Journal of Cellular Biochemistry 27:303-316 (1985)

Immunopurification, Characterization, and Nature of Membrane Association of Human Melanoma-Associated Oncofetal Antigen gp87 Defined by Monoclonal Antibody 140.240

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A melanoma-associated oncofetal antigen, gp87 (a p97-like molecule), defined by the monoclonal antibody (MoAb) 140.240 has been purified to homogeneity from the spent medium of cultured melanoma cells by a two-step immunoadsorbent procedure. The first immunoadsorbent step using glutaraldehyde-insolubilized MoAb 140.240 (ascites fluid) resulted in a 13-fold enrichment with 93% recovery in the bound material. In the second immunoadsorbent step constructed by the purified IgG2a of MoAb 140.240 (culture fluid) coupled to CNBr-activated Sepharose 4B the bound material from the first step was further purified resulting in a 330-fold purification with 90% recovery. SDS-polyacrylamide gel electrophoretic analysis of the final purified material revealed a single band migrating as a polypeptide with an approximate molecular weight of 87 Kd, consistent with the size of the molecule immunoprecipitated by MoAb 140.240 from lysates of radiolabelled melanoma cells. Preliminary amino acid analysis indicates a particularly high proportion of phenylalanine in gp87. We have also compared gp87 with two well defined antigens, HLA-A,B,C (integral membrane protein) and "94K" melanoma/carcinoma-associated antigen (peripheral membrane protein) with respect to antigen extractability from melanoma cells using phosphate-buffered saline, 0.1 M urea, 3 M NaCl, or nonionic detergent (NP-40). The results showed that whereas 94K antigen was extractable by each of the four different solutions, gp87, similar to HLA-A,B,C antigens, could only be extracted with NP-40, strongly suggesting that gp87 is an integral melanoma cell component.

Key words: melanoma, melanoma-associated oncofetal antigen, gp87, monoclonal antibody, purification

Malignant melanoma has been extensively studied as a model system of human tumors because of the possibility that immunological factors are involved in suscepti-

Received October 31, 1983; revised and accepted October 9, 1984.

bility to or outcome of this malignancy [for review see 1]. Melanoma-associated antigens have been detected by a variety of serological methods using patients' sera as well as xenoantisera. However, their usefulness in molecular characterization of the antigens has been hampered largely by limited availability and difficulty in standardization of antisera following a series of absorptions required for removal of unwanted antibodies and by the generally low titer and avidity of absorbed antisera. In the past eight years a rapid advancement in the production of monoclonal antibodies through the hybridoma methodology [2] has made a thorough analysis of melanoma surface antigens possible. By this approach, over 30 antigens on the melanoma cell surface have been described [for review see 1,3,4]. Nevertheless, a number of these antigens have been characterized in detail in terms of their structural and functional properties. A notable example is p97, a cell surface glycoprotein described by Brown et al [5–7].

We have recently produced a mouse monoclonal antibody, designated MoAb 140.240, which was raised against a cultured human melanoma line [8]. In our initial serological screening, this antibody reacted with most cultured and fresh human melanomas and tissue homogenates of some fetuses, but it does not react with a wide variety of other neoplastic or adult normal tissues as determined by direct and absorption testings in binding assays [8–10]. We have identified the structure bearing the epitope on melanoma cells and in fetal tissues (mostly restricted in small intestine) recognized by MoAb 140.240 to be an 87 Kd glycoprotein molecule (gp87).

Our most recent serological tests on three normal melanocyte culture strains derived from newborn foreskins have revealed that 10–30% of these culture cells are also reactive with MoAb 140.240. These observations suggest that gp87 may be a human normal differentiation-linked gene product whose expression is enhanced in the malignant state of human melanocytes. This antigen can therefore be classified as one of the melanoma-associated oncofetal (differentiation) antigens.

We have also investigated the biosynthetic profile of gp87 in cultured melanoma cells through pulse-chase labelling experiments and tunicamycin studies [10,11]. This antigen is initially synthesized in a form of 77 Kd precursor polypeptide (p77) that immediately undergoes N-asparagine–linked glycosylation to form an intermediate, nonsialylated 83 Kd glycopolypeptide (gp83). gp83 is then further modified and glycosylated to form a final product, 87 Kd sialoglycopolypeptide (gp87), which is normally expressed on the melanoma cell surface. A better understanding of the structural and functional properties of gp87 depends ultimately on detailed knowledge of its molecular structure. To this end, it is essential to obtain a sufficient quantity of highly purified gp87. The present study was therefore undertaken to purify gp87 to a high degree of homogeneity from the spent medium of cultured melanoma cells using two successive immunoadsorbents, the first made of glutaraldehyde-insolubilized MoAb 140.240 (ascites fluid) and the second constructed by coupling the IgG2a fraction of MoAb 140.240 (culture fluid) to Sepharose-4B. Amino acid composition of the purified gp87 was documented. In addition, attempts were made to investigate the nature of physical association of gp87 with the melanoma plasma membrane by examining the extractability of this antigen from the melanoma cell surface under various dissociating conditions.

MATERIALS AND METHODS

Cells and Culture Method

Human melanoma cell line CaCL 78-1 [8] was used. Cells were maintained in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS) without antibiotics at 37°C in 100% humidified atmosphere of 5% CO_2 and 95% air. The cultures were shown to be free of mycoplasma by cytochemical DNA staining at monthly intervals [12].

Preparation of Spent Medium Concentrate

Monolayer culture cells were prepared by seeding 5×10^6 cells in 30 ml of MEM + 10% FCS in each of the culture flasks with a surface culture area of 175 cm^2 (A/S NUNC, Roskilde, Denmark). When cultures reached confluency at day 4, the medium was discarded and cells were washed twice with phosphate-buffered saline (PBS), pH 7.4. Thirty milliliters of fresh MEM without FCS was added to each flask and the cultures were incubated at 37°C for 72 hr. The spent medium was then collected and centrifuged at 10,000g for 20 min. The cell-free supernatant was concentrated 50- to 80-fold in an Amicon ultrafiltration unit (Amicon Corp., Lexington, MA) using a YM5 membrane (5,000 molecular weight exclusion limit). The concentrated material was centrifuged in a B60 International Preparative Ultracentrifuge (IEC, Needham Heights, MA) at 136,000g for 1 hr. The clear supernatant was dialyzed against PBS containing 0.02% sodium azide and stored at −70°C until use.

Monoclonal Antibodies

Three monoclonal antibodies, MoAb 140.240, MoAb MAS 032, and MoAb 376.96S, were used. MoAb 140.240 produced in our laboratory has been previously described [8–10] and its use in antigen analysis is described in this paper. MoAb MAS 032 purchased from Sera-Lab has been shown to recognize a shared determinant of HLA-A,B,C antigens [13]. MoAb 376.96S, kindly supplied by Dr. S. Ferrone (Department of Pathology, Columbia University, New York, NY), has been reported to recognize a 94 Kd molecule present on melanoma as well as carcinoma cells [14].

Mixed Hemadsorption Assay

The mixed hemadsorption assay was performed as previously described [8]. Briefly, sufficient target cells were seeded to yield approximately 100 cells attached to each well of microtest plates (EDB, N-1495/1-63118, A/S NUNC) after overnight incubation. After the plates were washed with phosphate-buffered saline (PBS, 8 gm NaCl, 0.2 gm KH_2PO_4, 0.2 gm KCl, and 1.15 gm Na_2HPO_4 in 1,000 ml distilled water, pH 7.4) containing 0.2% gelatin, 10 μl of test antibody was added to each well and the plates incubated for 2 hr at room temperature. After washing, 10 μl of indicator sheep red blood cells (SRBC) was added to each well and the plates were incubated for 1 hr at room temperature. Indicator SRBC were prepared using appropriate concentrations of mouse anti-SRBC antiserum followed by goat antimouse Ig antiserum. After washing, the plates were flooded with 0.25% glutaraldehyde to stabilize the rosettes for counting. Target cells were scored positive if 5 or more

SRBC were adherent to the test target cell surface. The endpoint titer was the highest dilution at which at least 10% of the target cells were positive.

Quantitative Absorption Analysis

Fractions were tested for antigenic activity by quantitative absorption analysis [8]. The working dilution of the antibody used for absorption was the concentration two doubling dilutions less than that which gave 50% positive cells. Aliquots of the test material containing increasing amounts of protein were mixed with 10 μl of diluted antibody and the final volume brought up to 100 μl with PBS containing 0.2% gelatin, thereby giving the final antibody concentration consistent with the predetermined working dilution. The mixtures were then incubated for 1 hr at 22°C and 2 hr at 4°C. Ten microliters of each mixture was then transferred to duplicate wells of a prewashed plate of target cells and the residual activity of antibody was tested against CaCL 78-1 melanoma target cells by the mixed hemadsorption assay. The percent inhibition of antibody reactivity relative to the unabsorbed antibody was plotted on an arithmetic scale against the amounts of material used for absorption on a logarithmic scale. The amount of material required to reduce 50% of antibody reactivity was calculated from the inhibition curve and was referred to the absorption dose 50 (AD_{50}) [8].

Immunoadsorbent Made of Glutaraldehyde-Insolubilized MoAb 140.240 (Ascites Fluid)

Ascites fluid was produced in pristane-primed Balb/c mice by i.p. injection of 10^7 cells of the hybridoma clone (140.240, 6P1-4P1) [8]. Ascites fluid was polymerized and made insoluble using 2.5% glutaraldehyde according to the procedure previously described [15]. Briefly, ascites fluid was concentrated to a protein concentration of 30 mg/ml by Aquacide IIA (Calbiochem, San Diego, CA). To 5 ml of the concentrated ascites contained in a Sorvall round bottom centrifuge tube (29 × 102 cm; Dupont Instrument, Newtown, CT), glutaraldehyde solution (2.5%, 1.5 ml) was added dropwise. Gel was formed gradually through polymerization and it was left for 3 hr at room temperature to complete insolubilization. The insolubilized protein was dispersed in 20 ml of PBS, pH 7.4, and homogenized in a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) using a PT-10ST probe. The gel was washed in PBS three times by centrifugation at 3,000g for 15 min at 4°C. The immunoadsorbent (gel) was then washed extensively in the eluting solution, 3 M KSCN, until the optical density of supernatant solution remained unchanged at 280 nm. The immunoadsorbent was subsequently washed twice in PBS, pH 7.4. The spent medium concentrate containing antigenic activity was mixed with the gel and the mixture was incubated at room temperature for 1 hr. The unbound material was removed by repeated washing with PBS by centrifugation described above and the bound material was eluted by suspending the gel with 4 ml of 3 M KSCN. The elution was repeated three times until no protein could be detected in the eluted solution. The bound (eluted) and unbound fractions were dialyzed against PBS, pH 7.4, before they were concentrated and tested for antigenic activity.

Purification of IgG2a Fraction From MoAb 140.240

IgG2a fraction of culture fluid of the hybridoma clone (140.240, 6P1-4P1) producing MoAb 140.240 was prepared by fractionation on protein A–Sepharose CL-

4B (Pharmacia, Uppsala, Sweden) as described by Ey et al [16]. Briefly, 500 ml of the culture fluid was recycled through a protein A–Sepharose column (1 × 5 cm) equilibrated with PBS (pH 8.0) containing 0.02% sodium azide and 0.005% Nonidet P-40 (NP-40). The column was washed with PBS and then eluted sequentially with 0.1 M sodium citrate/citric acid buffers of pH 6.0, 5.0, 4.0, and 3.0 at a flow rate of 0.5 ml/min, and fractions of 4 ml were collected. Protein elution profiles were determined by optical density measurement at 280 nm. Fractions comprising each peak were pooled, concentrated, and tested for antibody activity against melanoma CaCL 78-1 target cells by the mixed hemadsorption assay. The IgG2a fraction was concentrated in the Ig peak eluted with citrate buffer, pH 5.0. The IgG2a isotype in the purified fraction was confirmed by gel diffusion as well as by a modified mixed hemadsorption assay designed to detect a minute amount of mouse Ig class or subclass using rabbit antimouse Ig class or subclass antisera [17]. The purity and homogeneity of purified IgG2a were visualized by the staining pattern of SDS-PAGE.

Antibody Affinity Chromatography

The purified IgG2a fraction (25 mg) of MoAb 140.240 was coupled to CNBr activated Sepharose-4B as previously described [18]. The IgG2a–Sepharose-4B beads were then packed in a column (5 × 1.6 cm) and equilibrated with PBS (pH 7.4) at 4°C. Unbound material recovered from immunoadsorbent obtained with glutaraldehyde-insolubilized MoAb 140.240 was applied to the column. This immunoadsorbent column was allowed to stand at room temperature for 1 hr and at 4°C for 1 hr. Unbound material was then collected and the column was washed extensively with 50 column-volumes of PBS. The bound material was eluted with 3 M KSCN, dialyzed against PBS three times, and concentrated by Amicon ultrafiltration using XM50 membrane (50,000 molecular weight exclusion limit). The unbound material was treated in a similar manner.

Extraction of Surface Radioiodinated Melanoma Cells

Melanoma cells were surface labelled with ^{125}I according to the procedure described by Markwell and Fox [19]. Monolayer cultures were washed four times with 30 ml portions of phosphate-buffered saline (pH 7.4). Cells were scraped from cultured flask by the aid of a rubber policeman and pelleted by centrifugation (800g, 10 min). The cell pellet was resuspended in PBS, and 1 ml of the suspension (1×10^7 cells/ml) was added to glass scintillation vials that had been coated in advance with 100 μg of 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (IODO-GEN, Pierce Chemical Co., Rockford, IL). The reaction was initiated by addition of 500 μCi of $Na^{125}I$ to each vial and allowed to proceed for 10 min with gentle agitation at 23°C. Cells were then washed three times with PBS containing 5 mM NaI, divided into four equal parts, and pelleted by centrifugation. To one part was added 2 ml of PBS and the remaining three cell pellets were resuspended in 2 ml of PBS containing either 0.1 M urea, 3 M NaCl, or 0.5% (w/v) Nonidet P-40, respectively. The suspensions were incubated at 37°C for 45 min with occasional agitation and centrifuged at 800g for 20 min followed by recentrifugation of the supernatants at 100,000g for 1 hr. Urea extracts as well as saline extracts were dialyzed against PBS prior to analysis by immunoprecipitation and SDS-polyacrylamide gel electrophoresis.

Iodination of Soluble Antigen

To iodinate the purified glycoprotein antigen, the IODO-GEN technique described above was used except that instead of cell suspension 50 μg of the antigen preparation was added to the glass vial coated with IODO-GEN. After the reaction was stopped by adding a solution of NaI, the reaction mixture was run over a Sephadex G-25 column (0.5 × 2 cm) to remove free iodine. The unbound fraction was collected and counted and the specific activity of the labelled antigen was determined.

Metabolic Labelling of Melanoma Cells With ^{3}H-Glucosamine

Metabolic labelling of D-[1-6-^{3}H(N)]-glucosamine (32.5 Ci/mmol, New England Nuclear, Boston, MA) was performed by adding 250 μCi to melanoma (CaCL 78-1) cell culture grown in a 75 cm^2 flask in 10 ml of glucose-deficient MEM (Grand Island Biological Co., Grand Island, NY) supplemented with 2% FCS for 18 hr. Labelled cells were scraped from the culture flask, centrifuged (1,000g, 10 min), and washed three times with PBS, pH 7.4. The cell pellet was extracted with 1 ml of lysing buffer (0.5% NP-40 in PBS containing 0.1 mM phenylmethylsulfonyl fluoride and 0.1 mM benzamidine hydrochloride) and cell debris was removed by centrifugation at 100,000g for 1 hr.

Determination of Protein Content

Protein concentration was determined by the BIO-RAD protein assay (BIO-RAD Laboratories, Richmond, CA) using bovine serum albumin as a standard.

Immunoprecipitation Procedure

Immunoprecipitation was performed by mixing 50 μl of monoclonal antibody or normal mouse serum (NMS) with 0.5 ml portions of the labelled cell extracts for 90 min at 4°C. Three hundred microliters or a 1:10 dilution of protein A–Sepharose CL-4B (Pharmacia Fine Chemicals, Inc., Quebec, Canada) in PBS was then added. After additional mixing for 30 min, immune complexes adsorbed to protein A beads were pelleted and washed five times by centrifugation (200g, 30 sec) with 1 ml of 0.5% NP-40 in PBS. Immune complexes were then dissociated by heating (100°C, 2 min) the beads in 35 μl of double-strength SDS-sample buffer [20].

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-polyacrylamide slab gels (1.5 mm thick) were prepared according to the method of Laemmli [20]. Samples were analyzed under reducing conditions by electrophoresis on gradient gels, 7.5–15% (v/v) polyacrylamide, at a current of 10 ma for 18 hr. After electrophoresis the gel was processed by scintillation autoradiography [21], and the radiolabelled bands were detected on Kodak XAR-5 X-ray film. ^{14}C-labelled protein molecular weight markers (New England Nuclear) were included in the gel.

For staining, gels were fixed and stained with 0.25% (w/v) Coomassie brilliant blue in 25% (v/v) methanol and 10% (v/v) acetic acid at 23°C overnight. The stained gels were destained with 37% methanol in 10% acetic acid for 6 hr and subsequently with the mixture of acetic acid/methanol/water (1:1:8) until the background became

clear. Low-molecular-weight standards (Pharmacia) were used for the determination of molecular weight of the test samples.

Amino Acid Analysis

Fifty micrograms of purified gp87 was hydrolyzed with 1.0 ml constantly boiling HCl at 110°C for 24 hr in an evacuated sealed tube. The hydrolysate was analyzed with a Beckman automatic amino acid analyzer, Model 121, using norleucine as the internal standard.

RESULTS

Purification of gp87 From Spent Medium of Cultured Melanoma Cells

We have previously shown that the antigenic activity [8] and the molecular structure [10,11] bearing the epitope recognized by MoAb 140.240 can be detected by quantitative absorption and immunoprecipitation analysis respectively in material spontaneously shed into medium by a number of melanoma cell lines. In view of these findings and of the much less complex nature of shed material compared to material extracted from cell membrane or intact cells, we chose the spent medium as a source of starting material for antigen purification. Two successive immunoadsorbents were used in the purification protocol. Step 1 immunoadsorbent was made of glutaraldehyde-insolubilized antibody (ascites fluid) and step 2 was constructed by coupling the purified antibody IgG2a (culture fluid) to Sepharose-4B. We then monitored the material recovered from each step of the purification process for antigenic activity by quantitative absorption of MoAb 140.240. Results are illustrated in Figure 1. A stepwise enrichment of antigenic activity detected by MoAb 140.240 was

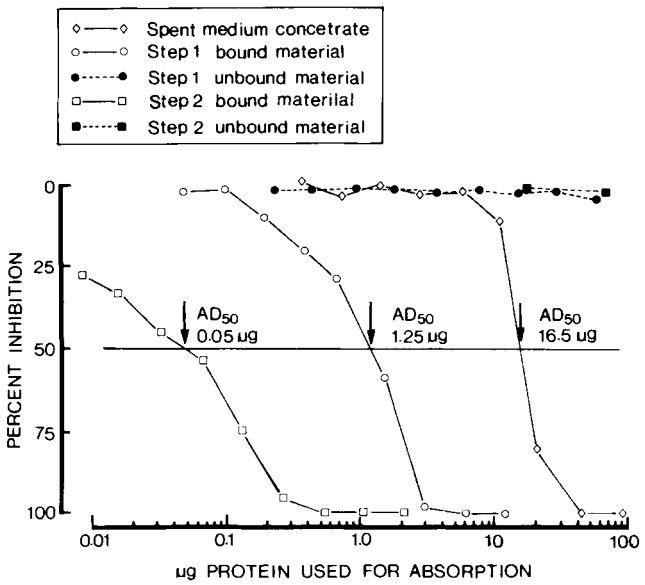

Fig. 1. Quantitative absorption studies on two-step purification of gp87 by immunoadsorbent using glutaraldehyde-insolubilized MoAb 140.240 ascites fluid (step 1) and purified IgG2 of MoAb 140.240 culture fluid–Sepharose 4B affinity chromatography (step 2). See Table I for further details.

achieved, as evident by a marked decrease in AD_{50} values from the spent medium concentrate, the bound material of step 1 immunoadsorbent, to the bound material of step 2 immunoadsorbent, respectively (ie, AD_{50} 16.5 μg, 1.25 μg, and 0.05 μg/100 μl of antibody at a dilution of 1:160). The unbound material recovered from either step 1 or step 2 immunoadsorbent failed to alter the antibody reactivity, indicating no detectable antigenic activity in these two samples.

Table I summarizes the concentration and antigen yield during the sequential purification of gp87. The antigen was enriched 13-fold with a 92.5% recovery in the bound material eluted from step 1 immunoadsorbent. The further purification of this antigenic fraction through step 2 immunoadsorbent resulted in a 330-fold increase in specific activity with a 90.4% recovery of antigens over the spent medium concentrate.

In order to directly visualize the degree of antigen purity, Coomassie brilliant blue staining of SDS-polyacrylamide gel electrophoretic patterns of material recovered from each purification step was set up. Whereas the original spent medium concentrate contained a variety of protein bands (Fig. 2A, lane a), only four distinct bands including one with an apparent molecular weight of 87,000 was detected in the bound material eluted from step 1 immunoadsorbent (Fig. 2A, lane b). The bound material recovered from step 2 contained only one band consistent with an 87,000 molecular weight (Fig. 2A, lane c).

To substantiate that the purified material was indeed the gp87 molecule recognized by MoAb 140.240, the purified antigen labelled with ^{125}I (no immunoprecipitation) and the immunoprecipitates obtained with NP-40 lysate of CaCL 78-1 melanoma cells metabolically labelled with ^{3}H-glucosamine were analyzed on the same SDS-PAGE (Fig. 2B). The iodinated antigen band (lane a) corresponds to the

TABLE I. Summary of Purification of gp87 Antigenic Activity From Spent Culture Medium of CaCL 78-1 Melanoma Cells Using Immunoadsorbent of Glutaraldehyde-Insolubilized MoAb 140.240 Followed by Antibody–Sepharose 4B Affinity Chromatography

Isolation step	Volume (ml)	Total protein (mg)	AD_{50}[a] (μg)	Specific activity (units/mg)[b]	Total units recovered	Purification factor	% of yield
Spent medium concentrate	4.0	55.5	16.5	60.6	3,363.2	(1.0)	(100)
Immunoadsorbent 1 (glutaraldehyde-insolubilized MoAb 140.240 [ascites fluid], bound material)	6.5	3.89	1.25	800.1	3,112.0	13.2	92.5
Immunoadsorbent 2 (MoAb 140.240 [IgG2a purified from culture fluid]–Sepharose 4B affinity chromatography, bound material)	0.17	0.152	0.05	20,000.0	3,040.0	330.2	90.4

[a] AD_{50}, amount of protein required to inhibit 50% of antibody (100 μl, 1:160 dilution) binding activity.
[b] The AD_{50} value is taken to represent one unit of antigenic activity. The number of units in 1 mg of protein (specific activity) can be calculated, eg, if AD_{50} is 16.5 μg/100 μl antibody, the specific activity will be 1,000/16.5 = 60.6 units/mg.

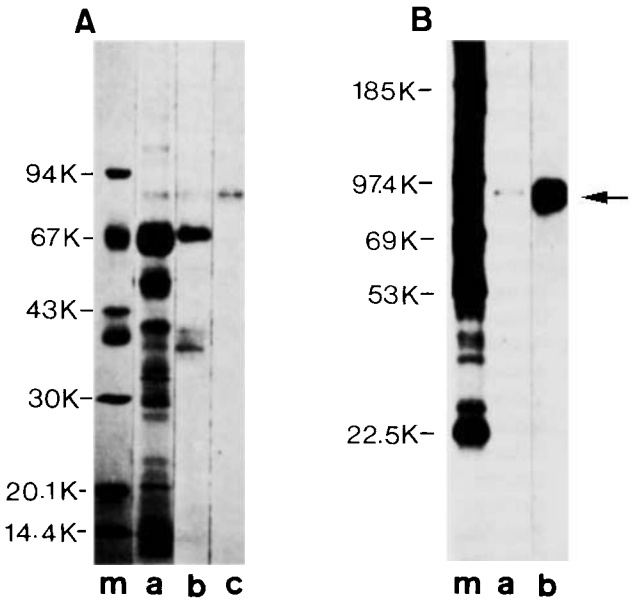

Fig. 2. A) Coomassie brilliant blue staining profiles of SDS-PAGE of material isolated from spent medium of cultured melanoma cells (CaCL 78-1) at different purification stages. Lane a, spent medium concentrate; lane b, bound material eluted from step 1; lane c, bound material eluted from step 2; lane m, molecular weight markers (purchased from Pharmacia). B) SDS-PAGE of ^{3}H-glucosamine-labelled antigen (from cultured melanoma cells, CaCL 78-1) immunoprecipitated by MoAb 140.240 (lane a) and of ^{125}I-labelled purified gp87 (lane b). ^{14}C-labelled molecular weight markers (New England Nuclear) are shown in lane m.

band of ^{3}H-glucosamine-labelled antigen (lane b), thus indicating that the purified antigen is gp87. Furthermore, since the gel electrophoresis using the iodinated purified antigen reveals no other obvious protein contaminants, gp87 preparation obtained by the present procedure may be considered to have fulfilled stringent criteria for its purity.

Amino Acid Composition of gp87

The composition of the supernatant from the acid hydrolysis of the purified gp87 is given in Table II. The percentage of composition in terms of nmol of each detectable amino acid relative to the total amount of amino acids was calculated. A wide range of amino acids are present in gp87 with a particularly high proportion of phenylalanine.

Nature of Association of gp87 With the Melanoma Cell Plasma Membrane

Proteins bound to mammalian cell membranes have been classified as peripheral or integral according to the criteria specified by Singer and Nicolson [22]. Peripheral proteins can be readily solubilized from cell membranes under mild dissociating conditions, such as high or low salt concentration. Proteins integral to the membrane on the other hand require more drastic treatments with reagents, such as detergents,

TABLE II. Relative Amino Acid Composition of gp87 Purified From Spent Medium of CaCL 78-1 Melanoma Cells

Amino acid	Percent nmol
Lysine	8.13
Histidine	1.83
Arginine	4.14
Aspartic acid	8.20
Threonine	5.05
Serine	6.66
Glutamic acid	10.38
Glycine	7.61
Alanine	6.73
Valine	5.00
Methionine	1.20
Isoleucine	2.97
Leucine	6.93
Tyrosine	2.47
Phenylalanine	21.69
Cysteine	1.01

to dissociate them from the cell membrane. To examine whether gp87 was integral or peripheral in its association with melanoma cell plasma membrane, CaCL 78-1 melanoma cells were surface-labelled with ^{125}I and extracted with either PBS, 0.1 M urea, 3 M NaCl, or 0.5% NP-40. Equal volumes of each of the extracts were then immunoprecipitated with MoAb 140.20, MoAb MAS 032, and MoAb 376.96S. MoAb 376.96S has been reported to recognize a 94 Kd melanoma/carcinoma-associated antigen [14] that has been shown to be a peripheral membrane protein [23]. The immune complexes were isolated by protein A-Sepharose and analyzed on SDS-PAGE. The autoradiograph of the gel showed that gp87, HLA-A,B,C antigens, and the 94 Kd antigen (molecular weight estimation is slightly higher in our results) (Fig. 3) could be extracted from surface-radioiodinated melanoma cells with the nonionic detergent NP-40. The 94 Kd melanoma-associated peripheral surface antigen could also be solubilized from the cells with PBS, 0.1 M urea, or 3 M NaCl, as previously documented [23]. In contrast, gp87, similarly to HLA-A,B,C antigens, which are known to be integral in their association with cell membranes [24], was detected only in the NP-40 extracts of the surface labelled cells. These data strongly suggest that gp87 is integral rather than peripheral in its association with the melanoma cell plasma membrane.

DISCUSSION

We have developed a protocol for the efficient purification of gp87 molecules from the spent medium of cultured human melanoma cells. One advantage of using monoclonal antibody immunoadsorbent as an early step in the purification of gp87 is to bypass the more conventional multiple-step procedures involving ion exchange and molecular sieve chromatography. The size and charge heterogeneity of the glycoprotein and the need for continued presence of detergent to keep glycoprotein in soluble

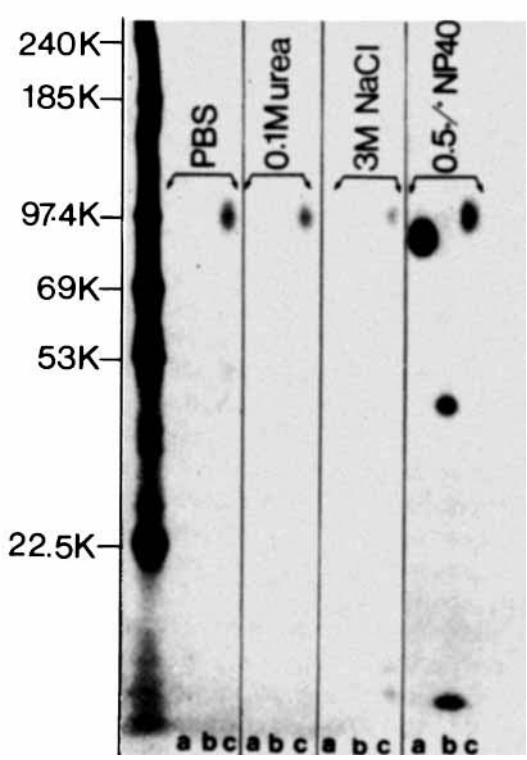

Fig. 3. Solubilization of melanoma plasma membrane-associated gp87, HLA-A,B,C antigens, and "94K" antigen with various dissociation agents. ^{125}I-surface-labeled CaCL 78-1 melanoma cells were extracted with PBS, 0.1 M Urea, 3 M NaCl, or 0.5% NP-40 for 45 min at 37°C. After dialysis of extracts containing urea or NaCl overnight against PBS, equal parts of extracts were immunoprecipitated with MoAb 140.240 (lane a), MoAb MAS 032 (lane b), and MoAb 376.96S (lane c). Immunoprecipitates were electrophoresed in a 7.5–15% polyacrylamide gradient SDS-slab gel. The gel was processed by scintillation autoradiography. Molecular weight markers: (methyl-^{14}C)-methylated filamin (240,000), α_2-macroglobulin (185,000), gammaglobulin (150,000 with subunits of 53,000 and 22,500), phosphorylase B (97,400), and bovine serum albumin (69,000) purchased from New England Nuclear.

form seem to restrict the efficacy of such conventional purification procedures. An additional advantage of the glutaraldehyde-insolubilized monoclonal antibody (ascites) is that it may be reused several times as an effective immunoadsorbent following extensive washing after each use. The spent medium was chosen as a source of starting material because gp87 was shed by melanoma cells [8–11] and because the spent medium compared to membrane preparations is much less complex in terms of protein compositions. We detected a reasonably high antigenic activity in the spent medium with an AD_{50} of 16.5 μg/100 μl antibody; this figure is comparable to what we have previously obtained (10–20 μg) with shed material recovered from a number of melanoma cell lines [8]. Having sequentially passed the spent medium concentrate on the two differently prepared MoAb 140.240 immunoadsorbents, we have achieved a remarkably high antigen recovery (ie, over 90%). We chose glutaraldehyde-treated ascites fluid of MoAb 140.240 for the first immunoadsorbent because a relatively

large quantity of the starting material (55.5 mg) was available [25,26]. This step eliminated 95% of the contaminant proteins (Table I). The predominant protein of the four distinct bands seen in the bound material recovered from this immunoadsorbent appears to be albumin (Fig. 2A, lane b). Two possibilities for the inability of the immunoadsorbent to eliminate albumin component may be considered. First, a high content of albumin-rich material contained in the spent medium might have overloaded the immunoadsorbent despite the fact that melanoma cells had been washed and then incubated with FCS-free medium for 72 hr prior to collection of spent culture fluid. Second, the ascites fluid used as the source of antibody in the immunoadsorbent might have contained antibodies to FCS, which is rich in albumin, as the MoAb 140.240–secreting hybridoma cells had been grown in 10% FCS containing medium before they were used for injection into pristane-primed Balb/c mice. We have obtained preliminary results to indicate that ascites fluid of MoAb 140.240 resulting from direct injection of hybridoma cells maintained in culture containing FCS is able to immunoprecipitate the albumin component of ^{125}I-labelled FCS, whereas ascites fluids obtained from animals injected with hybridoma cells that had been serially passaged through the animal reduced the ability to detect the albumin component (T. Kanamaru and S.-K. Liao, unpublished results). This finding tends to support the second possibility.

To avoid any anti-FCS reactivity in MoAB 140.240 and to more effectively isolate the antigen from the small quantity of antigen-containing preparation available (3.89 mg), we decided to use an immunoadsorbent constructed by coupling IgG2a fraction purified from MoAb 140.240 (culture fluid), which is known not to contain anti-FCS reactivity, as the second step of purification. This step was highly effective in purifying the antigen from the bound material recovered from step 1 immunoadsorbent. The isolated gp87 was obtained in a high degree of purity (a single band in SDS-PAGE analysis) and a high yield (90.4% recovery) in the bound fraction of step 2 immunoadsorbent. Thus the technique described is capable of rapidly preparing milligram quantities of this antigen. The use of this technique should aid in defining the further structural and functional properties of gp87.

Analysis of the amino acid composition in the hydrolysate of the purified gp87 showed a wide range of amino acids with a particularly high content of phenylalanine. The amino acid composition of gp87 documented here will be useful to compare with that of gp87 purified from melanoma cell membrane, normal melanocytes, or fetal tissues, as well as those of possibly identical antigens identified by other laboratories.

Among the human melanoma-associated antigens so far detected by murine monoclonal antibodies, the 97,000 molecular weight cell membrane glycoprotein (p97) described by Brown et al [5–7,27,28] is probably the best characterized. p97 is related to transferrin and lactoferrin as revealed by its partial amino acid sequence [5,6]. Like members of the transferrin family, p97 is capable of binding iron [6], and the gene for p97 is known to localize in chromosome 3 [7]. Using sequential immunoprecipitation and blocking experiments with different monoclonal antibodies, Brown et al [6] have shown that gp95, a melanoma-associated antigen described by Dippold et al [29] is identical to p97. We have taken a similar approach to determine whether or not gp87 defined by MoAb 140.240 and p97 defined by MoAB 95.6 obtained from the Hellströms' group are also identical using melanoma cells as the antigen source. We have found that gp87 and p97 are the same molecules identified by these two antibodies through recognizing two distinct epitopes (M.J. Khosravi,

J.P. Brown, and S.-K. Liao, submitted for publication). Thus gp87, gp97, and gp95 independently described by the three laboratories with their own monoclonal antibodies are in fact structurally identical. It is noteworthy, however, that different monoclonal antibodies capable of immunoprecipitating a same cell surface antigen may not necessarily give similar binding patterns when a panel of various cell types are simultaneously tested [3]. For instance, our serological studies by direct binding and adsorption analysis have clearly revealed that, though both MoAb 140.240 and MoAb 96.5 react strongly with 6/6 melanoma cell lines, MoAb 96.5 (but not MoAb 140.240) also reacts with 5/9 nonmelanoma cell lines tested. It is therefore important to select an antibody or antibodies, from available monoclonal antibodies, that are directed to the same cell surface molecule for the best suitability of a specific purpose, eg, immunohistochemical screening, development of sensitive immunoassays for antigen quantitation, preparation of immunotoxins or tumor imaging reagents.

To characterize gp87 further in terms of its physical association with plasma membranes of malignant melanoma cells, we showed that gp87 was different from the documented peripheral membrane protein 94K, but was not unlike the well known integral HLA-A,B,C antigens with respect to susceptibility to extraction by PBS, 0.1 M urea, 3 M NaCl, or NP-40 (Fig. 3). These results and our recent demonstration of an inability to extract gp87 and HLA-A,B,C antigens by noncytolytic concentrations of 1-butanol [30], which has been previously shown to solubilize "peripheral" rather than "integral" membrane proteins [31,32], strongly suggest that gp87 is an integral membrane component. A more sophisticated analytical approach using photoreactive probes [33] may be required to confirm this contention. In view of the relatively firm membrane association of gp87, and its expression on the membrane of melanoma cells [8,10], gp87 may be released by the process of membrane shedding such as that described for histocompatibility antigens [34]. A process similar to the antigen shedding from cultured melanoma cells may well operate in vivo. Though the biological significance of shedding of gp87 from melanoma cells remains unclear, quantitative measurement of this antigen level in sera of melanoma patients may have direct clinical applications in the diagnosis and prognosis of malignant melanoma. Finally, the highly purified antigen described in this paper will be useful in the development of highly sensitive serological assays for antigen or antibody detection in clinical specimens.

ACKNOWLEDGMENTS

This study was supported by a grant (MA 5429) from the Medical Research Council of Canada to S.-K.L. Dr. S.-K. Liao is an Associate of the Ontario Cancer Treatment and Research Foundation. Dr. M.J. Khosravi is the recipient of a Leukemia Research Scholarship from Leukemia Research Fund, Toronto. We thank Mr. J. Smith and Mrs. A. Fenyes for technical assistance. Thanks are also due to Dr. S. Ferrone for generously providing us with the monoclonal antibody 376.96S to study.

REFERENCES

1. Dent PB, Liao S-K: In Reisfeld RA, Ferrone S (eds): "Melanoma Antigens and Antibodies." New York: Plenum Publishing Corp, 1982, p 101.
2. Köhler G, Milstein C: Nature 256:495, 1975.

3. Dippold WG, Lloyd KO, Houghton AN, Li LTC, Ikeda H, Oettgen HF, Old LJ: In Mitchell MS, Oettgen HF (eds): "Hybridomas in Cancer, Diagnosis and Treatment." New York: Raven Press, 1982, p 173.
4. Hellström KE, Hellström I, Brown JP: Springer Semin Immunopathol 5:127, 1982.
5. Brown, JP, Hewick RM, Hellström I, Hellström KE, Doolittle RF, Dreyer WJ: Nature 296:171, 1982.
6. Brown JP, Nishiyama K, Hellström I, Hellström KE: J Immunol 127:539, 1981.
7. Plowman Gd, Brown JP, Enns CA, Shroder J, Nikinmaa B, Sussaman HH, Hellström KE, Hellström I: Nature 303:70, 1983.
8. Liao S-K, Clarke BJ, Khosravi M, Kwong PC, Brickenden A, Dent PB: Int J Cancer 30:573, 1982.
9. Liao S-K, Gallie BL: Invest Ophthalmol Vision Sci 24 (Suppl):295, 1983.
10. Liao S-K, Khosravi MJ, Kwong PC, Dent PB: In Cohen E, Singal DP (eds): "Non-HLA Antigens in Health, Aging and Malignancy." New York: Alan R. Liss, Inc., 1983, p 125.
11. Khosravi JM, Dent PB, Liao S-K: Int J Cancer 35:73–80, 1985.
12. Dent PB, Cleland GB, Liao S-K: Cancer Immunol Immunother 8:27, 1980.
13. Barnstable CJ, Bodmer WF, Brown G, Galfie G, Milstein C, Williams AF, Ziegler A: Cell 14:9, 1978.
14. Imai K, Wilson BS, Bigott A, Natali PG, Ferrone S: JNCI 68:761, 1982.
15. Liao S-K, Rahman AFR, Kwong PC, Dent PB: J Immunol Methods 27:111, 1979.
16. Ey PL, Prowse SJ, Jenkin CR: Immunochemistry 15:429, 1978.
17. Liao S-K, Kwong PC, Dent PB, Clarke BJ: J Immunoassay 2:227, 1981.
18. Khosravi M, Liao S-K, Thomson DMP, Dent PB: Transplantation 35:258, 1983.
19. Markwell MAK, Fox CF: Biochemistry 17:4807, 1978.
20. Laemmli UK: Nature 227:680, 1970.
21. Bonner WM, Laskey RA: Eur J Biochem 46:83, 1974.
22. Singer SL, Nicolson GL: Science 175:720, 1972.
23. Wilson BS, Imai K, Natali P-G, Kay NE, Cavaliere R, Pellegrino MA, Ferrone S: In Reisfeld RA, Ferrone S (eds): "Melanoma Antigens and Antibodies." New York: Plenum Press, 1982, p 401.
24. Owen MJ, Kissonerghis AM, Lodish HF: J Biol Chem 255:9678, 1980.
25. Jaton J-C, Brandt DC, Vassalli P: In Lefkovits I, Pernis B (eds): "Immunological Methods." New York: Academic Press, 1979, p 43.
26. Ternynck T, Avrameas S: In Ruoslahti E (ed): "Immunoadsorbents in Protein Purification." Baltimore: University Park Press, 1976, p 29.
27. Brown JP, Woodbury RG, Hart CE, Hellström I, Hellström KE: Proc Natl Acad Sci USA 78:539, 1981.
28. Brown JP, Wright PW, Hart CE, Woodbury RG, Hellström KE, Hellström I: J Biol Chem 235:4980, 1980.
29. Dippold NG, Lloyd RO, Li LTC, Ikeda H, Oettgen HF, Old LJ: Proc Natl Acad Sci USA 77:6114, 1980.
30. Liao S-K, Smith JW, Kwong PC: Cancer Immunother 17:95, 1984.
31. Legrue SJ, Allison JP, Macek CM, Pellis NR, Kahan BD: Cancer Res 41:3956, 1981.
32. Price MR, Campbell DG, Robins RA, Baldwin RW: Eur J Cancer Clin Oncol 19:81, 1980.
33. Leblanc P, Capone J, Gerber GE: J Biol Chem 257:14586, 1982.
34. Plesser YM, Markson Y, Weiss DW, Brautbar C, Doljanski F: Cell Mol Biol 29:227, 1983.