	Yes
Collagen/fibronectin binding	Yes	Yes
GAG content		
Heparan sulfate	Yes	Yes
Chondroitin sulfate	Yes	Yes
Density (g/ml)	>1.6	>1.6
Hydrodynamic size ( $K_{av}$ in Sepharose 4 B)	0.330	0.32-0.35
$M_r$ of core protein (Daltons on SDS-PAGE)	~53,000	~53,000

\* Part of this data has been already published (Rapraeger et al., 1985; Jalkanen et al., 1985; Koda et al., 1985).

The rapid rate at which PG is lost from cells after cell suspension was used to evaluate whether the immunoreactive PG that appears in the medium is lost from the cell surface. Newly confluent cells were removed from dishes by scraping on ice in PBS-EDTA, washing, and suspending in nutrient medium at 37°C in polycarbonate tubes. Radioimmunoassay was used to assess the kinetics of appearance of immunoreactive PG in the medium and the amount of PG remaining on the cell surface, assessed by trypsinizing cells at various times after suspension (Fig. 7). Immunoreactive PG appeared rapidly in the medium, reaching maximum levels 80 min after cell suspension. Cell surface PG was lost at a rate that was nearly identical to the rate at which PG appeared in the medium and remained low as long as the cells were suspended (Fig. 7). Total PG, accounting for the PG appearing in the medium plus the PG retained on the cells, slowly decreased and became ~75% of the original amount of cell surface PG at 80 min, indicating that some of the cell surface PG may be degraded after suspension. These kinetic data indicate that the shed PG is derived from the cell surface PG.

The PG that is shed from the cells upon suspension was compared with the immunoreactive PG in conditioned medium. These PGs appeared identical by several criteria, sharing these properties with the trypsin-released ectodomain (Table I). Thus, the medium PG is apparently derived from the cell surface PG by cleavage of the ectodomain from the lipophilic domain.

### Discussion

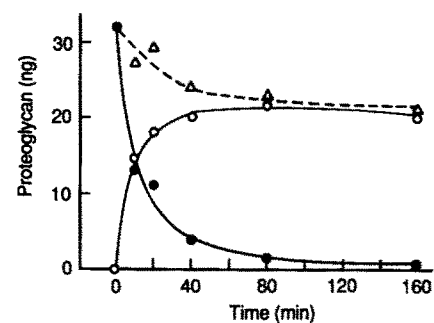
The cell surface PG on mouse mammary (NMuMG) epithelial cells consists of an ectodomain that binds via its GAG chains to interstitial collagens, and fibronectin, and a lipophilic domain that is presumably intercalated into the plasma membrane (Rapraeger et al., 1986). The current data show that the PG is released from the cell surface as a nonlipophilic PG that is indistinguishable from the ectodomain produced by trypsin treatment of the cells. Thus, the PG is released from cells by cleavage of its lipophilic domain from its ectodomain. The similarity of the trypsin-released ectodomain and the PG released from the cell surface suggests that the PG is released by a protease.

We have proposed that the cell surface PG is a receptor for

interstitial matrix materials that is involved in maintaining epithelial cell shape (Bernfield et al., 1985). Prior data suggest that when the cell surface PG binds cells to these matrix materials, the matrix molecules cross-link the PG in the plane of the membrane, linking it to the intracellular cytoskeleton (Rapraeger et al., 1986). In this way, matrix molecules, which are insoluble and cross-linked in vivo and too large for endocytosis, may stabilize cell and tissue shapes. This mechanism, called matrix anchoring, implies that matrix-mediated changes in cell shape can be induced either by disruption of the matrix or loss of the matrix receptor itself. The current results show that the matrix-binding region of the cell surface PG, the ectodomain, is released from the cells by cleavage from its membrane-associated domain, providing a mechanism by which the cells can loosen their attachment to the matrix.

### Purified Immunoreactive Medium PG Is Indistinguishable from the Ectodomain of the Cell Surface Proteoglycan

From prior work it is clear that medium conditioned by



**Figure 7.** Immunoreactive PG is shed from the cell surface upon suspension. Newly confluent NMuMG cells were washed and scraped on ice in calcium- and magnesium-free PBS-EDTA, centrifuged, and resuspended. Aliquots were taken for trypsinization and the remainder incubated for varying times in complete culture medium at 37°C. The amounts of immunoreactive PG in the medium (open circles) and released by trypsin (solid circles) were measured on duplicate samples from each time point with a radioimmunoassay (see text). Open triangles represent the sum of the PGs on the cell surface and in the medium.

NMuMG cells contains a heterogeneous mixture of PGs and GAGs (David and Bernfield, 1981) and that the monoclonal antibody 281-2 recognizes a site on the ectodomain of the cell surface PG (Jalkanen et al., 1985). Therefore, during the purification of  $^{35}\text{S}$ -PG from conditioned medium, immunoreactivity was used to select those fractions that were related to the cell surface PG. Chromatography on DEAE-cellulose and isopycnic centrifugation removed most other proteins and immunoaffinity chromatography removed other detectable contaminants including GAG chains and nonreactive PGs. These latter molecules were more polydisperse and smaller in hydrodynamic size than the immunoreactive medium PG, possibly representing modification, partial degradation, or a distinct PG, and represented the majority of the  $^{35}\text{S}$ -labeled materials in the medium. This sequence of steps resulted in  $>10^5$ -fold purification and has been routinely used to process 6-10 liters of conditioned medium at a time. The procedure yields a core protein that behaves as a single species upon PAGE and  $\text{NH}_2$ -terminal amino acid analysis (unpublished data), suggesting that it is not further degraded after release.

The PG purified from the medium has all of the properties of the ectodomain released from the cell surface by mild trypsin treatment of the cells. Both contain heparan and chondroitin sulfate chains, have a core protein that migrates at an  $M_r$  of  $\sim 53$  kD, bind to matrix molecules, react with mAb 281-2, and are nonlipophilic. Occasionally, a slight difference was noted between these PGs in hydrodynamic size on Sepharose 4B, the medium PG being slightly smaller. However, no differences were noted upon PAGE of their core proteins. Thus, the occasional difference in size is either due to loss of GAG chains from the medium PG, presumably resulting from inadequately rapid neutralization of the triethylamine used to elute this PG from immunoaffinity columns, or the medium PG loses a variable amount of GAG upon shedding.

The intact cell surface PG is lipophilic and can be readily intercalated into liposomes (Rapraeger and Bernfield, 1983), but the immunoreactive medium PG and the trypsin-released ectodomain are not lipophilic. This lack of lipophilicity was documented both by an inability to be intercalated in liposomes and to bind to silicone-treated paper, a hydrophobic matrix. The binding of PG to silicone-treated paper correlated with the ability to be intercalated into liposomes, suggesting that this method of assessing lipophilicity may also be applicable to other membrane proteins. A potential advantage of this hydrophobic matrix is demonstrated by its ability to serve as a transfer medium from polyacrylamide gels.

### ***Immunoreactive Medium PG Is Derived from the Cell Surface PG***

When the NMuMG cells were detached from the culture dish, suspended, and incubated at  $37^\circ\text{C}$ , the cells rapidly lost PG from their surfaces. Simultaneously, and with the same kinetics, an immunoreactive PG appeared in the medium. This medium PG accounted for  $>70\%$  of the PG lost from the cell surface. The remainder may have been internalized and degraded or may have lost its immunoreactivity upon release from the cells. The immunoreactive PG released was nonlipophilic and had a core protein with the same relative molecular mass as the PG accumulating in conditioned me-

dium. These results are most consistent with the conclusion that the immunoreactive medium PG is derived directly from the cell surface PG. Indeed, we will subsequently report that an affinity-purified serum antibody raised against the immunoreactive medium PG recognizes the cell surface PG (Jalkanen et al., 1988).

Release of heparan sulfate-rich PG into the medium has been described for a variety of cultured cells. For example, rat ovarian granulosa cells release to the culture medium only a small proportion of cell surface PG in a form that is indistinguishable from the parent molecule; the bulk is internalized and degraded (Yanagishita and Hascall 1984 *a, b*). Heparin is reported to release cell surface heparan sulfate from cultured hepatoma cells (Kjellman et al., 1981). D-Mannose-6- $\text{PO}_4$  and D-inositol-2- $\text{PO}_4$ , among several phosphate esters, will release cell surface heparan sulfate proteoglycan from a rat hepatocyte cell line (Bienkowski and Conrad, 1984; Ishihara et al., 1987) and this released proteoglycan can then be taken up by the cells (Ishihara et al., 1986, 1987). A cell surface receptor for the PG apparently does not exist on NMuMG cells; no PG can be displaced by heparin (Rapraeger and Bernfield, 1983), by D-mannose-6- $\text{PO}_4$  or by *myo*-inositol-2- $\text{PO}_4$  (Peters, M., and M. Bernfield, unpublished results) and none is taken up when the cells are incubated with conditioned medium (David and Bernfield, 1979) or with the purified immunoreactive medium PG (Peters, M., and M. Bernfield, unpublished results). Release from the cell surface, however, is not the sole process by which the NMuMG cells lose cell surface PG. When the cells are suspended, only  $\sim 70\%$  of the PG initially present on the cell surface is shed into the medium (cf. Fig. 7).

The immunoreactive medium PG may be released from the cells by a proteolytic mechanism. Although mild trypsin treatment of the cells has been routinely used to generate the ectodomain, chymotrypsin is equally effective and yields an apparently identical PG (Rapraeger and Bernfield, 1985). The similarity in hydrodynamic size of these proteolytically generated PGs and in relative mass of their core proteins suggests that there is an especially susceptible site for proteolysis on the cell surface PG. The relative molecular mass of the core protein of the immunoreactive medium PG is the same as that of the proteolytically generated ectodomain, suggesting that some endogenous protease acting at the susceptible site may cause the release of the PG.

The ectodomain is released from NMuMG cells during growth in monolayer culture and this release is accelerated upon cell detachment and suspension. The data recall the report of Kraemer and Tobey (1972), which showed that cell surface heparan sulfate is lost from Chinese hamster ovary cells when the cells are rounding just before mitosis and that this heparan sulfate accumulates in the medium. A similar release of the cell surface PG from NMuMG cells when they become round during passage through the cell cycle could account for the shedding of the PG observed here in monolayer cultures. Induced rounding of cells causes the release of endogenous proteases, including a metalloproteinase that is known to degrade proteoglycans (Chin et al., 1985).

### ***Significance of the Release of the Ectodomain***

The cell surface PG has several properties consistent with a function as a matrix receptor that physically links the intra-



cellular cytoskeleton to the interstitial matrix, stabilizing cell shapes and tissue morphology. The binding to the matrix is high affinity, e.g., the  $K_d$  for the interaction between the ectodomain and fibrillar type I collagen is  $\sim 10^{-9}$  M, suggesting that the association is stable under physiological conditions (Koda and Bernfield, 1984). The induced release of the ectodomain upon cell detachment and suspension may reflect a physiological mechanism in which change in cell shape leads to loss of the cell surface PG and thus to a change in the stability of the cell-matrix association. Although cell suspension leads to complete loss of the cell surface PG, less dramatic or localized changes in shape might lead to a more selective loss of PG.

When the cells are induced to lose their cell surface proteoglycan by detachment and suspension, the ectodomain is rapidly released, yet although the cells remained viable, no replacement at the cell surface was noted over a period of several half-lives. This observation suggests that deposition of the PG at the cell surface may be inhibited in rounded cells, and may occur only when cells are adherent. A possibly analogous phenomenon has been described by Vanucchi et al., (1982), who showed changes upon cell adhesion from chondroitin sulfate to heparan sulfate at the cell surface. Because the ectodomain is released and the cell surface PG is not replaced when the cells are suspended, change in cell shape may be involved, in some way, with PG metabolism at the cell surface.

Heparin and heparin-like molecules are known to exert a wide variety of effects on cell behavior. Most of these are effects on mesenchymal cells, e.g., inhibition of growth (Hoover et al., 1980) and modulation of the synthesis and secretion of extracellular matrix components (Majack et al., 1985). Release of the cell surface PG in a soluble form could be a process by which epithelial cells modify the behavior of closely associated mesenchymal cells.

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