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Immunology

Unique glycoprotein–proteoglycan complex defined by monoclonal antibody on human melanoma cells

(melanoma antigen complex/biosynthesis)

T. F. BUMOL AND R. A. REISFELD

Department of Molecular Immunology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, California 92037

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ABSTRACT A monoclonal antibody, 9.2.27, with a high specificity for human melanoma cell surfaces has been utilized for biosynthetic studies in M21 human melanoma cells to define a unique antigenic complex consisting of a 250-kilodalton N-linked glycoprotein and a high molecular weight proteoglycan component larger than 400 kilodaltons. The 250-kilodalton glycoprotein has endoglycosidase H-sensitive precursors and shows a lower apparent molecular weight after treatment with neuraminidase. The biosynthesis of the proteoglycan component is inhibited by exposure of M21 cells to the monovalent ionophore monensin; this component can be labeled biosynthetically with $^{35}SO_4$, is sensitive to β-elimination in dilute base, and is degraded by both chondroitinase AC and ABC lyases, suggesting that it is a chondroitin sulfate proteoglycan. These data demonstrate that the antigenic determinant recognized by monoclonal antibody 9.2.27 is located on a glycoprotein–proteoglycan complex which may have unique implications for the interaction of glycoconjugates at the human melanoma tumor cell surface.

The development of monoclonal antibodies to tumor cell surface components has provided specific probes to establish the molecular nature of these antigens. Antigenic determinants with unique biochemical properties defined by monoclonal antibodies specific for colorectal carcinoma have recently been identified as glycolipids (1). Several additional monoclonal antibody reagents have defined components that appear to be either cell-surface or secreted glycoproteins of human tumors (2–4). The high specificity of these monoclonal antibody probes and their ability to immunoprecipitate antigens selectively from complex soluble tumor extracts permits the biochemical analysis of these potentially tumor-associated gene products.

This laboratory has recently developed a monoclonal antibody, 9.2.27, that is directed against an antigen highly associated with the surface of all melanoma cell lines and frozen sections of fresh surgical melanoma specimens thus far examined (4).

In this study, we utilized the 9.2.27 monoclonal antibody in a combination of biosynthetic and enzymatic digestion studies to define a unique cell surface antigen that consists of a glycoprotein–proteoglycan complex expressed on the surface of human melanoma cells.

MATERIALS AND METHODS

Cells. The M21 human melanoma cell line, originally derived from a metastatic melanoma lesion (5), was maintained in long-term tissue culture in RPMI-1640 medium supplemented with 10% fetal calf serum (GIBCO), 2 mM L-glutamine, and 50 μg of gentamycin sulfate per ml at 37°C in a humidified 5% CO_2/95% air atmosphere. Cells used for all experiments exceeded 90% viability by trypan blue exclusion.

Antisera. Monoclonal antibody 9.2.27 was developed in this laboratory from a fusion of the nonsecreting 653 variant of the murine P3-X63–Ag8 myeloma cell line with spleens of BALB/c mice previously primed with 4 M urea extracts of M21 melanoma cells. The specificity of this reagent is reported elsewhere (4). Spent tissue culture fluid from this hybridoma was the source of antibody for the studies described.

Biosynthetic Labeling and Pulse–Chase Studies. M21 melanoma cells were biosynthetically labeled with radioactive amino acids by incubation in RPMI-1640 Selectamine media (GIBCO) which contained only 25% of the normal amount of unlabeled amino acid to be radiolabeled. These media were supplemented with 10% dialyzed fetal calf serum and gentamycin sulfate (50 μg/ml). M21 cells were intrinsically labeled for the time periods indicated and separated from labeling media by centrifugation. The cell pellets were extracted in RIPA lysis buffer (0.01 M Tris·HCl/0.15 M NaCl/1% Triton X-100/1% deoxycholate/0.1% $NaDodSO_4$) with 1% Trasylol added (Sigma) as described (6) and used for indirect immunoprecipitation analysis.

In some biosynthetic labeling experiments, monensin (Calbiochem), a monovalent ionophore that can inhibit cell surface expression of membrane glycoproteins (7–10), was added to the culture medium at the concentrations indicated in the text by dilution of a 10 mM stock solution in absolute ethanol. The highest ethanol concentration in these labeling experiments was 0.01%, and it had no effect on control glycoprotein biosynthesis.

Pulse–chase studies were initiated by methionine starvation of M21 cells at 2×10^6 cells per ml for 30 min at 37°C in methionine-free RPMI-1640 Selectamine supplemented with 10% dialyzed fetal calf serum. [^{35}S]Methionine (1 mCi in phosphate-buffered saline; 1 Ci = 3.7×10^{10} becquerels) was then added to initiate a 10-min pulse at 37°C, followed by a 1-min centrifugation step and resuspension in chase medium (RPMI-1640 containing 10% fetal calf serum with no radioactive methionine). Aliquots (1-ml) were removed at the time points indicated and the cells were centrifuged and extracted in RIPA lysis buffer as described above. Samples were then frozen at −20°C for storage.

Indirect Immunoprecipitation and $NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Indirect immunoprecipitation of biosynthetically labeled antigens was achieved with Sepharose-protein A (Sigma) immunoadsorbents (IAD). Monoclonal antibody 9.2.27 was initially preadsorbed to a 10% (vol/vol) Sepharose-protein A suspension in PTO (phosphate-buffered saline, pH 7.2/0.5% Tween 20/0.1% ovalbumin) for 2 hr at 4°C with rotation. This IAD was then washed three times by 1000 × g

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Abbreviations: IAD, immunoadsorbent; Dal, dalton; HMW C, high molecular weight component.

centrifugation with PTO prior to the addition of biosynthetically labeled RIPA extracts of M21 melanoma cells. The IAD and labeled RIPA lysates were allowed to react overnight at 4°C with continual rotation. The IAD–antigen complex was then washed three times by centrifugation in PTO followed by a wash in phosphate-buffered saline prior to elution of radiolabeled antigens with 100 μl of electrophoresis sample buffer [0.01 M Tris·HCl, pH 6.8/0.1% $NaDodSO_4$/0.1% 2-mercaptoethanol/10% (vol/vol) glycerol] (11). Electrophoresis of immunoprecipitated antigens was carried out as described (12) and followed by fluorography of slab gels to visualize antigens according to Bonner and Laskey (13), with Kodak XRP-5 film.

Molecular weights of the glycoproteins under study were estimated by comparing the migration distances of unknown components to those of human erythrocyte ghost membrane proteins according to molecular weights established by Steck (14) and to ^{14}C-labeled protein standards obtained from New England Nuclear. These included [^{14}C]myosin [200,000 dalton (Dal)], [^{14}C]phosphorylase B (92,500 Dal), ^{14}C-labeled bovine serum albumin (68,000 Dal), and [^{14}C]ovalbumin (43,000 Dal). Ferguson plot analyses were also performed with seven acrylamide concentrations (4.5–7.5%) to examine and confirm molecular weight estimates initially based on data obtained at a single acrylamide gel concentration (15, 16). At this time, however, these estimates still remain extrapolations from our highest molecular weight standard [spectrin band I, 240,000 Dal (14)] determined by $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions.

Enzymatic Digestions and β-Elimination Reactions. In the pulse–chase studies, the detergent extracts analyzed at each time point were divided into two fractions which were immunoprecipitated on Sepharose-protein A-9.2.27 IgG IADs. Prior to analysis on $NaDodSO_4$/polyacrylamide gel electrophoresis, the washed immunoprecipitates were resuspended in 50 μl of 20 mM sodium citrate buffer (pH 5.5) with or without the addition of 0.01 unit of endoglycosidase H [endo-β-*N*-acetylglucosaminidase H purified from the culture filtrate of chitin-grown *Streptomyces plicatus* by the method of Tarentino *et al.* (17)] obtained from Health Research (Albany, NY) and incubated for 1 hr at 37°C. Fifty microliters of 2X sample buffer was then added and the samples were analyzed by $NaDodSO_4$/5% polyacrylamide gel electrophoresis.

In some experiments, washed 9.2.27 immunoprecipitates were incubated with 50 μl of sodium phosphate buffer (pH 7.6) or with the same buffer containing 0.05 unit of chondroitin ABC lyase (EC 4.2.2.4) or chondroitin AC lyase (E.C. 4.2.2.5) (Miles) according to Saito *et al.* (18) or 10 units of *Clostridium perfringens* neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18) (type VIII, Sigma). In each case, after 1-hr incubation at 37°C, 50 μl of 2X sample buffer was added, antigens were eluted, and samples were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis.

In additional experiments, 9.2.27 immunoprecipitates were subjected to a β-elimination reaction in 0.1 M NaOH for 15 hr at 4°C (19). After this time, the reaction mixture was neutralized with dilute HCl, 50 μl of 2X sample buffer was added, and samples were analyzed as described above.

Materials. L-[^{35}S]Methionine (1035 Ci/mmol), L-[4,5-^{3}H(N)]leucine (42 Ci/mmol), and $Na_2{}^{35}SO_4$ (110 mCi/mmol of SO_4) were obtained from New England Nuclear. All other materials were reagent grade or better.

RESULTS

Initial studies on the nature of the antigen defined by monoclonal antibody 9.2.27 were performed with biosynthetically labeled M21 melanoma cells that had been exposed to the monovalent ionophore monensin. Recent experiments have shown that monensin affects the cell surface expression and intracellular transport of several glycoproteins and the biosynthesis of proteoglycans (7–10), and we were interested in examining possible biosynthetic precursor components.

When [^{3}H]leucine-labeled cells were extracted in RIPA lysis buffer and analyzed by indirect immunoprecipitation with the 9.2.27 antibody followed by electrophoresis and fluorography (Fig. 1), two components were seen in control immunoprecipitates: one of 250 kDal and a larger component, of >400 kDal, that just entered the 5% polyacrylamide gel. In contrast to these results, RIPA lysates of cells treated with either 1, 0.1, or 0.01 μM monensin exhibited a single component at 250 kDal recognized by the 9.2.27 monoclonal antibody. Results identical to these have been obtained with [^{35}S]methionine- and [^{3}H]glucosamine-labeled M21 cells and with the M14 melanoma cell line (data not shown). Thus, the antigenic determinant recognized by this antibody is located on the 250-kDal component and does not require the presence of the high molecular weight component (HMW C) for recognition by the monoclonal antibody.

Fig. 2 illustrates the results obtained when pulse-chase biosynthetic studies were performed on [^{35}S]methionine-labeled M21 cells to investigate any possible precursor/product biosynthetic relationships between the 250-kDal component and the HMW C. Aliquots taken at the indicated times were also

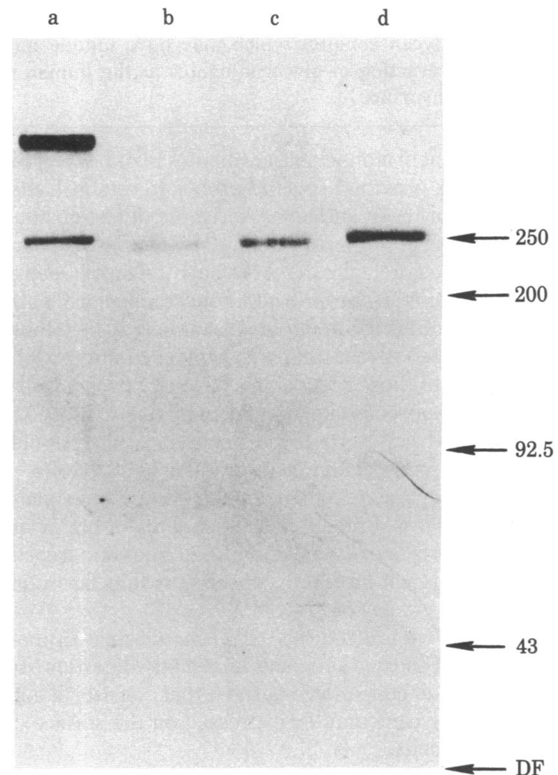

FIG. 1. Indirect immunoprecipitation analysis with 9.2.27 monoclonal antibody, showing effects of exposure of M21 melanoma cells to monensin. M21 melanoma cells were seeded in parallel cultures at cell densities of 0.5×10^6/ml and exposed to monensin for 18 hr. Control cells (lane a) and cells exposed to monensin at 1 (lane b), 0.1 (c), and 0.01 (d) μM were then labeled with 0.5 mCi of [^{35}S]methionine in RPMI-1640 medium with 25% of the normal concentration of methionine for an additional 12 hr in the presence of monensin. Antigens in RIPA lysates were analyzed by indirect immunoprecipitation and $NaDodSO_4$/5% polyacrylamide gel electrophoresis. The migration of standards ($M_r \times 10^{-3}$) is indicated on this 72-hr-exposure fluorograph. DF, dye front.

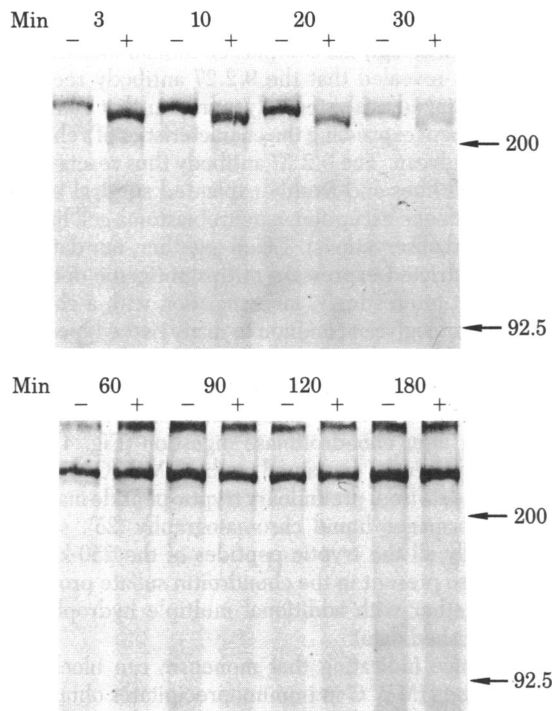

FIG. 2. Pulse–chase biosynthesis studies on the 9.2.27 monoclonal antibody-defined antigenic complex. M21 melanoma cells were pulsed with 1 mCi of [^{35}S]methionine and chased for 3 hr in nonradioactive medium. Cells were collected at times indicated in the chase, extracted in RIPA lysis buffer, and immunoprecipitated with the 9.2.27 monoclonal antibody. Two immunoprecipitates at each time point are shown: −, control antigenic profile; +, immunoprecipitates digested with endoglycosidase H for 1 hr at 37°C prior to electrophoresis on NaDodSO$_4$/5% polyacrylamide gel. The migration of standards (M_r × 10^{-3}) is indicated in these 96-hr-exposure fluorographs.

examined for their sensitivity to endoglycosidase H which cleaves high-mannose, nonprocessed oligosaccharides at N-linked asparagine glycosylation sites (20, 21). This step was included to examine the kinetics of biosynthesis and transport of these molecules within the cell to delineate biosynthetic events associated with the Golgi apparatus where resistance to endoglycosidase H is conferred on glycoproteins (21, 22).

A 240-kDal component initially was detected at early times (3, 10, and 20 min) of the chase by specific immunoprecipitation analysis with monoclonal antibody 9.2.27. This molecule was sensitive to endoglycosidase H as judged by the presence of a faster-migrating component in enzymatically digested immunoprecipitates examined at the same times. These data indicate the presence of high-mannose oligosaccharides in the precursor component recognized by 9.2.27. At 30 min the 250-kDal component initially appeared in both control and enzymatically treated immunoprecipitates, indicating that some endoglycosidase H-resistant molecules existed at this stage of the chase studies. The HMW C appeared initially at this time and became readily apparent at 60 min into the chase. At this point, the 250-kDal component seemed completely resistant to endoglycosidase H and appeared together with the HMW C as visualized in RIPA extracts of long-term biosynthetically labeled M21 melanoma cells (see Fig. 1, lane a). The complete biosynthesis of this monoclonal antibody-defined antigenic complex occurred between 30 and 60 min into the chase, and the appearance of the HMW C presumably occurred during and after processing of N-linked oligosaccharides of the 250-kDal component in the Golgi apparatus as judged by the loss of endoglycosidase H sensitivity. Thus, the pulse–chase studies indicate that monoclonal antibody 9.2.27 recognizes a determinant on an N-linked 250-kDal glycoprotein and its precursors early in the chase and that the expression of this determinant does not necessarily require the presence of the HMW C that appears at later steps in the biosynthesis of this complex.

Further experiments involved the use of $^{35}SO_4$-labeled M21 human melanoma cells to resolve the nature of the HMW C recognized by monoclonal antibody 9.2.27. Immunoprecipitates from control cell populations revealed a strong band of $^{35}SO_4$-labeled material that was specifically recognized by the monoclonal antibody in the absence of a 250-kDal glycoprotein component (Fig. 3). Thus, the HMW C apparently is heavily sulfated but the 250-kDal component is not. The migration of this component in the polyacrylamide gel is identical to that observed for the HMW C in immunoprecipitates from ^{3}H- and ^{35}S-labeled M21 melanoma cells (Figs. 1 and 2). Treatment of this immunoprecipitate in a β-elimination reaction (19) resulted in a complete breakdown of the HMW C, indicating a high degree of alkali labile O-linkage groups (Fig. 3). These data eliminate the possibility of the HMW C being related to N-linked fibronectin glycoproteins found in melanoma cells which in our hands migrate considerably faster than the HMW C or the 250-kDal component (i.e., as the 240-kDal component) on NaDodSO$_4$/5% polyacrylamide gel electrophoresis under reducing conditions.

Parallel immunoprecipitates from monensin-treated $^{35}SO_4$-labeled cells did not demonstrate the HMW C even with extensive overexposure of fluorographs, indicating a potential biosynthetic block in this component induced by monensin (Fig. 3, lane a).

The above data suggest strongly that the HMW C is proteoglycan in nature because of its high molecular weight, high de-

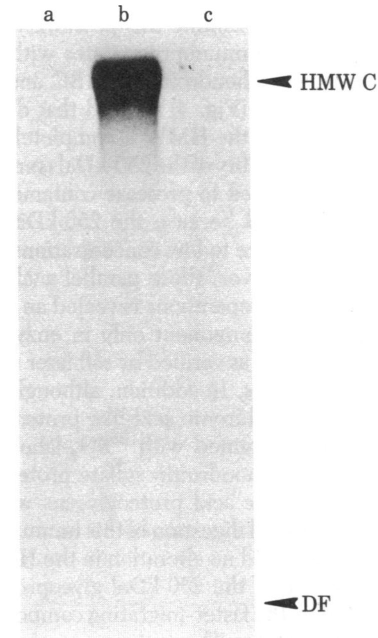

FIG. 3. Immunoprecipitation analysis of $^{35}SO_4$-labeled detergent extracts of M21 melanoma cells with monoclonal antibody 9.2.27. Control M21 cells (lane b) and parallel cultures exposed to μM monensin (lane a) for 18 hr were labeled with 1 mCi of $^{35}SO_4$ for an additional 12 hr in complete medium. Cells were extracted and analyzed by indirect immunoprecipitation. The washed immunoprecipitates from control cell extracts were divided into two fractions, one (lane c) of which was subjected to β-elimination (19). Electrophoresis was on NaDodSO$_4$/5% polyacrylamide gels. This fluorograph represents a 96-hr exposure. DF, dye front.

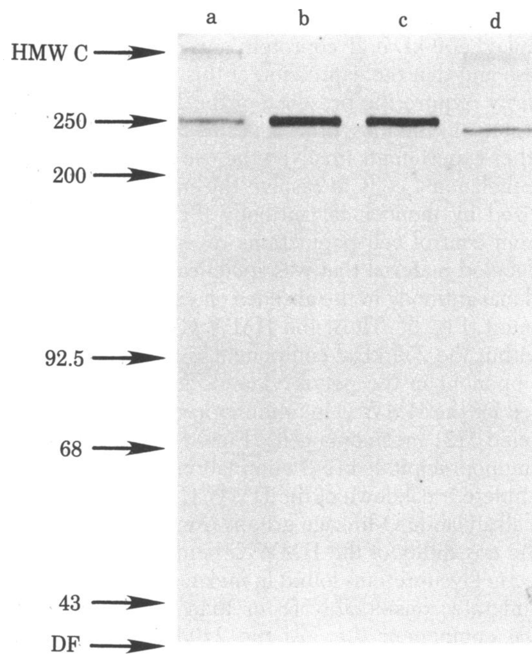

FIG. 4. Enzymatic digestion studies on the 9.2.27 monoclonal antibody-defined antigenic complex. [^{3}H]Leucine-labeled M21 melanoma cells were extracted in RIPA lysis buffer and these extracts were used in indirect immunoprecipitation analysis. Washed immunoprecipitates were treated with buffer (control, lane a), chondroitinase ABC (lane b), chondroitinase AC (lane c), or neuraminidase (lane d) for 1 hr at 37°C prior to analysis by NaDodSO$_4$/5% polyacrylamide gel electrophoresis. Migration of standards ($M_r \times 10^{-3}$) is indicated; DF, dye front.

gree of sulfation, and susceptibility to β-elimination. Additional experiments designed to examine this possibility critically involved the digestion of immunoprecipitates with the proteoglycan-specific enzymes chondroitinase ABC and chondroitinase AC (18). The results (Fig. 4) showed that digestion with these enzymes eliminated the HMW C completely but did not affect the presence or mobility of the 250-kDal component. This finding cannot be attributed to protease contamination of the enzyme preparations used because the 250-kDal component itself is exquisitely sensitive to low concentrations of proteases (unpublished data). However, these parallel analyses of identically labeled antigenic preparations revealed an increased intensity of the 250-kDal component only in enzymatically digested immunoprecipitates as verified by soft laser densitometric scans of these fluorographs. In addition, although these chondroitinases can digest hyaluronic acid-like proteoglycans (18), identical results were obtained with $^{35}SO_4$-labeled material, suggesting strongly that chondroitin sulfate proteoglycans and not nonsulfated hyaluronic acid proteoglycans were digested (data not shown). A parallel digestion of this immunoprecipitate with neuraminidase showed no alteration in the HMW C; however, the apparent size of the 250-kDal glycoprotein was decreased as indicated by the faster-migrating component present in this immunoprecipitate. Thus, the monoclonal antibody 9.2.27 recognizes a 250-kDal, N-linked, sialyated, glycoprotein that either associates with a chondroitin sulfate-like proteoglycan in melanoma cells or itself provides the core glycoprotein for the biosynthesis of the chondroitin sulfate proteoglycan in the Golgi apparatus of human melanoma cells.

DISCUSSION

A combination of biosynthetic and enzymatic digestion studies of the antigenic determinant recognized by the antimelanoma monoclonal antibody 9.2.27 has allowed us to identify a unique glycoprotein–proteoglycan complex on human melanoma cells. These studies revealed that the 9.2.27 antibody recognizes a 250-kDal, N-linked, sialyated, glycoprotein that can associate with a component expressing the characteristics of a chondroitin sulfate proteoglycan. The 9.2.27 antibody thus reacts only with melanoma cell lines and freshly explanted surgical melanoma specimens with one exception, a neuroblastoma cell line LA-N-2 (unpublished observations). Taken together, our data suggest not only a restricted expression of this antigenic determinant but also, most interestingly, an association with a chondroitin sulfate-like proteoglycan common to many tissue types (23, 24).

Although additional studies are required to understand the precise relationship of the 250-kDal component and the proteoglycan HMW C seen in immunoprecipitates, the increase in the former after chondroitinase digestion (Fig. 4) suggests that this component is contained in the HMW C. This idea is supported by data from preliminary tryptic peptide map analysis by high-performance liquid chromatography (25), suggesting that essentially all the tryptic peptides of the 250-kDal component are also present in the chondroitin sulfate proteoglycan molecule together with additional multiple hydrophilic peptides (unpublished data).

Our evidence indicating that monensin can block the appearance of the HMW C in immunoprecipitates obtained with monoclonal antibody 9.2.27 (Figs. 1 and 3) correlates with findings by Tanzer and colleagues (10) that monensin can affect the biosynthesis of proteoglycans in chondrocytes. The pulse–chase analyses of the endoglycosidase H-treated antigen complex also provide evidence that the appearance of the HMW C is linked kinetically to biosynthetic functions of the Golgi apparatus, the proposed site for the glycosyltransferases involved in proteoglycan biosynthesis (23). Thus, our data taken together strongly suggest that the 250-kDal glycoprotein may be a "core" protein onto which chondroitin sulfate proteoglycan side chains are added (23).

In this regard, current information on the biosynthesis of chondroitin sulfate proteoglycans suggests that a core protein ranging from 200 to 370 kDal serves as the site for the addition of 20 or more chondroitin sulfate oligosaccharides to form the high molecular weight chondroitin sulfate proteoglycan (23, 24, 26). In one case, the core protein itself was a glycoprotein, a fact that agrees with our hypothesis suggesting that the 250-kDal component of human melanoma cells also may serve as a core glycoprotein for a chondroitin sulfate proteoglycan (27). At this point we envision that the monoclonal antibody recognizes both the free pool of core protein and chondroitin sulfate proteoglycan monomer. This hypothesis is strengthened by recent reports describing large pools of free proteoglycan core protein determined by immunoprecipitation analysis (26).

Little is known about the biosynthesis of proteoglycans in metastatic human melanoma. However, alterations in the cell surface expression of proteoglycans have been described in B16 mouse melanoma and several other human tumor systems (28, 29). In fact, chondroitin sulfate-like proteoglycans have been implicated in these cell surface changes (28, 29).

The unique specificity of the 9.2.27 monoclonal antibody for a 250-kDal, N-linked, sialyated, glycoprotein associated with a common chondroitin sulfate proteoglycan suggests that the tumor cell may express unique or modified gene products capable of serving as acceptors or core glycoproteins for common proteoglycan side chains. This type of alteration could account for changes in the proteoglycan makeup of the membranes and extracellular matrix of tumor cells previously described (28, 29) and provides a mechanism for significant structural changes on the tumor cell surface. Further studies on the distribution of

this antigenic determinant in normal and malignant tissues are necessary to verify this hypothesis. However, the approaches outlined here, combining the use of the 9.2.27 monoclonal antibody with biosynthetic and enzymatic studies, have permitted us to identify and obtain structural information of this glycoprotein–proteoglycan complex at the melanoma cell surface without the biochemical isolation of these components. This information will facilitate the design of studies specifically targeted to delineate the functional roles these components may have in the biology of malignant human melanoma.

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