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Immunology

Identification of a cell surface protein, p97, in human melanomas and certain other neoplasms

(monoclonal antibody/tumor antigen)

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ABSTRACT **BALB/c mice were immunized with a human melanoma cell line, SK-MEL 28, and their spleen cells were fused with mouse NS-1 myeloma cells. Hybrid cells were tested in an indirect ^{125}I-labeled protein A assay for production of antibodies that bound to surface antigens of SK-MEL 28 melanoma cells but not to autologous skin fibroblasts. One hybridoma, designated 4.1, had the required specificity. It was cloned and grown in mice as an ascites tumor. The monoclonal IgG_1 antibody produced by the hybridoma was purified from the ascites fluid and labeled with ^{125}I. The labeled antibody bound, at significant levels, to approximately 90% of the melanomas tested and to approximately 55% of other tumor cells, but not to three B-lymphoblastoid cell lines or to cultivated fibroblasts from 15 donors. Immunoprecipitation and sodium dodecyl sulfate gel electrophoresis were used to detect the target antigen in ^{125}I-labeled cell membranes of both cultivated cells and tumor biopsy samples. A protein with a molecular weight of 97,000 was identified. This protein, designated p97, was present in both cultured cells and biopsy material from melanomas and certain other tumors, but it was not detected in eight different samples of normal adult epithelial or mesenchymal tissues obtained from five donors.**

Many human neoplasms express tumor-associated antigens (1). With few exceptions, however, methodological difficulties have hampered attempts to purify and to characterize these antigens. Monoclonal antibodies produced by hybridomas (2) offer great promise as a means of identifying tumor-associated antigens (3). The antibodies can also be used to purify the antigens by immunoprecipitation for subsequent biochemical characterization.

Antigens associated with human melanomas have been studied extensively. Melanoma patients have been found to mount both cell-mediated and humoral immune reactions to their tumors (4, 5), and serological studies with melanoma patients' sera indicated the presence of two classes of melanoma-associated antigens (6). Antigens of one class are each restricted to a single melanoma. Those of the other class are shared by many melanomas and a small fraction of other tumors.

Yeh and coworkers (7) recently established three hybridomas that secrete antibodies to an antigen that is present in greatest amount on cells of the immunizing melanoma and is also detectable on cells from about 10% of other melanomas but not on cells from other tumors, B cells, or fibroblasts. Koprowski *et al.* (8) have obtained hybridomas that produce antibodies to antigens shared by many melanomas. Recent data (9), however, indicate that at least some of these antibodies also bind to normal human cells.

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In this paper we describe the isolation of a hybridoma, 4.1, that secretes antibody recognizing an antigen present on cells from the immunizing human melanoma (SK-MEL 28), on approximately 90% of the allogeneic melanomas, and on approximately 55% of other tumors tested. The antigen is a protein with a molecular weight of 97,000. It is localized at the cell surface, and it can be detected in tumor biopsy materials.

MATERIALS AND METHODS

Cells. The melanoma cell line SK-MEL 28, which was used to immunize mice, was obtained from the Sloan–Kettering Institute for Cancer Research through the courtesy of M. Bean. Various other normal and neoplastic cell lines were used. Some of these were previously described (7). Others include melanoma lines M2028, M1975, M1923, M1916, M1766, M1688, M1152, M1151, M1101, M1079, M1013, M933, M919, M908, M902, M894, M740, M603, and M342; lung carcinomas L1849, L1152, L828, and L812; breast carcinomas Br1202, Br988, Br893, and Br587; kidney carcinomas K994, K992, K752, and K195; ovary carcinomas O695, O555, and O138; colon carcinomas C2042, C975, C750, C675, and C531; endometrial carcinomas E854 and E318; liposarcoma Li919; rhabdomyosarcoma R705; cecum carcinoma Ce449; stomach carcinoma S927; bladder carcinoma B907; normal skin fibroblast lines F1823, F1697, F1688, F1075, F893, F826, F740, F675, and F603; and osteogenic sarcoma Os998; all these lines have been cultured in our laboratory. Osteogenic sarcoma Os906 was obtained from J. Fogh (Sloan–Kettering Institute for Cancer Research, New York), bladder carcinomas B1038 and B1039 came from P. Perlmann (Stockholm Univ., Sweden), glioblastoma G821 was from M. Bean (Virginia Research Center, Seattle, WA). Breast carcinoma Br926 was obtained from R. Herberman (National Cancer Institute, Bethesda, MD) and melanoma M646 came from N. Levy (Duke University, Durham, NC). B-lymphoblastoid cell lines Som 2, PA 3, and SF were provided by J. Hansen (Fred Hutchinson Cancer Research Center, Seattle, WA). Adult human surgical tissues were provided by R. Jones (University of Washington Hospital, Seattle, WA), R. Quint (Swedish Center Hospital, Seattle, WA), and L. Hill (Virginia Mason Hospital, Seattle, WA).

The various target cell lines were grown in 5% CO_2 in air in Waymouth's culture medium buffered with $NaHCO_3$ and supplemented with 30% fetal calf serum, 1% nonessential amino acids, 1 mM sodium pyruvate, and 2 mM L-glutamine (7).

P3-NS1/1-Ag4-1 (NS-1) is an azaguanine-resistant BALB/c myeloma line, which was kindly provided by C. Milstein (Medical Research Council Laboratory of Molecular Biology,

Abbreviation: P_i/NaCl, phosphate-buffered saline.

Cambridge, England). NS-1 cells were grown as described (7).

Growth, Selection, and Cloning of Hybridoma 4.1. Five 3-month-old BALB/c mice were immunized with two intraperitoneal inoculations of 10^7 viable SK-MEL 28 melanoma cells 4 weeks apart. Four days after the second immunization, their spleen cells (4×10^8) were fused with NS-1 myeloma cells (9×10^7) and seeded into 480 microtest wells. Hybrids were grown in selective medium as described (7).

Radioiodination of Antibody. Approximately 20 μg of antibody was incubated at 0°C with 1 mCi of $Na^{125}I$ (1 Ci = 3.7×10^{10} becquerels), 4 ng of lactoperoxidase, and 15 ng of H_2O_2 for 10 sec. The reaction was stopped by the addition of 500 μl of phosphate-buffered saline, pH 7.2 (P_i/NaCl), and the ^{125}I-labeled antibody was purified by gel filtration on a column of Sephadex G-25 superfine that had been pretreated with 1 ml of 2% bovine serum albumin and then equilibrated in P_i/NaCl. The specific activity of the ^{125}I-labeled antibody was approximately 2×10^7 cpm/μg. The ^{125}I-labeled antibody was diluted with an equal volume of 2% bovine serum albumin in P_i/NaCl and aliquots were frozen at −70°C.

Binding of ^{125}I-Labeled Monoclonal Antibody to Human Cells. ^{125}I-Labeled antibody 4.1 (2×10^6 cpm, 100 ng) was incubated with 5×10^4 cells in a final volume of 200 μl of medium containing 1% bovine serum albumin, at 37°C for 1 hr. The cells were washed three times in 4 ml of 0.1% bovine serum albumin in P_i/NaCl and then transferred to 12 × 75 mm tubes for ^{125}I determination. Adherent cells were detached by trypsinization for 5 min prior to use in the assay.

Membrane Preparations. Adherent cultured cells were detached by brief trypsinization and washed with RPMI 1640 culture medium containing 15% fetal calf serum and then with P_i/NaCl. Two million cells were resuspended in 10 ml of 1 mM $NaHCO_3$, incubated at 0°C for 20 min, and then disrupted with 10 strokes of a Dounce homogenizer. Nuclei were removed by centrifugation at 2000 × *g* for 5 min at 4°C. The supernatant was centrifuged at 300,000 × *g* for 10 min at 4°C and the pellet containing membranes was incubated for 20 min at 0°C in 0.5 ml of 20 mM Tris·HCl buffer, pH 8.0, containing 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40 (Particle Data, Elmhurst, IL). The extract was centrifuged at 300,000 × *g* for 10 min and the supernatant was passed through a column of Sephadex G-25 superfine pretreated with 1 ml of 2% bovine serum albumin and equilibrated with ice-cold buffer used to extract the membranes. Solubilized membrane preparations were stored at −70°C in small aliquots.

Tissue or tumor biopsy material (50 mg) was minced with scissors and homogenized in a Dounce homogenizor in 5 ml of ice-cold 1 mM $NaHCO_3$ containing 0.5 mM phenylmethylsulfonyl fluoride (Calbiochem) and 0.5 mM EDTA. The membrane fraction was purified as described above except that the membrane pellet was extracted with 1–2 ml of buffer, and that prior to labeling with ^{125}I, a portion of the extract was absorbed to 10 mg of *Staphylococcus aureus* in order to remove some of the endogenous immunoglobulin.

Radioiodination of Membrane Extracts. Approximately 10 μg of membrane protein was incubated with 0.5 mCi of $Na^{125}I$ and 10 μg of chloramine-T in 500 μl of P_i/NaCl at 0°C for 20 min. The reaction was stopped by addition of 10 μg of sodium metabisulfite. The ^{125}I-labeled membrane components were purified by gel filtration on a column of Sephadex G-25 superfine equilibrated with 20 mM Tris·HCl buffer, pH 8.0, containing 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM NaI, and 2% bovine serum albumin (immunoprecipitation buffer). The ^{125}I-labeled proteins were stored at −70°C.

Immunoprecipitation of ^{125}I-Labeled Membrane Antigens. The ^{125}I-labeled membrane extract was added to 10 mg of *S. aureus* to remove immunoglobulins and proteins binding nonspecifically to the bacteria. The bacteria were removed by centrifugation and $NaDodSO_4$ was added to the supernatant to give a final concentration of 0.2%. Five micrograms of antibody 4.1 was incubated with 50–200 × 10^6 cpm of ^{125}I-labeled membrane extract in a volume of 200 μl for 1 hr at 0°C. Five microliters of goat anti-mouse IgG serum was added, and the incubation was continued for 10 min. Immune complexes were adsorbed to 2 mg of *S. aureus*. The bacteria were washed three times with 1 ml of immunoprecipitation buffer containing 0.2% $NaDodSO_4$ and twice with 1 ml of 2 mM Tris·HCl/10 mM NaCl/0.1 mM EDTA/0.05% Nonidet P-40, pH 8.0 (10). The bacteria were incubated in 60 μl of $NaDodSO_4$/polyacrylamide gel electrophoresis sample buffer containing 2-mercaptoethanol (11) at 100°C for 10 min and pelleted by centrifugation, and 50 μl of the supernatant was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (11). ^{125}I-Labeled human serum albumin, heavy and light chains of mouse IgG, and ribonuclease A were used as molecular weight markers. The gels were dried and autoradiographed at −70°C with preflashed Kodak XR-2 film and a Rarex B mid speed intensifying screen (GAF, New York, NY) (12).

RESULTS

Testing of Hybridoma Media. Hybridomas formed from NS-1 cells and the spleen cells of mice immunized with SK-MEL 28 melanoma cells were seeded in test wells and 20 days later samples of medium from each well were tested in an autoradiographic modification of an indirect ^{125}I-labeled protein A assay (13) with SK-MEL 28 melanoma cells and autologous fibroblasts as targets. One hybridoma, designated 4.1, which secreted antibody that bound to SK-MEL 28 but not to the fibroblasts, was selected for further study. It was cloned twice by a previously described method (7). The class of the antibody produced by hybridoma 4.1 was determined to be IgG_1 by an indirect ^{125}I-labeled *S. aureus* protein A assay using rabbit anti-mouse immunoglobulin sera directed toward specific immunoglobulin classes (7).

Purification of Monoclonal Antibodies from Ascites Fluid. Hybridoma 4.1 was inoculated intraperitoneally (10^7 cells per mouse) into pristane-primed BALB/c mice, where it grew as an ascites tumor. The ascites fluid was collected 2 weeks after tumor inoculation. It contained antibody that gave significant binding to SK-MEL 28 cells at dilutions up to 1:100,000 in the indirect ^{125}I-labeled protein A assay (7).

The antibody was purified by affinity chromatography on protein A coupled to Sepharose CL-4B by a procedure described by Ey *et al.* (14). Four milliliters of the ascites fluid was passed through the affinity column and the adsorbed IgG was eluted with buffer of decreasing pH (14). Twelve milligrams of IgG, containing most of the original antibody activity, eluted at pH 6, which is the reported pH of elution for IgG_1 (14). Analysis of the purified antibody by $NaDodSO_4$/polyacrylamide gel electrophoresis revealed a heavy immunoglobulin chain with a molecular weight of about 50,000 and two light chains with molecular weights of about 25,000. Antibody 4.1 was subsequently purified from spent medium of *in vitro* hybridoma cultures and gave identical $NaDodSO_4$/polyacrylamide gel electrophoresis patterns. The larger of the two light chains is believed to be the NS-1 myeloma light chain.

Binding of ^{125}I-Labeled Antibody to Human Cells. Purified antibody, labeled with ^{125}I, was tested for binding to surface antigens of various cells. The results (Fig. 1) from binding assays with 25 melanoma cell lines, 35 other tumor cell lines, 15 dif-

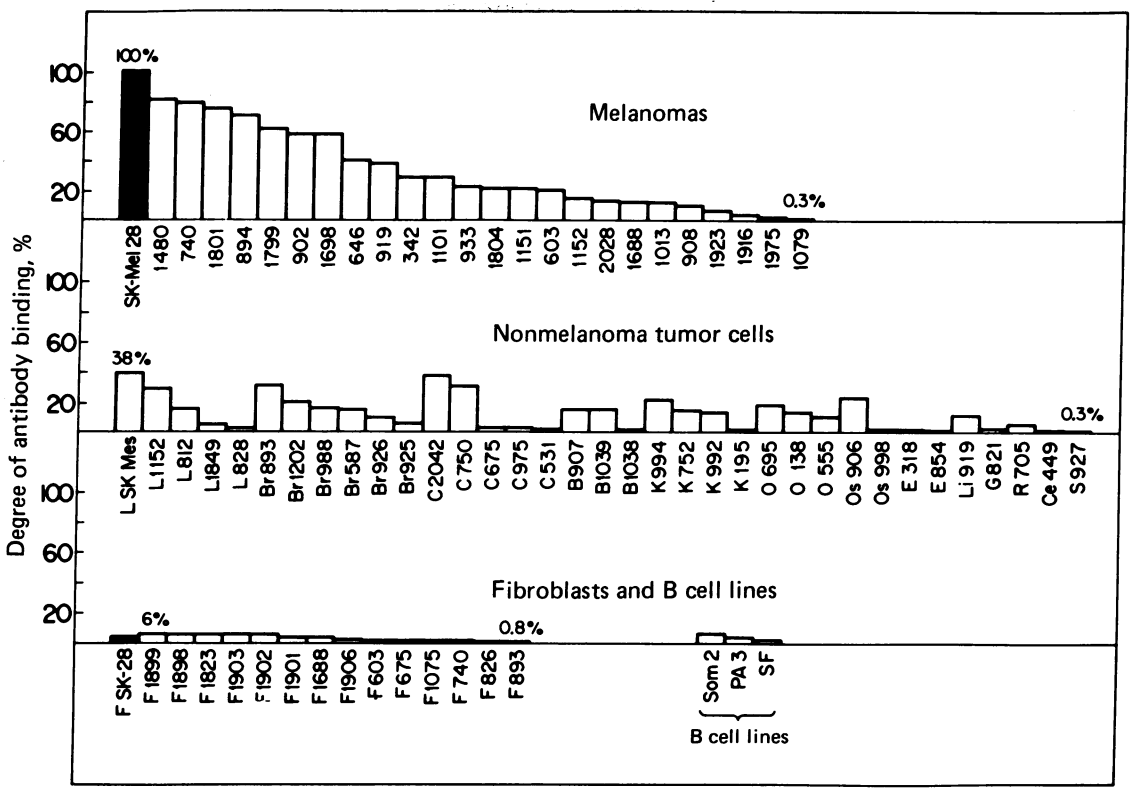

FIG. 1. Binding of ^{125}I-labeled antibody to human cell lines. Cells (5×10^4) were incubated at 37°C for 1 hr with approximately 100 ng (2×10^6 cpm) of ^{125}I-labeled antibody 4.1 in a final volume of 200 μl of medium containing 1% bovine serum albumin. The cells were washed three times in 4 ml of 0.1% bovine serum albumin in P_i/NaCl and then transferred to 12 × 75 mm tubes for ^{125}I determination. Adherent cells were trypsinized for 5 min prior to use in the assay. Trypsinization of cells for as long as 15 min had little effect on the level of antibody binding. ^{125}I-Labeled antibody 4.1 binding to cells is expressed as percentages relative to the level of binding to SK-MEL 28 melanoma cells, which was arbitrarily taken as 100% under the assay conditions. Values represent the average of duplicate samples. Approximately 10^5 cpm (5 ng) of ^{125}I-labeled antibody 4.1 bound to 5×10^4 SK-MEL 28 melanoma cells. Black bars indicate the immunizing melanoma cell and autologous fibroblast cell lines. Abbreviations used for nonmelanoma tumor cells are: B, bladder; Br, breast; C, colon; Ce, cecum; E, endometrial carcinoma; G, glioblastoma; K, kidney; L, lung; Li, liposarcoma; O, ovary; Os, osteogenic sarcoma; R, rhabdomyosarcoma; S, stomach carcinoma; F indicates fibroblast cell lines.

ferent samples of explanted fibroblasts, and 3 B-lymphoblastoid cell lines indicate that antibody 4.1 bound at significant levels (cells binding at least 10% of the antibody that was bound by SK-MEL 28 cells) to the cell surface of approximately 90% of the melanomas and approximately 55% of other types of tumor cells tested, but not to a measurable degree to any of the fibroblasts and B-lymphoblastoid cell lines. Detachment of adherent cells by trypsinization (even for as long as 15 min) had no effect on antibody binding.

Immunoprecipitation of the Antigen Identified by Antibody 4.1. Antibody 4.1 was incubated with ^{125}I-labeled membrane proteins from SK-MEL 28 cells. Antigen–antibody complexes were isolated by adsorption to *S. aureus* and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Autoradiography revealed a single polypeptide chain (Fig. 2*A*). The electrophoretic mobility of the protein was identical to that of rabbit muscle phosphorylase *b*, whose amino acid sequence has been determined and which has a molecular weight of 97,400 (15). The antigen recognized by antibody 4.1 is thus a 97-kilodalton polypeptide, which we have designated p97. Its molecular weight is the same when membranes are prepared without prior trypsinization of cells.

Autologous fibroblasts were also tested (Fig. 2*B*). We could not detect p97 in these cells even when immunoprecipitation incubations of fibroblast membrane extracts contained 20×10^7 cpm compared to 5×10^7 cpm for the melanoma membrane extract. A control serum rabbit anti-human β_2-microglobulin (DAKO PATTS, Copenhagen, Denmark), was positive with cell membranes from both fibroblasts and melanoma. Immunoprecipitation tests of ^{125}I-labeled cell membranes from melanomas other than SK-MEL 28 and from some nonmelanoma tumors also revealed the presence of p97 (Fig. 2 *C* and *D*).

Detection of p97 in Tumor Biopsy Materials. To test for p97 in tumor biopsy materials, we assayed ^{125}I-labeled membrane preparations from biopsies of four melanomas and from one breast carcinoma. The antigen p97 was identified in two melanomas and also weakly in the breast carcinoma (Fig. 3).

Eight normal adult human tissues (skin, muscle, fascia, lung, placenta, ovary, fallopian tube, and uterus) obtained as fresh surgical material from five donors were tested by immunoprecipitation for the presence of p97. No p97 was detected in any of these tissues even when the gels were autoradiographed for 5 days in order to reveal any weak bands. A control serum (rabbit anti-human β_2-microglobulin) was clearly positive.

DISCUSSION

We have isolated and cloned a hybridoma, 4.1, that produces an IgG_1 antibody that binds to SK-MEL 28 melanoma cells but not to autologous fibroblasts. Extensive studies using ^{125}I-labeled antibody showed that the antibody binds at significant levels to cells from approximately 90% of the melanomas and to approximately 55% of other tumors tested, but not to 15 different samples of skin fibroblasts or 3 human B-lymphoblastoid cell lines. The antigen defined by hybridoma 4.1 is a polypeptide

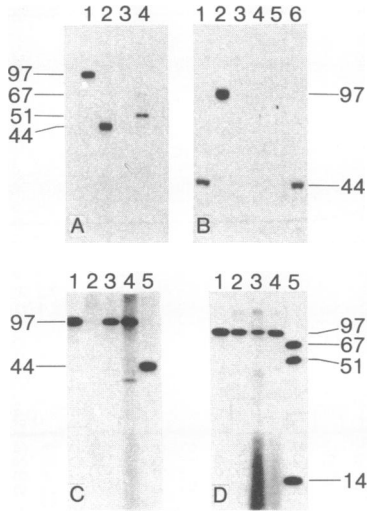

FIG. 2. Autoradiographs of immunoprecipitations of ^{125}I-labeled membrane proteins from human cells analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. Immunoprecipitation samples contained 50–200 × 10^6 cpm of ^{125}I-labeled cell membrane extracts and 5 μg of antibody 4.1 or rabbit anti-human β_2-microglobulin as indicated, and all contained 5 μg of goat anti-mouse IgG. (*A*) Immunoprecipitation of ^{125}I-labeled membrane preparation from SK-MEL 28 melanoma cells (5 × 10^7 cpm). Incubations, indicated by gel track number, included: 1, antibody 4.1; 2, rabbit anti-human β_2-microglobulin (band observed is histocompatibility antigen HLA); 3, medium control; 4, ^{125}I-labeled human serum albumin and mouse immunoglobulin heavy chain, 67 and 51 kilodaltons, respectively. (*B*) Immunoprecipitation of ^{125}I-labeled membrane preparations from SK-MEL 28 melanoma cells (tracks 1 and 2; 5 × 10^7 cpm) or autologous fibroblast cell line (tracks 4–6; 20 × 10^7 cpm); 1, rabbit anti-human β_2-microglobulin (HLA observed); 2, antibody 4.1; 3, NaDodSO$_4$/polyacrylamide gel electrophoresis sample buffer; 4, medium control; 5, antibody 4.1; 6, rabbit anti-human β_2-microglobulin. (*C*) Immunoprecipitation of ^{125}I-labeled membrane preparations from several melanomas. All incubations included antibody 4.1. Track 1, SK-MEL 28 melanoma membrane extract (5 × 10^7 cpm); 2, autologous fibroblast membrane extract (20 × 10^7 cpm); 3, M1801 melanoma cell membrane extract (5 × 10^7 cpm); 4, M1923 melanoma cell membrane extract (20 × 10^7 cpm); 5, SK-MEL 28 melanoma membrane extract (5 × 10^7 cpm) incubated with 1 μl of rabbit anti-human β_2-microglobulin (band observed is HLA). (*D*) Immunoprecipitation of ^{125}I-labeled membrane preparations from nonmelanoma tumor cell lines. All incubations included antibody 4.1. Track 1, SK-MEL 28 melanoma cell membrane extract (5 × 10^7 cpm); 2, SK-MES lung carcinoma (5 × 10^7 cpm); 3, Br988 breast carcinoma (10 × 10^7 cpm); 4, K994 kidney carcinoma (20 × 10^7); 5, human serum albumin (67 kilodaltons), mouse immunoglobulin heavy chain (51 kilodaltons), and ribonuclease A (14 kilodaltons) as molecular weight markers. Numbers to the side of the autoradiographs indicate molecular mass in kilodaltons. All gels contained 13% polyacrylamide except *B*, which contained 9%.

with a molecular mass of approximately 97 kilodaltons. It has hence been designated p97.

Identification of p97 by immunoprecipitation assays on biopsy material from two melanomas and one breast carcinoma establishes that the antigen is expressed *in vivo*. The widespread occurrence of p97 in nonmelanoma tumor cells clearly establishes that it is not melanoma-specific. As a group, however, melanomas bound more antibody 4.1 than did other types of tumors. Thus, whereas 9 of 25 melanoma cell lines bound 40% or more ^{125}I-labeled antibody 4.1, with binding to SK-MEL 28 cells arbitrarily chosen as the 100% level, none of the 35 nonmelanoma tumors attained this level of antibody binding. Interestingly, among the melanomas tested, the immunizing cell, SK-MEL 28, bound the greatest amount of ^{125}I-labeled antibody 4.1 even though the antigen is present on nearly all of the

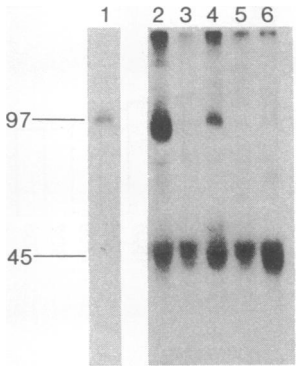

FIG. 3. Autoradiograph of immunoprecipitation of ^{125}I-labeled membrane protein (5 × 10^7 cpm in each incubation) from tumor biopsy materials analyzed by NaDodSO$_4$/9% polyacrylamide gel electrophoresis. All incubations included antibody 4.1. Immunoprecipitation, indicated by gel track number, included: 1, SK-MEL 28 melanoma cell membrane extract; 2, M1801 melanoma tumor biopsy cell membrane extract (The slightly faster electrophoretic mobility of the protein band may be due either to overloading of sample or to limited proteolysis during the membrane preparation. This band was absent in both positive and medium controls.); 3, M2028 melanoma biopsy cell membrane extract; 4, M2040 melanoma biopsy cell membrane extract; 5, M1876 melanoma biopsy cell membrane extract; 6, M2027 breast tumor biopsy cell membrane extract (faint detectable band at 97 kilodaltons). Numbers to the side of the autoradiograph indicate the molecular mass in kilodaltons. The bands migrating at 45 kilodaltons may represent actin that binds nonspecifically to *S. aureus*. They are observed also in both the negative and positive controls.

melanomas. SK-MEL 28 melanoma cells also bound more hybridoma antibody 4.2 or 4.3 (obtained from the same cell fusion that produced hybridoma 4.1) in the indirect ^{125}I-labeled protein A assay than did 12 allogeneic melanomas (data not shown).

We did not detect p97 in eight different normal adult human tissues tested with immunoprecipitation assays. However, more normal tissues must be examined and more quantitative tests (such as absorptions and competition radioimmunoassays), as well as membrane immunofluorescence tests on biopsy material, are needed before one can make any conclusions about its degree of tumor specificity. It is possible that some normal adult and fetal cells express p97 in amounts undetectable by the approaches used in this study. Nonetheless, the data presented herein establish that p97 is a protein that is present, *in vivo*, in many melanomas and in some other neoplasms and, therefore, it should be considered for further investigation.

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