

The Melanoma Proteoglycan: Restricted Expression on Microspikes, a Specific Microdomain of the Cell Surface

H. Jacques Garrigues,*‡ Michael W. Lark,* Stephanie Lara,* Ingegard Hellström,*‡ Karl Erik Hellström,*‡ and Thomas N. Wight*

*Department of Pathology, University of Washington; and ‡Oncogen, Seattle, Washington 98195.
Address correspondence to Dr. Wight.

Abstract. A cell surface chondroitin sulfate proteoglycan associated with human melanomas and defined by mAb's F24.47 and 48.7 has been characterized biochemically and localized by indirect immunogold electron microscopy. These antibodies recognize distinct epitopes on the intact proteoglycan. In addition, mAb 48.7 also recognizes an epitope on a 250,000-D glycoprotein and is therefore similar to antibody 9.2.27 (described by Bumol, T. F., and R. A. Reisfeld, 1982, *Proc. Natl. Acad. Sci. USA.*, 79:1245-1249). Furthermore, it was shown that the glycosaminoglycan chains released by alkaline borohydride treatment of the proteoglycan recognized by mAb 48.7 had a size of ~60,000 D. Since the intact proteoglycan was estimated to be 420,000 D, there are probably three chondroitin sulfate chains attached to the 250,000-D core glycoprotein. Furthermore, an oligosaccharide fraction containing 42% of the ³H activity (glucosamine as precursor) was isolated.

Immunolocalization studies using whole-mount electron microscopy revealed that the chondroitin sulfate proteoglycan was present almost exclusively on micro-

spikes, a microdomain of the melanoma cell surface. These processes were present as 1-2- μ m structures on the upper cell surface and as longer (up to 20 μ m) structures at the cell periphery. Peripheral microspikes were involved in the initial interactions between adjacent cells and formed complex footpads that made contact with the substratum. Immunogold-labeled cells were also thin sectioned and the specific localization of the chondroitin sulfate proteoglycan antigen was quantitated. The data confirmed the results of whole-mount microscopy and demonstrated a statistically significant association of the antigen with the microspike processes as compared with other areas of the cell surface. By using two different mAb's (48.7 and F24.47) that recognize epitopes on either the core glycoprotein or the intact proteoglycan, respectively, we have demonstrated that both molecules have the same restricted distribution at the cell surface. The specific localization of the antigen to microspikes at the cell surface suggests it may play a role in cell-cell contact and cell-substratum adhesion, which could be important in the metastatic process.

PROTEOGLYCANS are present throughout the interstitial matrix of all tissues and are associated with such specialized structures as basement membranes and/or basal laminae (18). Furthermore, these molecules appear to be associated with the plasma membranes of all cells, either as peripheral cell surface components bound via a cell membrane receptor(s) or intercalated within the plasma membrane itself (23, 46).

Cell surface proteoglycans have been implicated in a variety of cellular events, such as proliferation (29, 35, 48), migration (10, 14), and adhesion (3, 5, 10, 31), and it has been proposed that they may influence cellular behavior by acting as receptors for components of the extracellular matrix and for various circulating molecules (23). Proteoglycans are known to bind to several components of the extracellular matrix, including fibronectin (50, 53, 67), laminin (52, 65), and collagen (32, 53, 54), and also other proteoglycans (15). It

has also been shown that a membrane-intercalated heparan sulfate proteoglycan isolated from mammary epithelial cells may interact with polymerized actin (47). When this molecule was cross-linked with an antibody, aggregation of clustered antigen was inhibited by cytochalasin D, suggesting that the proteoglycan was attached to the cytoskeleton (46). Similarly, actin and proteoglycan codistribute in cultured fibroblasts and have been isolated together at focal adhesion sites (37, 66). It has been suggested, based on these observations, that changes in the organization of the extracellular matrix may be transmitted to the cytoskeleton through membrane-intercalated proteoglycans, thus ultimately influencing cell shape and behavior (23, 46).

The metabolism of proteoglycans is altered in transformed cells, and these alterations have been implicated in the abnormal growth behavior of such cells (26). For example, neoplastic cells synthesize elevated levels of chondroitin sulfate

Sepharose CL-4B / TNEN

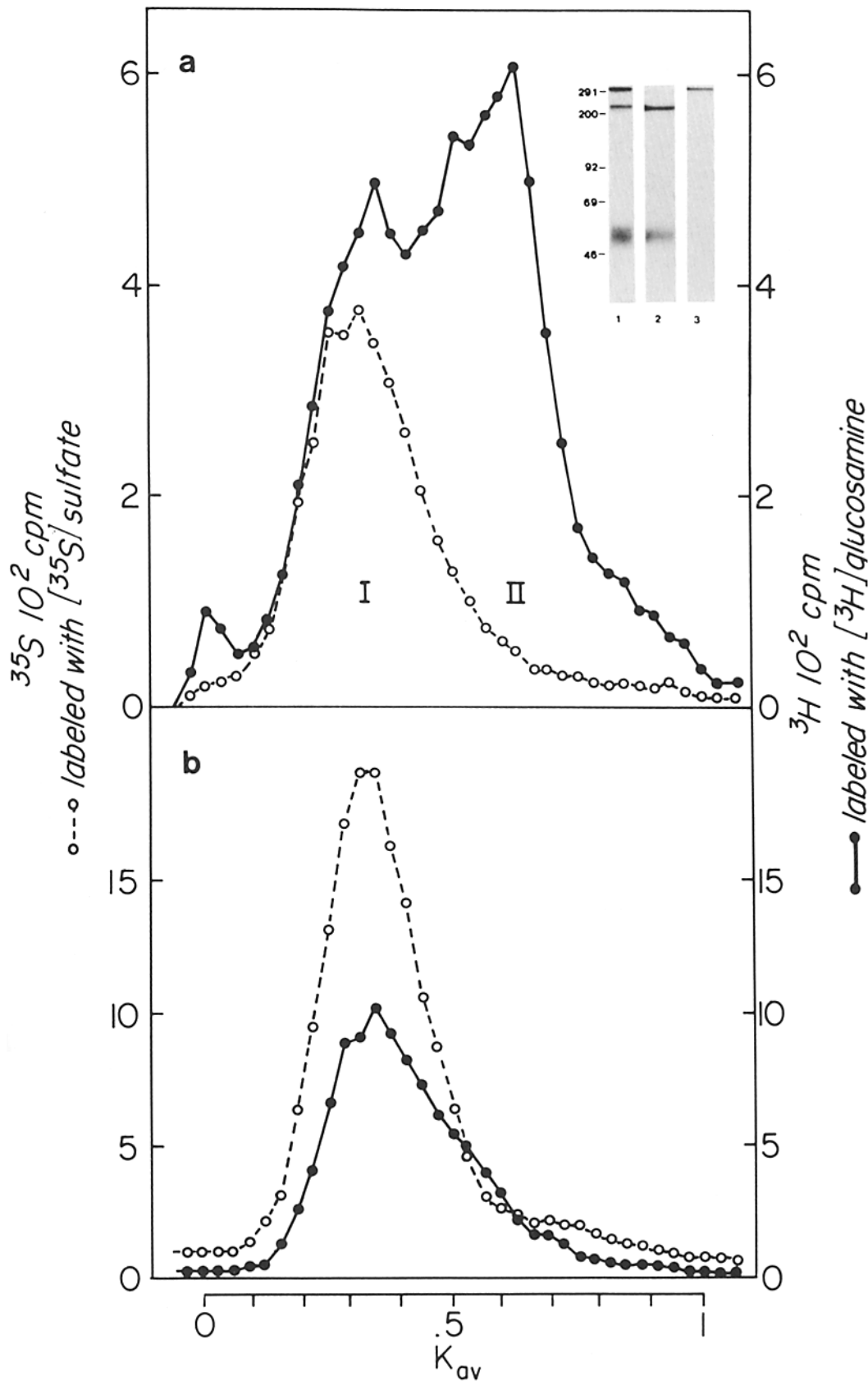


Figure 1. Gel filtration and electrophoretic analyses of $[^{35}\text{S}]$ sulfate- and $[^3\text{H}]$ glucosamine-labeled melanoma antigens. Detergent-solubilized cell lysates were prepared from labeled M 2669-C1 13 and M 1477 melanoma cells. The antigens defined by mAb's 48.7 and F24.47 were purified by affinity chromatography and analyzed on a Sepharose CL-4B column or by SDS PAGE. (a) mAb 48.7 affinity-purified

proteoglycan (CSPG)¹ (11, 20, 27, 33, 34, 55) and viral transformation can block proteoglycan synthesis (43). There is evidence that neoplastic cells can synthesize proteoglycans that are chemically modified, e.g., undersulfated (30, 40, 56, 63), or structurally altered, e.g., keratan sulfate glycosaminoglycan chains (39, 44). Such modified proteoglycans may account for the fact that transformed cells fail to assemble a pericellular matrix as compared with their normal counterparts (16, 21, 25, 57, 58).

Recent studies using several different mAb's have identified a CSPG associated with human malignant melanoma. These molecules are believed to play a role in cell-cell interactions and may be important in growth regulation (6, 7, 22, 49, 61, 62, Yang, H. M., U. S. Garrigues, K. E. Hellström, S. Hakomori, and I. Hellström, manuscript submitted for publication). Two mAb's (48.7 and F24.47) that recognize an epitope on either the core glycoprotein or the intact proteoglycan have been used to purify these melanoma-associated antigens. Thus, we have proposed a model for the structure of the CSPG based on biochemical analyses. Furthermore, we have used the antibodies to localize the core glycoprotein and the proteoglycan antigens at the ultrastructural level and report, for the first time, that they are present primarily on microspikes, a microdomain of the cell surface that is involved in cell attachment and cell-cell interactions.

Materials and Methods

Materials

Materials were purchased from the following sources: RPMI-1640 medium and anti-pleuropneumonia-like organisms agent from Gibco, Grand Island, NY; defined FBS serum from HyClone Laboratories, Sterile Systems, Inc., Logan, UT; tissue culture roller bottles from Corning Glass Works, Corning Science Products, Corning, NY; gold (5 nm)-labeled goat anti-mouse IgG from E. Y. Laboratories, Inc., San Mateo, CA; gold (40 nm)-labeled goat anti-mouse IgG from Janssen Pharmaceutica, Piscataway, NJ; vinyl microtiter plates from Costar, Data Packaging Corp., Cambridge, MA; EPON 812 from Ted Pella, Inc., Irvine, CA; carrier-free [³⁵S]sulfate (379 mCi/mmol), D-[6-³H]glucosamine (39 Ci/mmol), [³⁵S]methionine (>800 Ci/mmol), and EN³HANCE from New England Nuclear, Boston, MA; NP-40 from Particle Data, Inc., Elmhurst, IL; benzamidine hydrochloride, 6-aminohexanoic acid, and XAR-5 film from Eastman Kodak Co., Rochester, N.Y.; phenylmethylsulfonyl fluoride PMSF, *N*-ethylmaleimide and diethylamine from Sigma Chemical Co., St. Louis, MO; EDTA from MC&B Manufacturing Chemists, Inc., Gibbstown, NJ; Affi-Gel 10 and ¹⁴C-labeled molecular mass markers from Bio-Rad Laboratories, Richmond, CA; DEAE-Sephacel, Sepharose CL-4B, and CL-6B from Pharmacia Fine Chemicals, Piscataway, NJ; NaBH₄ from Wilshire Chemical Co. Inc., Gardena, CA; chondroitinase AC and ABC from Miles Laboratories, Inc., Elkhart, IN; mouse myeloma Pl.17 from American Type Culture Collection, Rockville, MD; and Hep-212 well teflon slides from Meloy Laborato-

1. *Abbreviations used in this paper:* CSPG, chondroitin sulfate proteoglycan; TNEN, buffer consisting of Tris-HCl, NaCl, EDTA, and NP-40.

ries, Inc., Springfield, VA. Digitizer was a 4052 graphics system computer and 4956 graphics tablet from Tektronix, Inc., Beaverton, OR; rabbit anti-mouse Ig from Calbiochem-Behring Corp., La Jolla, CA; Immuno-Precipitin (formalin-fixed *Staphylococcus aureus*) from Bethesda Research Laboratories, Gaithersburg, MD.

Cells and Culture Conditions

The cell line M 2669-CI 13 was established by explanting a human metastatic melanoma (4) and M 1477 was obtained from the Sloan-Kettering Institute for Cancer Research, New York, NY (12). Cells were maintained in RPMI-1640 medium supplemented with 15% inactive FBS, 1% penicillin and streptomycin, 1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 1% anti-pleuropneumonia-like organisms agent, and buffered with NaHCO₃. Cultures were grown to ~50% confluency at 37°C in a humidified atmosphere containing 5% CO₂ (68). The M 1477 cell line was tested for, and shown to be free of contamination by *Mycoplasma*, however, the status of M 2669-CI 13 was not known at the time of these experiments.

Antibodies

Two mAb's, 48.7 (22) and F24.47, were used as the primary probes for the localization and biochemical characterization studies. We have previously reported that mAb 48.7 is an IgG 1 that has a high degree of specificity for human melanomas and compound nevi, while normal tissues and other tumors either express none or very low levels of the antigen (22). mAb F24.47 is an IgG 2b that recognizes a different epitope on the antigen defined by mAb 48.7. This epitope has been referred to as PG^d (Yang, H. M., U. S. Garrigues, K. E. Hellström, S. Hakomori, and I. Hellström, manuscript submitted for publication). An IgG 2a immunoglobulin secreted by the mouse myeloma Pl.17 was used as a negative control antibody. For the immunocytochemical studies, an indirect staining protocol was used in which the secondary antibody, goat anti-mouse IgG, was conjugated to either 40 nm or 5 nm colloidal gold.

Antigen Purification

M 2669-CI 13 and M 1477 melanoma cells were grown to 50% confluency in 850 cm² roller bottles in RPMI medium that contained 50–75 μCi/ml [³⁵S]sulfate and 10 μCi/ml [³H]glucosamine, or 50 μCi/ml [³⁵S]methionine and 10 μCi/ml [³H]glucosamine. After 48 h of labeling, the medium was aspirated and the cells rinsed three times with warm (37°C) PBS, pH 7.2. The adherent cells were then released from the substratum by treatment with 0.5 mM EDTA, resuspended and washed three times in RPMI medium using repeated centrifugation (400 g). The pellet was resuspended in 20 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40 (referred to TNEN buffer) for 30 min at 4°C. The TNEN buffer also contained the following protease inhibitors: 0.05 M benzamidine hydrochloride, 0.16 M 6-aminohexanoic acid, 1 mM PMSF and 10 mM *N*-ethylmaleimide. The cell lysate was recentrifuged at 1,700 g to remove debris and the supernatant collected and centrifuged at 45,700 g for 30 min at 4°C. The supernatant was isolated and stored at -70°C for further analysis, and is referred to as the cell lysate.

The antigenic determinants defined by mAb's 48.7 and F24.47 were purified by affinity chromatography (24, 45, 49). The affinity columns were prepared by coupling 20 mg of normal mouse IgG, mAb 48.7, or mAb F24.47 to 1.0 ml of Affi-Gel 10. Cell lysates were sequentially applied to three columns consisting of 0.5 ml of Sepharose CL-4B, 0.5 ml normal mouse IgG Affi-Gel, and 0.5 ml mAb 48.7 Affi-Gel, respectively, in TNEN buffer at a flow rate of 2 ml/h. The first two columns were used to eliminate any debris or molecules that might stick nonspecifically to mAb 48.7 Affi-Gel column. The mAb 48.7 or F24.47 affinity columns were washed in four

antigen from M 2669-CI 13; (b) mAb F24.47 affinity-purified antigen from M 1477. The V₀ and V_i positions were identified using [³H]DNA and [³⁵S]sulfate, respectively. Analysis by SDS PAGE (7.5% gel) resolved the mAb 48.7-defined antigen into a high molecular mass component >400,000 D, a 250,000-D component, and a 52,000-D component (SDS PAGE inset, lane 1). The antigen was chromatographed on DEAE-Sephacel and a portion that did not bind to the column was recovered as the DEAE-nonbinding fraction (lane 2). This fraction had an identical elution profile as peak II when analyzed by gel filtration chromatography (data not shown). The remainder of the sample bound to the column and was eluted with 0.8 M NaCl/TNEN buffer as the DEAE-binding fraction (lane 3). This fraction also had an identical elution profile as peak I when analyzed by gel filtration chromatography (data not shown). Molecular mass determinations are based on protein molecular mass marker standards: myosin (200 kD), phosphorylase B (92 kD), BSA (69 kD), ovalbumin (46 kD), and [α1(III)]₃ collagen (291 kD).

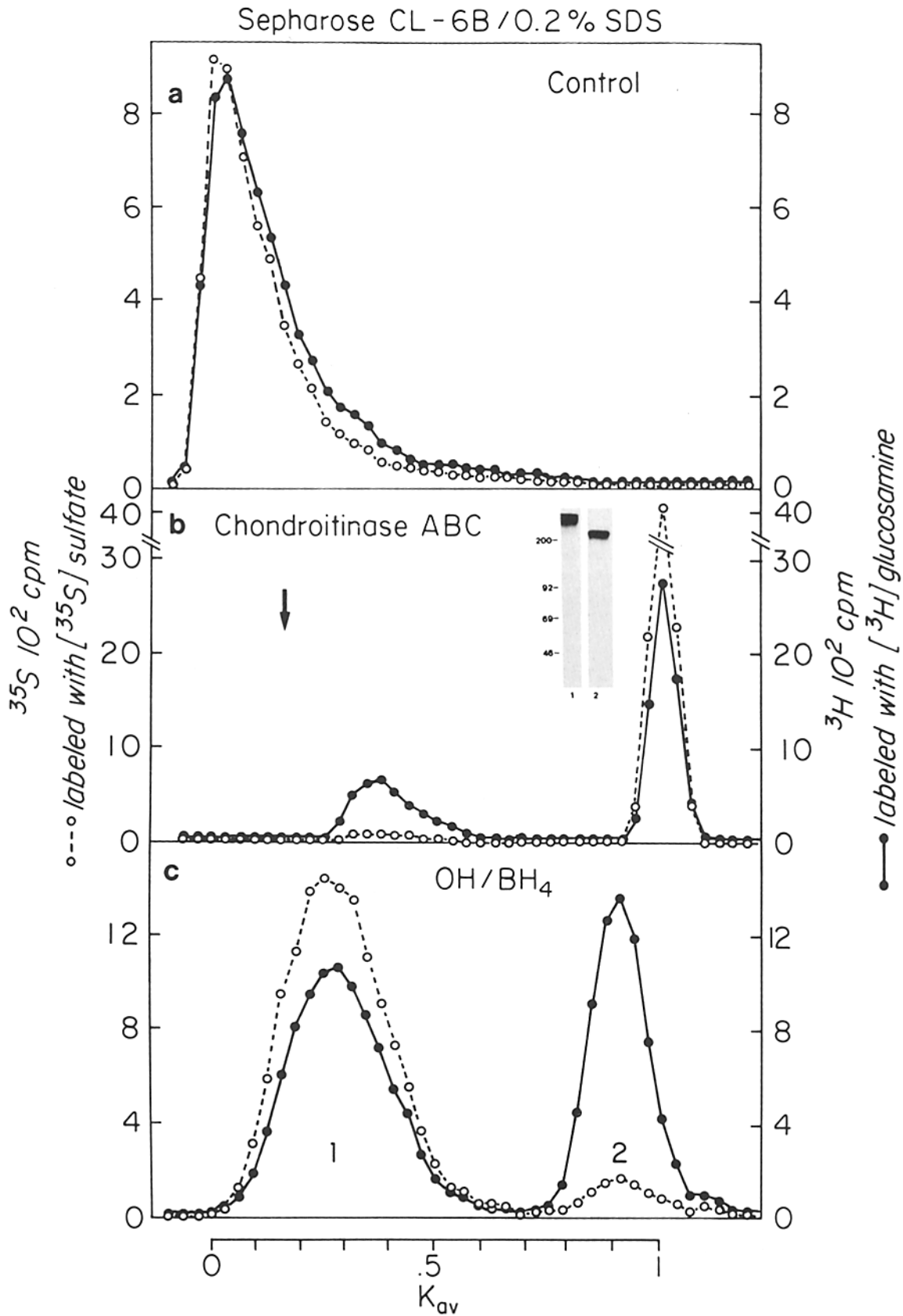


Figure 2. Proteoglycan analysis of peak I from M 2669-C1 13 melanoma cells. A control aliquot of the [^{35}S]sulfate- and [^3H]glucosamine-labeled peak I isolated as the DEAE-binding fraction (see legend for Fig. 1) was handled as a control (a), digested chondroitinase ABC (b), or treated with alkaline borohydride (c). All samples were brought to 0.2% SDS and chromatographed on identical Sepharose CL-6B columns. Arrow (b) indicates the elution position of a collagen standard [$\alpha 1(\text{III})_3$] that has a molecular mass of 291,000 D. The V_0 and V_i positions were identified as described in the legend to Fig. 1. Core protein analysis by SDS PAGE. M 2669-C1 13 cells were labeled

steps as follows: (a) with 2.5 ml TNEN; (b) with 20 ml TNE + 0.2% NP-40; (c) with 1.5 ml TNE + 0.1% NP-40; and (d) with 2.5 ml TNE + 0.2% NP-40 (49). The affinity-purified antigens were then eluted from the columns with 5 ml of 0.1 M diethylamine, pH 11.5, and immediately brought to neutral pH. Using this technique, it was possible to isolate ~3.3% of the total [³⁵S]sulfate-labeled macromolecules incorporated into the cell lysate.

For further purification, the antigen that eluted from the mAb 48.7 column was applied to a 0.2-ml DEAE-Sephacel column equilibrated with TNEN buffer. The nonbinding fraction was collected at a flow rate of 1 ml/h. After extensive washing, the DEAE-binding fraction was eluted by resuspending the resin in 0.4 ml of TNEN buffer containing 0.8 M NaCl for 1–2 h. The DEAE-binding and -nonbinding fractions were subsequently analyzed by SDS PAGE and gel filtration chromatography (see below).

Immunoprecipitation and Electrophoresis

The [³⁵S]methionine/[³H]glucosamine-labeled DEAE-binding fraction was immunoprecipitated as previously described (64). Briefly, the antigen fraction was preadsorbed with rabbit anti-mouse IgG (2% vol/vol) and fixed and heat-killed *S. aureus* (2% wt/vol). 10 µg of mAb 48.7 were then incubated with ~1.2 × 10⁵ cpm of preadsorbed antigen. Rabbit anti-mouse IgG was then added to the incubation mixture and the immune complexes were adsorbed to 1 mg of *S. aureus*. The immune complexes were washed three times in TNEN buffer that was supplemented with 10 mM sodium iodide, 10 mM sodium deoxycholate, 2% BSA, and 0.2% SDS, and washed once in TNEN (diluted 1:10 in H₂O). The pellets were then suspended in 100 µl of reducing Laemmli sample buffer (containing 2-mercaptoethanol) and boiled for 3 min. The bacteria were removed by centrifugation and the supernatant containing the immunoprecipitated antigen was analyzed by electrophoresis on 7.5% SDS polyacrylamide gels (36). In some experiments, [³⁵S]sulfate/[³H]glucosamine-labeled affinity-purified antigen and DEAE-binding or -nonbinding fractions were analyzed by SDS PAGE that had not been immunoprecipitated.

Gel Filtration Chromatography

To further analyze the molecular size of the melanoma antigen, aliquots of the affinity-purified material or the DEAE-binding fractions were analyzed by gel filtration chromatography. The columns used included: Sepharose CL-4B (0.7 × 100 cm) equilibrated with TNEN buffer and eluted with flow rate of 2.5 ml/h; and Sepharose CL-6B (0.7 × 100 cm) equilibrated with 0.2% SDS in 0.15 M sodium acetate, 1 mM CaCl₂, pH 7.4, and eluted with flow rate of 1.5 ml/h. Fractions (0.75 ml) were collected and assayed for radioactivity by scintillation counting; column yields were 85–95%.

Proteoglycan Analysis

To identify the proteoglycan nature of the DEAE-binding fraction, the fraction was digested with chondroitinase ABC or AC II (51). Enzymatic digestion was carried out by diluting 60 µl of the sample in an equal volume of digestion buffer containing 0.3 U of enzyme in 0.5 M Tris-HCl, 0.36 M sodium acetate, and 0.1% BSA, pH 6.8. The buffer also contained the following protease inhibitors: 10 µM EDTA, 10 µM *N*-ethylmaleimide, 5 µM PMSF, and 0.36 mM pepstatin. The samples were incubated at 37°C for 4 h, after which they were brought to 0.2% in SDS and analyzed on a Sepharose CL-6B (0.7 × 100 cm) column. Control samples were handled identically except that no enzyme was added. To determine the size of the core glycoprotein, the DEAE-binding fraction was immunoprecipitated with mAb 48.7 and *S. aureus* and digested by resuspending the washed precipitates bound to *S. aureus* in 50 µl chondroitinase ABC digestion buffer and incubated at 37°C for 4 h. The core glycoproteins were then analyzed by SDS PAGE.

To study the glycosaminoglycan chains of the proteoglycan, the DEAE-binding fraction that was isolated from M 2669-C1 13 cells and labeled with [³⁵S]sulfate and [³H]glucosamine was treated with 1 M NaBH₄ in 0.1 M NaOH for 18 h at 45°C (8). The reaction mixture was then neutralized with acetic acid and the glycosaminoglycan chains and oligosaccharides released from the core protein were analyzed by gel filtration chromatography on Sepharose CL-6B eluted with TNEN.

with [³⁵S]methionine and [³H]glucosamine. Peak I was isolated and then immunoprecipitated with mAb 48.7 and analyzed by SDS PAGE (7.5% gel). Lane 1, immunoprecipitated peak I; lane 2, immunoprecipitated peak I digested with chondroitinase ABC. Molecular mass determinations are based on protein molecular mass marker standards as described in Fig. 1.

Immunoelectron Microscopy

Single cell suspensions of M 1477 were prepared by trypsinization and plated at a concentration of 1 × 10⁴ cells/well in vinyl microtiter plates or 1.5 × 10³ cells/well on well teflon slides (Meloy Laboratories, Inc.). After a 24–48-h incubation, the cells were washed three times in warm (37°C) PBS, pH 7.2, and fixed for 2 h in warm fixative (0.5% paraformaldehyde and 0.65% glutaraldehyde) diluted in 0.1 M sodium cacodylate buffer, pH 7.4 (28). After fixation the cells were washed three times in cacodylate buffer and used for ultrastructural immunocytochemistry, either directly or after storage at 4°C.

mAb's 48.7 and F24.47 were isolated from hybridoma culture supernatant (10 µg/ml of antibody) or were purified from ascites fluid using Protein A-Sepharose. All antibodies were added to a diluent that contained 10% normal human serum and 3% normal goat serum in PBS. The hybridoma culture supernatants were diluted 1:1, while affinity-purified antibodies were diluted to 10–25 µg/ml. The 5-nm and 40-nm gold-labeled secondary antibodies were diluted to final concentrations of 2.7 and 2.5 µg/ml, respectively.

The immunogold staining procedure consisted of an initial preincubation of the fixed cells with normal human serum (diluted 1:5 in PBS) for 30 min at room temperature to reduce nonspecific background staining. The serum was then aspirated and replaced with mAb 48.7 or F24.47. After a 4-h incubation at room temperature, the primary antibody was removed and the cells were washed three times for 10 min in an agitated PBS bath. The secondary antibody, a goat anti-mouse IgG (conjugated to 40 nm gold for whole-cell mounts or 5 nm gold for thin sections) was incubated with the cells for 2–3 h. The cells were then washed as above, treated with 1% OsO₄ for 30 min and postfixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The fixed cells were dehydrate with graded ethanol and infiltrated with Epon 812 or dried at the critical point of CO₂. Thin sections of embedded cells were cut on an Ultratome III (LKB Instruments, Inc., Gaithersburg, MD), collected on Formvar-coated grids, and double stained with uranyl acetate and lead citrate. Dried specimens to be used for whole-mount microscopy were coated with carbon and replicas were subsequently floated onto H₂O and picked up on coated copper grids. All specimens were examined with an electron microscope (model 100 B; JEOL USA, Peabody, MA) at an accelerating voltage of 60 kV for the sectioned specimens and 100 kV for the whole-mount preparations.

The localization of the antigen defined by mAb 48.7 to specific cell surface structures was quantitated using transmission electron microscopy. The topographical features of M 1477 cells were divided into three groups that included microspikes, surface blebs, and underlying areas of smooth membrane (13, 60). Micrographs of immunogold-stained cells taken at the same magnification were used to generate the membrane length data. Measurement of the membrane lengths for each of the three groups was determined using a graphics tablet system computer (Tektronix, Inc.). The mean number of gold particles per micrometer of membrane per group was determined. The difference between the groups was compared by the Student's *t* test.

Results

mAb's 48.7 and F24.47 Recognize Two Different Epitopes on the Melanoma-associated CSPG Antigen

The antigens recognized by mAb's 48.7 and F24.47 were purified from detergent-solubilized cell extracts of M 2669-C1 13 and M 1477 melanoma cells by affinity chromatography. The mAb 48.7-defined antigen from M 2669-C1 13 cells that had been labeled with [³⁵S]sulfate and [³H]glucosamine was resolved into two major peaks of radioactivity when analyzed by Sepharose CL-4B chromatography, namely, a double-labeled ³⁵S/³H peak (peak I) that eluted at a *K_{av}* = 0.3 and a single-labeled ³H peak (peak II) that eluted at *K_{av}*

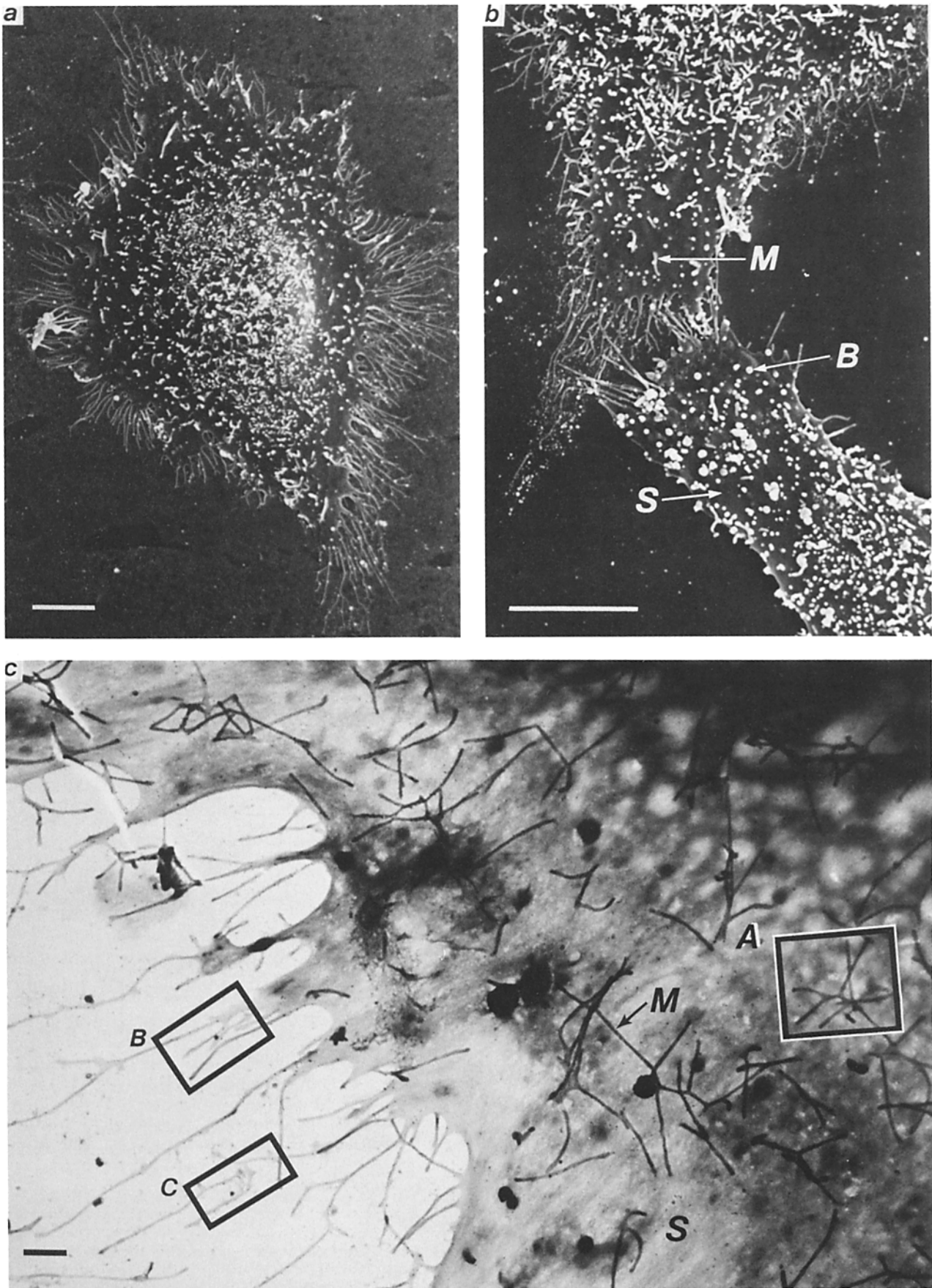


Figure 3. Cell surface topography of M 1477 melanoma. Cells were prepared for scanning electron microscopy (a and b) or as whole-cell mounts for transmission electron microscopy (c). The topography of M 1477 (for overview see a) was characterized by numerous upper

= 0.56 (Fig. 1 *a*). A similar analysis of an M 1477 cell lysate purified on a mAb F24.47 affinity column resulted in the isolation of only peak I, while the glycoprotein peak (peak II) was absent (Fig. 1 *b*). Previously, immunoprecipitation data using mAb 48.7 and M 1477 cell lysates demonstrated that these cells do express the glycoprotein component on the cell surface (22). The results presented here indicate that this glycoprotein component, however, is not recognized by mAb F24.47. Further studies demonstrated that peak I could be separated from peak II by DEAE-Sephacel ion exchange chromatography. Approximately 50% of the [³H]glucosamine-labeled material and all of the [³⁵S]sulfate-labeled material bound to the ion exchange resin and could be eluted with a high salt (0.8 M NaCl)-containing buffer. The DEAE-binding fraction contained only a high molecular mass component (Fig. 1, SDS PAGE inset, lane 3) that coeluted with peak I when analyzed by gel filtration chromatography (data not shown). A comparison of the elution position of peak I with that reported for other proteoglycans indicates that peak I had an approximate molecular mass of 420,000 D (19, 42). The material which did not bind to the ion exchange resin was labeled only with [³H]glucosamine and it coeluted with peak II when analyzed by gel filtration (data not shown). Analysis of the DEAE-nonbinding fraction by SDS PAGE indicated that it contained 250,000-D and 52,000-D components (Fig. 1, SDS PAGE inset, lane 2). The material migrating at 52,000 D most likely represented a component adsorbing nonspecifically to the affinity column, since it could not be immunoprecipitated from an aliquot of the affinity-purified antigen using mAb 48.7.

The fact that peak I labeled with [³⁵S]sulfate and [³H]glucosamine and bound to a DEAE-Sephacel column suggested that it was a proteoglycan. To investigate this further, an aliquot of the [³⁵S]sulfate- and [³H]glucosamine-labeled peak I (purified on a mAb 48.7 column) from M 2669-C1 13 cells was digested with either chondroitinase ABC (Fig. 2) or AC II (data not shown) and the digestion products separated by gel filtration on Sepharose CL-6B. An aliquot representing the untreated control eluted as a single peak in the void volume of the Sepharose CL-6B column (Fig. 2 *a*). After digestion with either enzyme, all of the [³⁵S]sulfate activity eluted at the column total volume, indicating that the glycosaminoglycan chains were completely degraded by both enzymes (Fig. 2 *b*). A small peak of [³H]glucosamine-labeled material eluted at a $K_{av} = 0.38$ and presumably represented the core protein that contained oligosaccharide side chains. Chondroitinase digests of [³⁵S]methionine-labeled peak I also eluted at this position (data not shown). A collagen standard, [α I(III)]₃, having a molecular mass of 291,000 D, eluted slightly before the core glycoprotein peak (see arrow, Fig. 2 *b*). To obtain a better estimate of the size of the core glycoprotein, an aliquot of peak I (labeled with [³⁵S]methionine and [³H]glucosamine) was immunoprecipitated with mAb 48.7, digested with chondroitinase ABC and

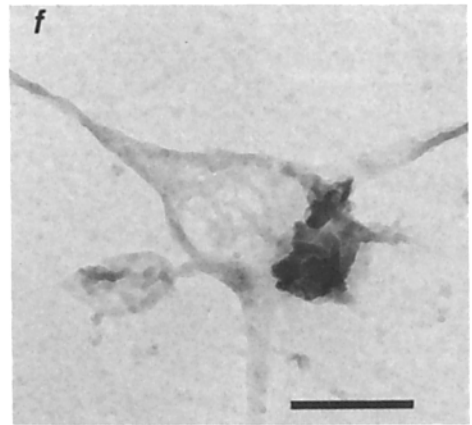
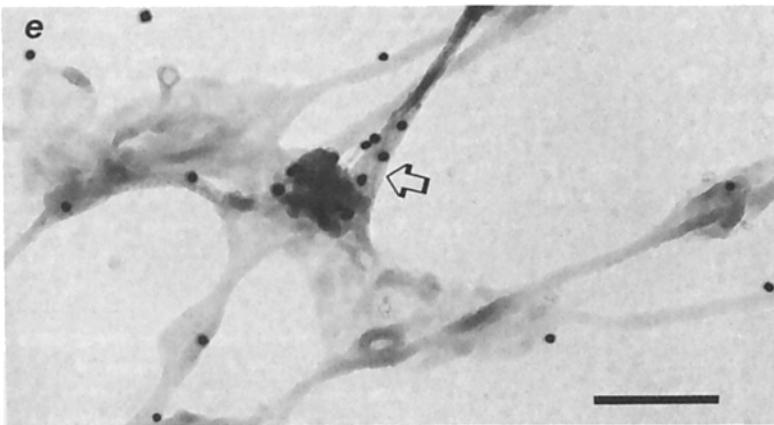
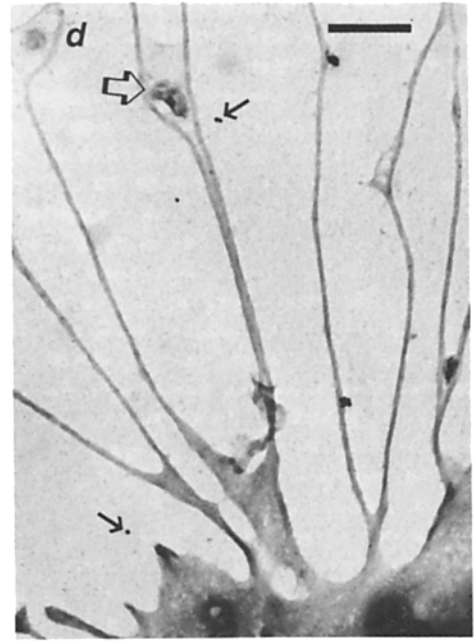
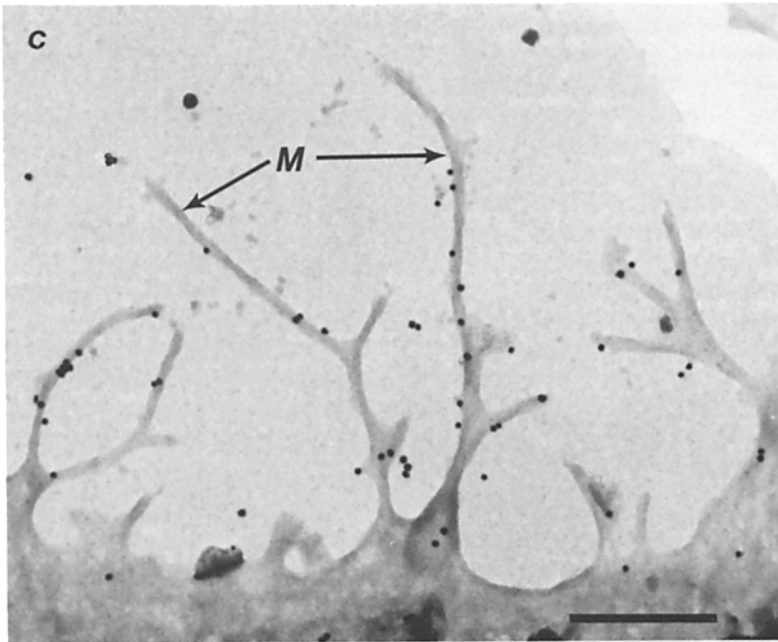
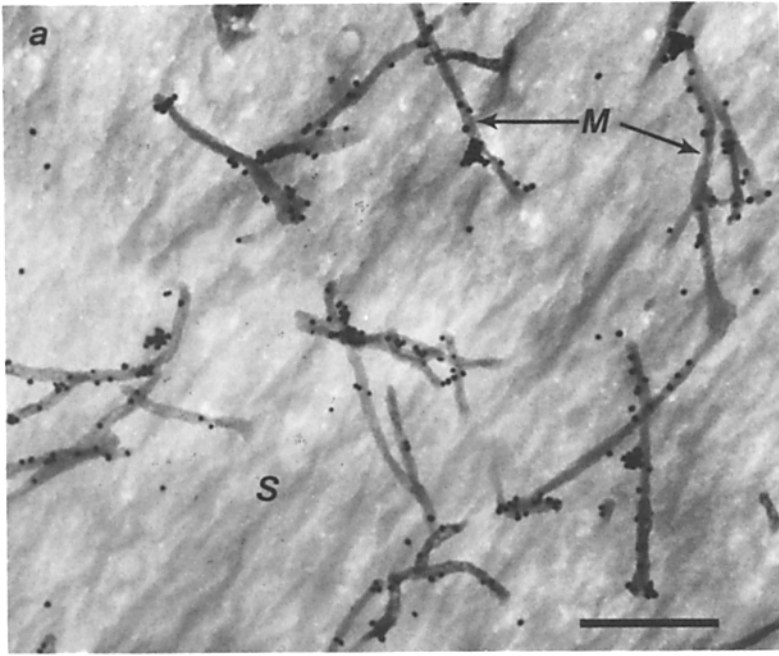
analyzed by SDS PAGE (Fig. 2 *b*, SDS PAGE inset). The nondigested fraction barely entered the 7.5% gel (lane 1). After chondroitinase ABC digestion, the radiolabeled band shifted to a position corresponding to 250,000 D (lane 2). This position is identical to that of peak II (Fig. 1, SDS PAGE inset, lane 2). This indicates that the 250,000-D component isolated as the DEAE-nonbinding fraction and the core glycoprotein of the CSPG are probably the same molecule. Peptide map analyses are consistent with the conclusion that the 250,000-D components and the core glycoprotein are similar (7, 49, 62). These data also demonstrate that mAb 48.7 recognizes an epitope present on the core glycoprotein as well as on the intact CSPG, while the epitope defined by mAb F24.47 is present only on the proteoglycan. Epitope analysis using mAb F24.47 suggests that the determinant contains galactose (Yang, H. M., U. S. Garrigues, K. E. Hellström, S. Hakomori, and I. Hellström, manuscript submitted for publication).

To determine the size of the glycosaminoglycan chains, an aliquot of the double-labeled peak I isolated from M 2669-C1 13 and purified on a mAb 48.7 column was treated with alkaline borohydride to release glycosaminoglycan and oligosaccharide chains (8). The glycosaminoglycan chains that were labeled with both [³⁵S]sulfate and [³H]glucosamine (peak 1) eluted at a $K_{av} = 0.29$ when chromatographed on Sepharose CL-6B (Fig. 2 *c*). The glycopeptide/oligosaccharide fraction that labeled only with [³H]glucosamine (peak 2) eluted at a $K_{av} = 0.91$. By comparison to a standard curve for chondroitin sulfate samples of known molecular mass, the elution position of peak 1 corresponds to an approximate molecular mass of 60,000 D (59). The data also indicate that 58% of the [³H]glucosamine radioactivity was incorporated into the glycosaminoglycan chains of peak 1. The remainder of the radioactivity released by alkaline borohydride treatment eluted in a position corresponding to oligosaccharide (9). The majority (88%) of the oligosaccharides were labeled with [³H]mannose, suggesting that they are attached to the core protein through *N*-linkage to asparagine (data not shown).

Ultrastructural Immunocytochemistry: the Melanoma-associated CSPG is Localized to a Specific Microdomain of the Cell Surface

The morphology of M 1477 melanoma cells was examined by scanning electron microscopy. Although there were large variations in cell shape and size, as would be expected from a rapidly dividing tumor cell line, typical cell surface features are illustrated in Fig. 3. At the periphery, M 1477 cells characteristically sent out delicate, finger-like projections that made contact with the substratum (Fig. 3 *a*). In densely populated cultures where cell-cell interactions were occurring, these processes were often involved in the initial events of cell-cell contact (Fig. 3 *b*). On the upper cell surface,

cell surface projections or microspikes (*M*), blebs (*B*), and areas of smooth membrane (*S*) (*a* and *b*). Microspikes were also present at the cell margin and were shown to be involved in the initial events of cell-cell contact (*b*) as well as cell-substratum contact (*a-c*). The high resolution transmission electron microscopy (*c*) clearly shows the upper surface microspikes as distinct structures; however, blebs were not so easily identified in these preparations. Three regions of the cell have been examined for the expression of the melanoma CSPG (see Fig. 4) namely, the upper cell surface (box *A*), the cell margin (box *B*), and cell-substratum attachment sites (box *C*). Bar, (*a* and *b*) 10 μ m; (*c*) 1 μ m.



shorter projections were observed as well as blebs and areas of smooth membrane (Fig. 3 *a* and *b*). To examine the cell surface in more detail, whole-cell mounts were prepared for transmission electron microscopy. Numerous 1–2- μm projections, which we refer to as microspikes, were easily distinguished from areas of smooth membrane on the upper cell surface (Fig. 3 *c* and Fig. 4). At the cell periphery, projections with a similar diameter (~ 80 nm) as microspikes were observed (Fig. 3 *c* and Fig. 4). These peripheral microspikes were generally much longer (up to 20 μm) than the upper surface structures and they formed complex footpads that were intimately associated with the substratum (Fig. 3 *c* and Fig. 4, *d*, *e*, and *f*). Bleb structures were more difficult to identify in these preparations; however, their presence was confirmed using thin sections (Fig. 5). To delineate the overall distribution of the proteoglycan defined by mAb F24.47, M 1477 melanoma cells were labeled by an indirect immunogold technique and prepared for whole-mount microscopy. Three areas of the cell surface were examined as shown in Fig. 3 *c*: (*A*) the upper surface, (*B*) the cell margin, and (*C*) cell-substratum contact points. The melanoma-associated CSPG antigen defined by mAb F24.47 was shown to have a restricted distribution and was localized predominantly to microspikes on the upper cell surface (Fig. 4 *a*). Similarly, peripheral microspike structures radiating from the cell margin were shown to express this antigen (Fig. 4 *c*). It was often noted that these structures usually formed branches after the microspike had extended ~ 5 μm from the cell margin. At these branch points, cellular material was laid down in the form of a footpad that made contact with the substratum. As shown in Fig. 4 *e*, these footpad structures also contain the melanoma-associated antigen. Control experiments were performed in which mAb F24.47 was replaced with an isotype-matched antibody secreted by mouse myeloma P1.17. Essentially no labeling of the M 1477 cells was observed (Fig. 4, *b*, *d*, and *f*).

To examine the cell surface in more detail and to assess whether both the core glycoprotein and the CSPG were localized to microspikes, melanoma cells were labeled using mAb 48.7, thin sectioned, and analyzed by transmission electron microscopy. The general topographical features of sectioned M 1477 cells were similar to those seen by whole-mount microscopy, except that surface blebs were more discernible in the sections. The immunolocalization results were also in agreement and showed that the antigen defined by mAb 48.7 was predominantly associated with the microspikes, while the blebs and underlying smooth cell surfaces were stained considerably less (Fig. 5). Also, the gold particles tended to be associated with an amorphous surface material and frequently occurred in clusters (Fig. 5 *a*). Further support for the specific localization of the antigen to microspikes was obtained by quantitating the number of la-

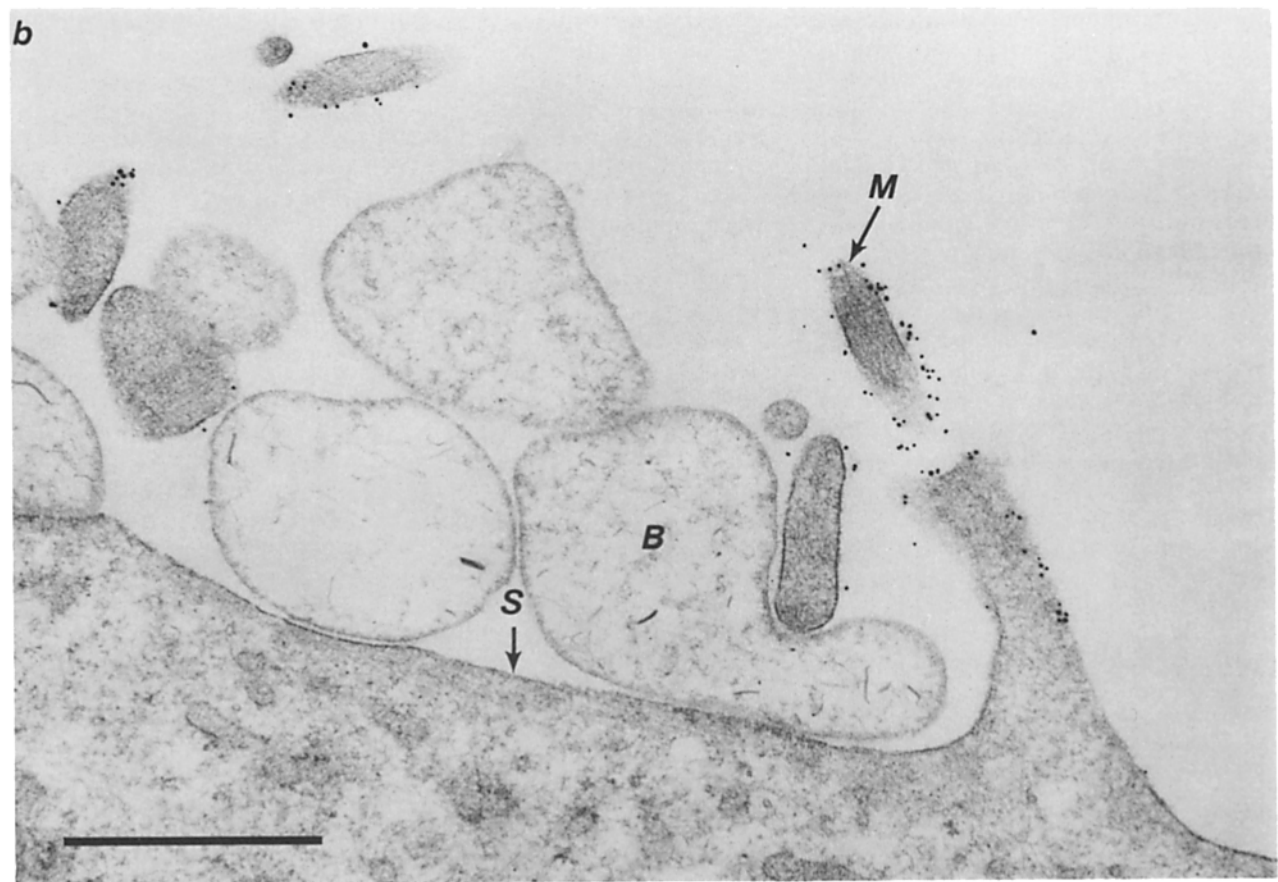
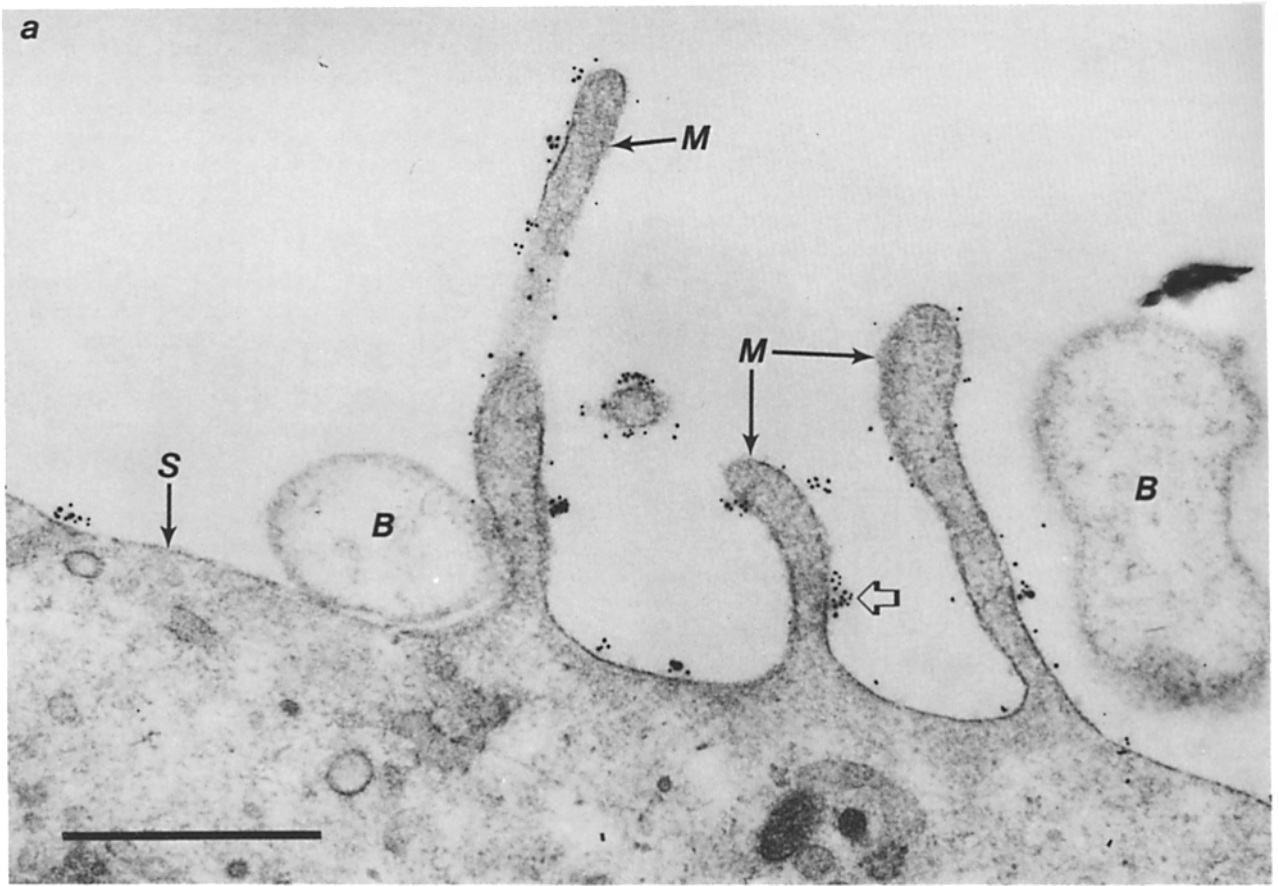
beled particles per micrometer of membrane length of either blebs, microspikes, or underlying cell surface. There were 39.5 particles/ μm of microspike membrane compared with 0.35 and 2.9 particles/ μm of bleb or underlying surface, respectively (Fig. 6); the first of these three groups was statistically significant from the other two ($P < 0.001$).

Discussion

The antigen recognized by mAb 48.7 is expressed primarily on melanomas and benign nevi (22) and is similar (identical?) to an antigen first described by Bumol and Reisfeld (6) and subsequently by others (7, 49, 61, 62; Yang, H. M., U. S. Garrigues, K. E. Hellström, S. Hakomori, and I. Hellström, manuscript submitted for publication). Ross et al. have proposed that this antigen consists of two core glycoproteins associated to form a homodimer of 520,000 D, and, that after the addition of glycosaminoglycan chains (30,000 D each), the completed proteoglycan had an estimated size of $7\text{--}10 \times 10^5$ D (49). Our results, using gel filtration chromatography under nondenaturing conditions, demonstrated that the core glycoprotein eluted in a slightly retarded position relative to a collagen standard (291,000 D) and had a molecular mass of 250,000 D when analyzed by SDS PAGE. The proteoglycan eluted at a K_{av} of 0.3 on Sepharose CL-4B, which gives an estimated molecular mass of 420,000 D, similar to the size of a CSPG isolated from chick embryo cartilage (42). Since the size of the glycosaminoglycan chains was estimated to be $\sim 60,000$ D by gel filtration chromatography, the addition of three glycosaminoglycan chains would account for the shift in molecular mass. It is not clear why our results differ from those of Ross et al. (49). It is possible the differences could be attributed to the different melanoma cell lines used, even though we have generated identical results using two different melanoma cell lines. It is probably not due to a difference in mAb's, since an antibody from Bumol and Reisfeld (6) recognizes the antigen defined by mAb 48.7 (Mosely, G. H., unpublished data) as well as that described by Ross et al. (49).

We do not yet know how the melanoma CSPG is associated with the cell surface. Three pieces of data suggest, however, that the core protein of the CSPG is membrane-associated and may be intercalated in the plasma membrane. First, Wilson et al. (62) have shown that the CSPG can be extracted from cells three times more effectively with buffer that contains detergent than with high or low salt buffers. Second, the proteoglycan can be extracted from cells with 4 M guanidine hydrochloride as large aggregates that can be dissociated in the presence of detergent (38, 41; Garrigues, H. J., unpublished observation). Third, when melanoma cells in culture were treated with mAb F24.47, there was a redistribution of the CSPG antigen at the cell surface (Garrigues, H. J., un-

Figure 4. Immunogold-labeled whole-cell mount of M 1477 melanoma cells. The cells were fixed with 0.5% paraformaldehyde and 0.6% glutaraldehyde and labeled using mAb F24.47 as the primary probe, followed by goat anti-mouse IgG conjugated to 40 nm colloidal gold as the secondary antibody. Three areas of the cell surface (equivalent to boxes *A*, *B*, and *C* of Fig. 3 *c*) were studied. On the upper cell surface, mAb F24.47 was localized almost exclusively to short (1–2 μm) microspike processes (*a*). Areas of smooth membrane were labeled much less. At the cell margin, longer (up to 20 μm) peripheral microspikes (*c*) as well as distant footpads (*e*) were shown to be enriched for the melanoma CSPG. Control experiments where mAb F24.47 was replaced by mAb P1.17 showed only occasional gold particles associated with corresponding areas of the cell surface (*b*, *d*, and *f*). *M*, microspike; *S*, smooth membrane surface; *open arrow*, footpad; *small arrow*, nonspecific label in *d*. Bar (*a–d*) 1 μm , (*e* and *f*) 0.5 μm .



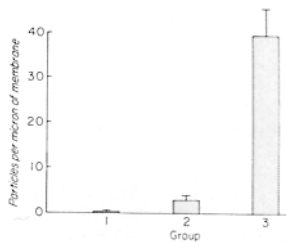


Figure 6. Immunogold quantitation. Membrane lengths for blebs (group 1), underlying smooth surface (group 2) and microspikes (group 3) were determined from electron micrographs of immunogold-labeled M 1477 cells (see Fig. 5). The mean number of gold particles per micrometer mem-

brane \pm SEM was calculated and the groups were compared by Student's *t* test. The *t* value for group 1 ($n = 16$) vs. group 3 ($n = 18$) = 6.46 ($P < 0.001$) and comparing group 2 ($n = 23$) vs. 3, $t = 7.18$ ($P < 0.001$).

published observation). Fourth, the ultrastructural data obtained in this study demonstrates that the CSPG is localized to microspikes, which constitute a specific microdomain of the cell surface. We have examined both whole-mount preparations and sections of cells labeled by the immunogold technique. In sections, the melanoma CSPG antigen was associated with an amorphous material that was observed on projections of the cell surface, and the antigen was often seen in clusters. The sectioned material, however, provided little information about the organization of these projections relative to the entire cell surface, nor was it possible to section remote cell attachment sites and accurately determine their orientation to the cell body. We therefore chose to study the localization of the CSPG antigen using whole-mount preparations where the orientation of surface domains is more easily interpreted. The results were identical for immunogold-labeled cells prepared by either procedure and demonstrated that the melanoma CSPG antigen is expressed almost exclusively on microspike projections located on the upper cell surface as well as radiating from the cell margin. This study also indicates that mAb 48.7 recognizes an epitope on the core glycoprotein and the intact CSPG. Both of these molecules are present on the cell surface since they can be iodinated by the lactoperoxidase procedure (22). Autoradiography of the immunoprecipitated antigens analyzed by SDS PAGE also indicated that they were present on the cell surface at an approximate molar ratio of 1:1 (22). It was not known, however, whether the proteoglycan and the core protein were localized to the same structure at the cell surface. Using mAb 48.7 and another antibody, mAb F24.47, which defines an epitope present only on the intact proteoglycan, we found identical staining patterns of the cell surface, implying that the core glycoprotein and the proteoglycan have the same restricted distribution on microspikes. This finding suggests that, for the melanoma-associated proteoglycan, the glycosaminoglycan chains probably do not play a role in determining its ultimate destination at the cell surface.

The functional significance of the specific localization of the melanoma-associated CSPG antigen to microspikes is unknown. Studies on cell motility have demonstrated that migrating cells send out microspikes that make initial contact with the substratum or with adjacent cells (1). For example,

when nontransformed 3T3 cells come into contact with each other, which is a process mediated by microspikes, the cells move apart, that is, they are contact inhibited (2), while tumor cells, which also have microspikes, are not contact inhibited. One possible interpretation of this is that the microspikes are somehow altered in transformed cells and the presence of tumor-associated CSPG might play a role in this alteration. Treatment of melanoma cells with an antibody to the CSPG antigen can block the early events of cell spreading on a basement membrane synthesized by endothelial cells (7), and can specifically inhibit anchorage-independent growth of melanoma cells in soft agar (17). These data suggest that the tumor-associated CSPG may mediate cell attachment and contact inhibition and may be an important potentiator of the metastatic process.

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Figure 5. Immunotransmission electron micrographs of M 1477 cells. Melanoma cells were immunostained as for Fig. 4. In this case the secondary antibody was conjugated to 5 nm gold. Note the specific localization of the mAb 48.7-defined antigen to microspikes (M). Fig. 5 b clearly demonstrates an absence of label on blebs (B) or on smooth membrane surface (S). These figures also demonstrate that the antigen appears to be clustered along various areas of the microspikes (open arrow). Bar, 1.0 μ m.

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