Identification of a Secreted M, 95,000 Glycoprotein in Human Melanocytes and Melanomas by a Melanocyte Specific Monoclonal Antibody

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ABSTRACT

We have isolated a monoclonal antibody, designated HMB-50, that is highly specific for melanomas and melanocyte derived lesions. The antibody recognizes melanomas, neonatal melanocytes, and junctional nevi but does not react with adult melanocytes, dermal nevi, or a variety of non-melanocyte derived neoplasms. In tissue culture, the antibody reacts with five of seven human melanoma lines and neonatal foreskin melanocytes but fails to recognize fibroblasts and a number of different carcinomas. HMB-50 identifies a M, 95,000 glycoprotein that is released into the growth medium by melanoma cells and neonatal melanocytes in vitro. This molecule is unrelated to antigens recognized by a variety of antimelanoma monoclonal antibodies isolated in other laboratories. The M, 95,000 glycoprotein has been purified by antibody affinity chromatography and a polyclonal rabbit antiserum raised that exhibits identical specificity to the monoclonal antibody. The M, 95,000 glycoprotein is rapidly released by melanoma cells (within 60 min) and one line produces relatively large quantities of the molecule (1 µg/10^8 cells/24 h). The molecule in normal melanocytes differs slightly in electrophoretic mobility compared to its counterpart in melanomas and this difference appears to result from posttranslational modification.

INTRODUCTION

Since the development of monoclonal antibody technology (1), malignant melanoma has been the subject of intensive study. Many laboratories have isolated antimelanoma monoclonal antibodies to use as diagnostic aids in surgical pathology (22—24). Because melanoma is part of the differential diagnosis of undifferentiated malignant neoplasms, we have attempted to isolate melanoma specific antibodies that could distinguish melanoma from carcinoma, lymphoma, sarcoma, and astrocytoma. We have recently described one such antibody, HMB-45, which is highly specific for melanoma and melanocyte derived lesions and works well on fixed, embedded tissue (25). We describe here a second antibody, HMB-50, that recognizes a M, 95,000 glycoprotein secreted by melanomas and neonatal melanocytes in vitro. We have presented some of these data in a previous publication (26). In this paper, we present additional data on the reactivity of this antibody with a variety of human melanoma cell lines, describe a rabbit polyclonal antibody made to the purified glycoprotein, demonstrate that this molecule differs from antigens detected by other anti-melanoma antibodies, and compare the glycoprotein in neonatal melanocytes and melanoma cells.

MATERIALS AND METHODS

Sources of Monoclonal Antibodies. HMB-50 was isolated as described using an extract of metastatic human malignant melanoma as the immunogen (25). HMB-50 is an IgG2a. Purified p97 and monoclonal antibodies to p97 were provided by Drs. Joe Brown and Charles Estin, Oncogen, Seattle, WA, and antibodies to the M, 250,000—500,000 proteoglycan complex and ganglioside G03 provided by Drs. Karl-Erik and Ingegerd Hellström, Oncogen, Seattle, WA. Dr. Soldano Ferrone, Department of Microbiology, New York Medical College, Valhalla, NY, contributed antibodies 376.92 and 465.12. Dr. Dirk Ruiter, Department of Pathology, University of Leiden, Leiden, Holland, provided antibodies PAL-M1 and PAL-M2.

Immunocytochemistry on Fixed Embedded Tissue. Immunocytochemistry on human tissue fixed in methanol-Carnoy's (methacarn fixative) or formaldehyde was performed as described (25). All tissue was obtained from Hospital Pathology, University Hospital, University of Washington.

Cell Culture of Normal Melanocytes and Sources of Cell Lines. To isolate melanocytes, two freshly obtained newborn foreskins were washed in PBS and serum free medium, freed of s.c. fat and parts of the dermis with iris scissors, and cut into 1-mm strips with a scalpel. The strips were incubated in 2.5 mg/ml collagenase (CLS Cooper Biomedical, Malverne, PA) in DME containing 10% newborn calf serum (5 ml) for 90 min at 37°C. The epidermis was separated from the dermis with forceps, and the epidermal fragments were suspended in 5 ml of trypsin-EDTA (Gibco, Grand Island, NY) and vigorously pipetted for 5 min with a Pasteur pipet. The trypsin solution was removed taking care not to include the epidermal fragments and 5 ml of DME containing 10% newborn calf serum was added to the tissue pieces. The epidermal fragments were again pipetted for 5 min and the liquid was added to the trypsin solution. The trypsin-DME were centrifuged for 5 min at 1100 x g, and the cells were suspended in complete MCDB 153 medium (27, 28) and plated in 35-mm dishes at 1—2 x 10^5 cells/dish. Medium was changed every 2 days and the cells reached confluence in 3—4 weeks. The cultures were then passaged 1:3 every 7—10 days. Subconfluent cultures at passages 2—5 were used for immunoprecipitation experiments.

The human melanoma Mel-1 was established from the same piece of tissue used as antigen to produce HMB-50 (25). Melanoma lines 919, 1688, 1804, 3092, 3090, and 1092 and the lung carcinoma line CaLu were obtained from Drs. Karl-Erik and Ingegerd Hellström, Oncogen, Seattle, WA. T-24 human bladder carcinoma cells were obtained from Dr. Robert Weinberg (Massachusetts Institute of Technology, Cambridge, MA). BT474 is a human breast carcinoma line provided by Dr. Helene Smith, Peralta Cancer Research Center, Oakland, CA, and the human colon carcinoma line CCL218 was obtained from the American Type Culture Collection, Rockville, MD. The source and description of the other human tissue culture lines have been described (22).

Immunofluorescence. Immunofluorescence on cultured cells was performed as described (25).

[^1] Methionine Labeling of Cells and Immunoprecipitation. Cells were incubated in methionine free RPMI containing 5% dialyzed fetal calf serum for 1—2 h prior to addition of label. [^2] Methionine (100—250 µCi/plate, 800 Ci/mmol; NEN, Wilmington, DE) was added in fresh methionine free RPMI and the cells were incubated with label for 4 h.

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[^3]The abbreviations used are: p97, M, 97,000 protein; G03, disialoganglioside; PBS, phosphate-buffered saline; DME, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; RIPA buffer, 1% Nonidet P-40-1% deoxycholate-0.1% SDS-10 mM Tris (pH 7.4)-0.15 mM NaCl.

[^4]Supported by NIH Grant CA28238.

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Medium was collected and centrifuged at maximum speed for 3 min in a tabletop microcentrifuge, and an aliquot was precipitated with 10% trichloroacetic acid to determine radioactive incorporation. The cell monolayer was washed 3 times with PBS and extracted for 10 min at room temperature with 1% Triton X-100 in PBS containing 0.09 mg/ml phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO). Medium was centrifuged and an aliquot was precipitated with 10% trichloroacetic acid.

For immunoprecipitation experiments, 3 × 10^5—3 × 10^6 cpm of labeled medium or 5 × 10^5—5 × 10^6 cpm of Triton extract was preclayed by incubating with 5 μl of nonimmune ascites or preimmune rabbit serum for 30 min at room temperature, followed by 100 μl of 10% Pansorbin (Calbiochem, La Jolla, CA) for another 30 min. The sample was centrifuged and the supernatant was incubated with 5 μl of HMB-50 ascites fluid or immune rabbit serum for 1 h followed by 100 μl of 10% washed Pansorbin for another 1 h. The material was centrifuged and the Pansorbin pellets were washed consecutively in RIPA buffer, high salt buffer (2 M NaCl-10 mM Tris, pH 7.4, 1% Nonidet P-40, 0.5% deoxycholate), low salt buffer (0.5% Nonidet P-40 in PBS), 1 M MgCl₂, 1 mM Tris (pH 7.4) and RIPA, and centrifuged, and SDS sample buffer was added. The samples were boiled for 3 min and centrifuged, and an aliquot was counted. Material was displayed on 8—16% polyacrylamide gels. Prestained molecular weight markers (BRL, Gaithersburg, MD) were used to determine the size of the antigens.

For sequential immunoprecipitation experiments (Fig. 8), aliquots of Mel-1 conditioned medium were preclayed and immunoprecipitated with the rabbit polyclonal anti-M, 95,000 serum or monoclonal antibody 465.12 as described above. The precipitates were solubilized in SDS sample buffer and the supernatants were then subjected to another round of immunoprecipitation with the same or different antibody.

Pulse-Chase Experiment. Subconfluent Mel-1 cells in 35-mm dishes were incubated in methionine free RPMI with 5% dialyzed fetal calf serum for 2 h. This medium was then replaced with 0.5 ml of methionine free RPMI, with 5% dialyzed fetal calf serum and 80 μCi of [35S]methionine. The cells were incubated at 37°C for 15 min (pulse label) after which the labeled medium was removed, the cells were washed 3 times with PBS, and methionine containing RPMI with 5% fetal calf serum added to the cells (chase). At 0, 15, 30, 60, and 120 min after removal of the radioactive medium, one plate was taken from the incubator, the conditioned medium collected and the monolayer extracted with 1% Triton X-100 in PBS, 0.09 mg/ml phenylmethylsulfonyl fluoride. Aliquots of culture medium and Triton extract were immunoprecipitated with HMB-50 as described above, and the precipitates displayed on 8—16% polyacrylamide gels.

Labeling with [3H]Glucosamine. One subconfluent 35-mm dish of Mel-1 was incubated in glucose free Dulbecco’s modified Eagle’s medium with 2% dialyzed fetal calf serum for 2 h. The medium was removed and replaced with fresh glucose free medium containing 25 μCi of [3H]glucosamine. The cells were incubated at 37°C for 6 h after which the medium was removed and an aliquot was immunoprecipitated with HMB-50 as described above.

Western Blot Experiments. Protein samples were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose paper (0.45 μm; Schleicher and Schuell, Keene, NH) overnight at 150 mA in 0.2 M Tris-0.15 M glycine-20% methanol. The nitrocellulose was incubated in 5% human serum in PBS for 1 h and then exposed to a 1:200 dilution of the rabbit polyclonal anti-M, 95,000 serum in 5% human serum in PBS for 1 h. The antibody containing solution was removed; the nitrocellulose was washed 4 times in Tris buffered saline and then incubated with a 1:300 dilution of glucose oxidase conjugated goat antirabbit IgG (Accurate Chemical, Westbury, NY) in 5% human serum in PBS for 1 h. The nitrocellulose was washed 4 times with Tris buffered saline and the reaction developed in 50 mM Tris, pH 8.1, containing 75 mM glucose, 1.65 mM nitroblue tetrazolium and 0.82 mM phenazine methosulfate.

Purification of HMB-50 and Coupling of Antibody to Sepharose. HMB-50 was purified from ascites fluid by Drs. Karl-Erik and Ingegerd Hellström by protein A affinity chromatography as described (29). Purified antibody (15 mg) was coupled to Sepharose CL-4B with cyanogen bromide as described (30). Nonimmune antibody-Sepharose was prepared by coupling nonimmune ascites fluid directly to Sepharose. This nonimmune antibody Sepharose was used to remove material that nonspecifically adhered to Sepharose before incubation with HMB-50-Sepharose.

Purification of M, 95,000 Protein. The M, 95,000 protein was purified from Mel-1 conditioned medium. Twenty confluent 100-mm dishes of Mel-1 cells were incubated with serum free Dulbecco’s modified Eagle’s medium (5 ml) for 24 h after which time the medium was removed and 5 ml of fresh serum free medium were added. Medium was collected for 5—7 days from the same cells. The conditioned medium was concentrated 50-fold in an Amicon filter with a PM10 membrane, incubated with nonimmune ascites-Sepharose for 2 h at 4°C, and centrifuged. The supernantant was added to HMB-50 Sepharose that had been washed 3 times in PBS just prior to use. After 3 h at 4°C the antibody Sepharose was pelleted by centrifugation and washed 4 times with RIPA buffer. The M, 95,000 material was eluted from the antibody-Sepharose with 0.05 M diethylamidine in H₂O for 5 min, dialyzed against 0.05 M NH₄HCO₃, and lyophilized.

Production of Polyclonal Serum. The lyophilized material was solubilized in PBS and 70 μg of protein were mixed with complete Freund’s adjuvant and injected s.c. at multiple locations on a male New Zealand White rabbit. Four weeks later, 35 μg of material in incomplete Freund’s were injected s.c. and the animal was bled 1 week after the booster injection. Blood was allowed to clot for 3 h at 4°C and centrifuged, and the serum was collected and frozen at −20°C.

Preeimmune serum was collected from the same animal prior to injection of purified antigen.

One Dimensional Chymotrypsin Peptide Analysis. Aliquots of Mel-1 and T₂₄ [35S]methionine labeled conditioned medium were immunoprecipitated as described above and the Pansorbin pellets were suspended in 50 μl of 0.4 M NaCl-0.05 M Tris, pH 7.5. Chymotrypsin (5 μg) was added and the samples incubated at 37°C for 15 min. SDS sample buffer (70 μl) was added, the samples boiled for 3 min and the peptides were displayed on a 16% polyacrylamide gel.

RESULTS

Antibody Reactivity on Tissue and Cultured Cells

The pattern of reactivity of HMB-50 was similar to that already described for antibody HMB-45 (25). HMB-50 recognized 9 of 17 melanomas with approximately 50% of the cell within a lesion being positive (Fig. 1). Reaction product was diffusely distributed throughout the cytoplasm of the positive cells (Fig. 1). The antibody was melanoma specific as it did not react with a variety of carcinomas, lymphomas, sarcomas, and astrocytomas (Table 1). HMB-50 did not detect melanocytes in normal adult skin but reacted with neonatal foreskin melanocytes (data not shown). Additionally, it was positive on the junctional components of benign nevi (26). Thus, this antibody can distinguish differences in subsets of normal melanocytes and benign nevi.

![Fig. 1. Avidin-biotin immunoperoxidase preparation of methacarn fixed human melanoma metastatic to a lymph node. Monoclonal antibody HMB-50. × 200.](image-url)
Five of seven human melanoma lines reacted strongly with the antibody, while a variety of carcinomas and human fibroblasts were negative (Table 2). Immunofluorescence experiments demonstrated a granular cytoplasmic pattern (Fig. 2).

Antigen Identification

Western blot analyses with HMB-50 were unrevealing; therefore we performed immunoprecipitation experiments using [35S]methionine labeled conditioned medium and Triton X-100 cell extracts. HMB-50 precipitated a M, 95,000 protein from melanoma conditioned medium and cell extracts and also recognized a M, 10,000 protein in cell extracts, but not conditioned medium (Fig. 3, Lanes 4 and 8). The M, 10,000 protein migrates just behind the dye front in these gels (8-16% gradient gels) and, if run on 17.5% gels, is far behind the dye front (26). The M, 10,000 protein has never been seen in immunoprecipitates of melanoma conditioned medium. Both the M, 95,000 and M, 10,000 proteins appear to be melanoma specific inasmuch as they are not found in conditioned medium or extracts from T24 cells (Fig. 3) or Hep3B, A431, HS0578T, or human fibroblasts (26).

Purification of the M, 95,000 Protein and Production of a Rabbit Polyclonal Antiserum

The M, 95,000 protein was purified to 90% homogeneity by antibody affinity chromatography (Fig. 4) and the purified material injected into a rabbit. The polyclonal serum was as specific as HMB-50, precipitating the M, 95,000 protein from Mel-1 conditioned medium and cell extracts and failing to detect any proteins in T24 cells (Fig. 5). It also identifies the M, 95,000 protein in Western blot experiments (see below; Fig. 7).

Table 1 Reactivity of HMB-50 on malignant tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Fraction positive</th>
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<tbody>
<tr>
<td>Breast carcinoma</td>
<td>0/5</td>
</tr>
<tr>
<td>Ovarian carcinoma</td>
<td>0/5</td>
</tr>
<tr>
<td>Colon carcinoma</td>
<td>0/5</td>
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<tr>
<td>Lung carcinoma</td>
<td>0/5</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>0/5</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>0/5</td>
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<tr>
<td>Astrocytoma</td>
<td>0/5</td>
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</tbody>
</table>

Table 2 Reactivity of HMB-50 on human cell lines

Cells were grown on glass slides, fixed in 2% paraformaldehyde, permeabilized with 1% Triton X-100 in PBS, and assayed by indirect immunofluorescence (25).

<table>
<thead>
<tr>
<th>HMB-50</th>
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<tbody>
<tr>
<td>Melanoma</td>
</tr>
<tr>
<td>Mel-1</td>
</tr>
<tr>
<td>919</td>
</tr>
<tr>
<td>1688</td>
</tr>
<tr>
<td>1804</td>
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<tr>
<td>3092</td>
</tr>
<tr>
<td>3050T</td>
</tr>
<tr>
<td>1092</td>
</tr>
<tr>
<td>Non-melanoma</td>
</tr>
<tr>
<td>HS0578T</td>
</tr>
<tr>
<td>T24</td>
</tr>
<tr>
<td>A431</td>
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<tr>
<td>HEp3B</td>
</tr>
<tr>
<td>BT474</td>
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<tr>
<td>CCL218</td>
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<tr>
<td>HEPG2</td>
</tr>
<tr>
<td>HS0746T</td>
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<tr>
<td>Calu</td>
</tr>
<tr>
<td>Fibroblasts</td>
</tr>
</tbody>
</table>

Fig. 2. Antibody HMB-50, indirect immunofluorescence on (a) Mel-1 cells and (b) HS0578T. Cells were fixed in 2% paraformaldehyde in PBS and permeabilized in 1% Triton in PBS. × 250. HS0578T is a human breast carcinosarcoma cell line.

To see if the antigens detected by these antibodies varied in different melanoma lines, labeled medium and cell extracts from 5 HMB-50 positive melanoma lines (Table 2) were tested with both HMB-50 and the polyclonal antiserum. Each antibody precipitated similarly sized antigens from conditioned medium (Fig. 6) and cell extracts (data not shown). Varying amounts of the protein were found in conditioned medium, and in one line (1092) the molecule migrated slightly faster (Fig. 6). The observation that HMB-50 and the rabbit polyclonal serum detect similar antigens is important because it argues that the melanocyte specificity of the monoclonal antibody results from the presence of the M, 95,000 glycoprotein in melanocytic cells and not from a melanocyte specific modification occurring on a M, 95,000 protein common to many types of cells.

Western blot analyses were used to compare the amount of M, 95,000 protein released into conditioned medium by the different melanoma lines. Aliquots of conditioned medium were tested along with known amounts of purified antigen and the relative intensities of the M, 95,000 bands compared. This assay is capable of detecting 20 ng of M, 95,000 protein per sample. Mel-1 cells produced approximately 1 μg protein/10⁶ cells/24 h while 1092 cells released one-half to one-third that amount. For the other lines, the assay was not sensitive enough to detect the antigen in the aliquot of conditioned medium. Thus, of the lines tested, Mel-1 cells released the greatest amount of the M, 95,000 molecule.

Comparison of the M, 95,000 Protein to Antigens Recognized by Other Antimelanoma Antibodies

p97. A number of antimelanoma antibodies have been described, some of which recognize glycoproteins similar in size to the M, 95,000 protein described here (4, 13, 15, 17, 31). We
MELANOCTYE DERIVED SECRETED M, 95,000 GLYCOPROTEIN

Fig. 5. Immunoprecipitation of T24 and Mel-1 conditioned medium and cell extracts by HMB-50 and polyclonal rabbit serum. Lanes 1–4, T24, Triton extracts; Lanes 5–8, Mel-1 conditioned medium; Lanes 9–12, Mel-1, Triton extracts. Lanes 1, 5, 9, rabbit preimmune serum; Lanes 2, 6, and 10, immune rabbit serum; Lanes 3, 7, and 11, nonimmune mouse ascites; Lanes 4, 8, and 12, HMB-50. NI, nonimmune; I, immune. The Mr 95,000 and Mr 10,000 proteins are not detected in T24 conditioned medium (data not shown). Gradient gel, 8–16%.

Fig. 6. Immunoprecipitation of [35S]methionine labeled conditioned medium from 5 melanoma cell lines with HMB-50 or polyclonal rabbit serum. Melanoma cell lines: Lanes 1 and 2, Mel-1; Lanes 3 and 4, 919; Lanes 5 and 6, 1688; Lanes 7 and 8, 1092; Lanes 9 and 10, 3092; Lanes 1, 3, 5, 7, and 9, HMB-50; Lanes 2, 4, 6, 8, and 10, rabbit polyclonal antiserum. Gradient gel, 8–16%. We do not know the nature of the high molecular weight material in Lanes 3–7. This material is present right at the junction of the separating gel and the 5% stacking gel.

have obtained samples of some of these antibodies and compared their reactivity with HMB-50. In “Western blot” experiments, a cocktail of 6 monoclonal antibodies to p97, the transferrin related glycoprotein (4, 13) did not recognize purified M, 95,000 glycoprotein (Fig. 7, Lane 3) and the rabbit polyclonal antibody to the M, 95,000 glycoprotein did not react with purified p97 (Fig. 7, Lane 5). Thus, these 2 glycoproteins are immunologically unrelated.

465.12 Monoclonal antibody 465.12 detects a M, 95,000 glycoprotein in the conditioned medium of T24 cells (15, 17, 31) and Mel-1 cells (data not shown). To show this molecule is different than the M, 95,000 glycoprotein described here, sequential immunoprecipitations were performed in which Mel-1 conditioned medium was precipitated with one antibody and then reprecipitated with the second antibody. If these molecules were antigenically related, then precipitation with the first antibody should remove all the M, 95,000 material. If the molecules are not related, then precipitation with one antibody should leave a population of M, 95,000 molecules in the con-
conditioned medium which can then be precipitated by the second antibody. Fig. 8 demonstrates that precipitation with 465.12 leaves a population of M, 95,000 molecules that are precipitated by the rabbit polyclonal anti-M, 95,000 serum (Fig. 8, Lane 2), and precipitation with the polyclonal serum leaves M, 95,000 molecules that are precipitated by 465.12 (Fig. 8, Lane 5). Sequential immunoprecipitations with the same antibody detect no residual M, 95,000 material in the second precipitation (Fig. 8, Lanes 3 and 6). Therefore, these 2 antibodies recognize distinct populations of M, 95,000 molecules.

To further prove these antigens are unrelated, we performed one dimensional chymotryptic digests on material precipitated from T24 and Mel-1 conditioned medium. The M, 95,000 proteins precipitated from T24 and Mel-1 conditioned medium by 465.12 have similar chymotryptic digests (Fig. 9, Lanes 1 and 2), while the protein precipitated by HMB-50 from Mel-1 conditioned medium manifests a different pattern (Fig. 9, Lane 3).

Other Antibodies

Antibodies to the M, 250,000–500,000 melanoma associated proteoglycan (5, 6, 13), ganglioside GD3 (32, 33), and other antimelanoma antibodies (3, 19) do not react with the M, 95,000 glycoprotein (data not shown).

Fig. 8. Sequential immunoprecipitation with polyclonal rabbit anti-M, 95,000 serum and monoclonal antibody 465.12 from Soldano Ferrone. After immunoprecipitation with one antibody, the supernatant was reprecipitated with the same antibody or the other antibody. Lane 1, immunoprecipitation with 465.12 alone; Lane 2, immunoprecipitation with 465.12 followed by rabbit anti-p95; Lane 3, 465.12 followed by another aliquot of 465.12; Lane 4, immunoprecipitation with rabbit anti-p95; Lane 5, rabbit anti-p95 followed by 465.12; Lane 6, rabbit anti-p95 followed by rabbit anti-p95; 8–16% gradient gel.

Fig. 9. One dimension chymotryptic digests of proteins recognized by monoclonal antibody 465.12 and HMB-50. [35S]Methionine labeled conditioned medium from T24 cells and Mel-1 cells was immunoprecipitated with 465.12 or HMB-50; the precipitates were digested with chymotrypsin for 15 min at 37°C and run on a 16% SDS-polyacrylamide gel. Lane 1, T24 conditioned medium, 465.12; Lane 2, Mel-1 conditioned medium, 465.12; Lane 3, Mel-1 conditioned medium, HMB-50.

Pulse-Chase Experiments

The M, 95,000 protein can be labeled with [3H]glucosamine, demonstrating that it is posttranslationally modified (Fig. 10). We therefore did a pulse-chase experiment to determine the extent of glycosylation, investigate the degree of posttranslational modification of the molecule, and determine the length of time required for the protein to appear in conditioned medium. After a 15-min pulse label and no chase, a M, 95,000
band was apparent in cell extracts (Fig. 11A). Following a 15-
min chase, the Mel-95,000 band migrated slightly faster, and 
higher molecular weight material (M, 105,000–110,000) appeared (Fig. 11A). After 60 min of chase, material smaller than 
the Mel-95,000 protein was noted. At 120 min, small quantities 
of labeled protein are detected in cell extracts, presumably 
because most of the labeled protein has been secreted by this 
time. Labeled material appears in conditioned medium after 60 
min (Fig. 11B), and both the Mel-95,000 and Mel-95,000–110,000 molecular weight species were found in conditioned 
medium.

To be sure we were not missing smaller or larger precursor 
molecules, we labeled for a shorter time (5 min) and precipitated 
with HMB-50 and the polyclonal antibody. The results were 
identical to those shown in Fig. 11 (data not shown). Only the 
Mel-95,000 peptide was precipitated and both the monoclonal 
and polyclonal antibodies precipitated molecules identical in 
size.

The Mel-10,000 molecule was not detected in these experi-
ments presumably because of the short labeling time (15 min). 
In continuous labeling experiments, 2 h incubation with [35S]-
methionine was required in order for the Mel-10,000 molecule 
to appear in immunoprecipitates of cell extracts (26).

Mel-95,000 Protein in Cultured Normal Melanocytes

To compare this molecule in normal and malignant melanocytes, 
normal melanocytes from neonatal foreskins were grown in vitro. These cells had the typical bipolar appearance of 
melanocytes in vitro (Ref. 34; data not shown) and were positive 
with HMB-50 (data not shown). Immunoprecipitation experi-
ments demonstrated that the Mel-95,000 protein is present in 
melanocyte conditioned medium but it migrates slightly faster 
than the protein from Mel-1 cells (Fig. 12, Lane 1). Triton 
extracts of labeled melanocytes contain 3 distinct bands around 
Mel-95,000 instead of the diffuse band seen in Mel-1 extracts 
(Fig. 12). The smallest band is identical in size to the molecule 
in conditioned medium.

These differences could result from true alterations in the 
size of the peptide or posttranslational modification. To distin-
guish between these possibilities, we labeled cells for 15 min 
and analyzed the cell extracts. The size of the peptide in Mel-1 
and melanocyte extracts was identical, with no differences 
detectable after immunoprecipitation with HMB-50 or the pol-
cyonal antiserum (Fig. 13). Thus, the differences in the Mel-
95,000 protein in melanocytes and Mel-1 cells are probably the 
result of posttranslational modification.

**DISCUSSION**

We describe here a highly specific monoclonal antibody that 
recognized melanomas, junctional nevi, and neonatal melanocytes 
but failed to react with adult melanocytes, dermal nevi, or 
a variety of non-melanocyte derived malignant neoplasms. 
HMB-50 detected a Mel-95,000 glycoprotein in the cytoplasm 
and conditioned medium of melanocytes and 5 melanoma cell 
lines and differences in the size of the molecule in normal and 
malignant melanocytes were demonstrated. One melanoma 
line, Mel-1, produced relatively large quantities of this glyco-
protein, approximately 1 µg/10⁶ cells/24 h.

Comparison with Antigens Detected by Other Antimelanoma 
Monoclonal Antibodies. A number of other laboratories have 
isolated antimelanoma monoclonal antibodies and some of 
these antibodies recognize antigens similar in size to the Mel-
95,000 glycoprotein described here. We have obtained samples 
of antibodies 376.96 (3), 465.12 (15), Pal-M2 (19), anti-p97 
antibodies (29), and anti-GD3 (32, 33) and antibodies to a Mel-
250,000–500,000 proteoglycan complex (5, 6, 13). We found 
that they do not recognize the Mel-95,000 molecule (Figs. 7 and 
8; data not shown). In addition, other antimelanoma antibodies 
differ from HMB-50 based upon tissue specificity (18, 35, 36), 
surface localization (4–14), and size of the antigen (37–40).
Mr 95,000 Glycoprotein. In short pulse experiments, the antigen is first detected as a Mr 95,000 molecule which then undergoes posttranslational modification to higher and lower molecular weight species. The pattern of posttranslational modification of this protein in melanomas and melanocytes appears to be different (Figs. 12 and 13; see below). The molecule is acidic (pI = 5.3–5.5; data not shown) and composed of a single peptide chain, inasmuch as no larger or smaller subunits are detected on reduced versus unreduced gels (data not shown). Tunicamycin, an inhibitor of N-glycosylation (41) does not inhibit secretion of the molecule except at toxic concentrations (data not shown).

The Mr, 95,000 glycoprotein is rapidly released into the culture medium and different melanoma lines produce varying quantities of the molecule. One line, Mel-1, secretes large quantities (1 μg/10⁶ cells/24 h) while another line, 3092, releases barely detectable amounts. While we have not proved that the molecule is truly secreted by melanoma cells, we believe it is for the following reasons: (a) it is rapidly released into the culture medium (within 60 min); (b) it is highly unlikely that cell death could account for the rapid appearance of the molecule in medium or the large amounts produced by Mel-1 cells. An alternate explanation to secretion is that the molecule is a surface glycoprotein that is rapidly released from the membrane by proteolytic cleavage. We have investigated this possibility by doing immunofluorescence experiments on fixed unpermeabilized cells, asking if HMB-50 gives a diffuse surface staining pattern. Diffuse surface staining was not observed; only focal rare scattered fluorescent patches were observed (data not shown). Thus, we believe this alternate explanation is unlikely and the molecule is actively secreted by the cells.

HMB-50 and the rabbit polyclonal antibody also precipitate a Mr, 10,000 protein from Triton extracts of melanoma cells and melanocytes (Fig. 12). This molecule has not been observed in conditioned medium; it is found only in cell extracts. Additionally, previous studies have shown that HMB-45, another melanocyte specific monoclonal antibody, recognizes the Mr, 100,000 antigen but not the Mr, 95,000 glycoprotein (26). The relationship of these molecules and antibodies to each other remains to be determined. The Mr, 10,000 protein may be a breakdown product of the Mr, 95,000 molecule. Alternatively, the Mr, 10,000 protein may be bound to the Mr, 95,000 molecule in the cell and come off the protein as it is released from the cell. We plan to investigate these possibilities in the future.

Mr, 95,000 Glycoprotein in Melanocytes versus Melanoma Cells. An interesting finding was the subtle differences in this molecule in normal melanocytes compared with melanoma cells. Melanocyte extracts contained 3 distinct bands around Mr 95,000 and the smallest molecular weight form was predominant in conditioned medium. Extracts of the 5 melanoma lines listed in Table 2 contained only the diffuse band demonstrated in Mel-1 cells; 3 distinct bands were not observed in any of these extracts (data not shown). This difference in size is probably the result of posttranslational modification because in short pulse experiments, the size of the protein in melanocytes and melanoma cells is identical (Fig. 13). It is possible that the molecule in normal cells undergoes different patterns of glycosylation and/or proteolysis compared to the malignant cells.

Function of Mr, 95,000 Glycoprotein. A major question is what is the function of this molecule? The molecule is larger than tyrosinase (Mr, 72,000) and the nerve growth factor receptor (Mr, 77,000) both of which are produced by melanoma cells (42–44). The presence of the molecule in melanocytic cells is probably not related to melanin production because both pigmented and nonpigmented melanomas react with antibody HMB-50. Similarly, the Mel-1 cell line is pigmented while 919,
1688, 3092, and 1092 are unpigmented, yet each produces and secretes the molecule. Another possibility is that the production of the molecule is related to the presence of melanosomes but this is unlikely because adult melanocytes and dermal nevi contain melanosomes (45) but do not react with HMB-50. Additionally, the cell line 919 contains very few melanosomes (data not shown) but synthesizes the molecule.

The M, 95,000 molecule may be an “oncofetal” antigen expressed only in fetal and neoplastic melanocytes. The observation that other melanoma associated antigens exhibit a similar pattern of expression (14, 46) supports this hypothesis. An alternate explanation is that the production of the M, 95,000 protein is related to the proliferative state of the cells, such that only growing cells produce the molecule. This hypothesis could explain the pattern of reactivity of HMB-50, since melanomas and neonatal melanocytes are proliferating while adult melanocytes are quiescent. Additionally, some investigators hypothesize that junctional nevus cells proliferate and then become quiescent when they move down into the dermis (47—49). This hypothesis could be tested by culturing adult melanocytes in vitro and asking if they produce the molecule.

We have demonstrated that cells in vitro secrete the molecule but it is not known if cells in vivo also secrete it. We are currently investigating this question by asking if the molecule can be detected in serum from melanoma patients. Such studies could have great clinical usefulness as they could lead to a diagnostic test for melanoma.

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Identification of a Secreted $M_r$ 95,000 Glycoprotein in Human Melanocytes and Melanomas by a Melanocyte Specific Monoclonal Antibody

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