Eur J Cancer Clin Oncol, Vol. 20, No. 9, pp. 1163–1175, 1984.
Printed in Great Britain.

0277-5379/84$03.00+0.00

Purification of Melanoma-associated Oncofetal Antigen gp87 from Spent Medium and Cell Homogenate of Cultured Human Melanoma Cells*

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Abstract—*A melanoma-associated glycoprotein with an apparent molecular weight of* 87,000 daltons (*gp87*) *defined by the monoclonal antibody* 140.240 *has been purified from the spent medium and from cell homogenates of cultured human melanoma cells through a three-step purification procedure. The procedure involves a DEAE-Sephadex A-25 ion-exchange column, Sephacryl S-200 gel filtration and antibody-Sepharose-4B-affinity chromatography. By this approach we achieved a* 1205*-fold increase in specific activity with a* 42% *antigen recovery from spent medium concentrate, and a* 3366*-fold increase in specific activity with a* 29% *antigen recovery from the cell homogenate. The antigen was purified from these two sources of starting material with a high degree of purity as visualized in a single protein band in SDS-polyacrylamide gel electrophoresis. Thus these procedures described offer an efficient approach to the purification of gp87 molecules for further biochemical studies.*

INTRODUCTION

A VARIETY of serological techniques have been used to demonstrate the existence of tumor-associated antigens in human malignant melanoma. The information generated from serological dissection of the patients' sera suggested that melanoma cells acquire both cytoplasmic as well as plasma membrane-bound neoantigens that do not appear to be expressed by normal tissues. Studies with conventional xenoantisera have led to the identification of various melanoma-associated antigens (MAAs) with diverse specificities [1, 2]. With a few exceptions, the molecular nature of these antigens detected remains unknown. The recent advancement in the production of monoclonal antibodies has facilitated the identification and characterization of MAAs and has confirmed the antigenic diversity of this malignant tumor. None of the antigens identified by this approach has been proven to be absolutely specific for melanomas, although several were found to be present in large amounts on most or all melanomas but only in trace amounts in non-melanoma cell types [3–9]. Availability of sufficient quantity of highly purified molecules is essential for structural and functional properties of antigens involved.

Recently a murine monoclonal antibody, designated MoAb 140.240, has been raised in our laboratory against human melanoma cells [10, 11]. In our initial studies this antibody reacts with virtually all melanomas and some fetal tissues, but does not react with a wide variety of other neoplastic or adult normal tissues. Subsequent serological testings on three highly pure normal human melanocyte cultures derived from different newborn foreskins have indicated that a proportion (10–30%) of these cells were also reactive with MoAb 140.240. We have identified the structure bearing the epitope recognized by antibody 140.240 to be an 87,000-dalton (87-kd) molecule detectable in melanoma cells and fetal intestine tissues. Immunochemical and bio-

Accepted 20 March 1984.
*This investigation was supported by a grant (MA5429) from the Medical Research Council of Canada to S.-K. Liao.
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chemical studies with cultured melanoma cells have shown that the antigen is a single-chain polypeptide which is initially synthesized in a form of a 77-kd precursor molecule [11, 12]. This precursor is rapidly converted to an intermediate 83-kd glycoprotein (gp83) by *N*-asparagine-linked glycosylation. The oligosaccharide units of gp83 are replaced by complex carbohydrates that contain as much as 2% sialic acid residues resulting in fully glycosylated polypeptide, 87-kd molecule (gp87). Thus it is more appropriate to term this molecule as gp87, a melanoma-associated oncofetal (differentiation) antigen. We have also demonstrated that the antigen is detectable in material spontaneously shed by cultured melanoma cells [10, 11]. In this report we describe purification of gp87 from cultured melanoma cells as well as from the spent medium of these cells by an approach involving ion-exchange, gel filtration and antibody-affinity chromatography.

MATERIALS AND METHODS

Cells

The human melanoma cell line CaCL 78-1 [13], established in our laboratory, and the colon carcinoma cell line HT-29 obtained from Dr. W. E. Rawls, McMaster University, Hamilton, Ontario, were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) without antibiotics at 37°C in a 100% humidified atmosphere of 5% CO_2 and 95% air. The culture was shown to be free of mycoplasma by monthly cytochemical DNA staining [14].

Monoclonal antibody

The MoAb 140.240 with an IgG2a isotype is the secretory product of a hybridoma constructed by fusion of the mouse myeloma cells, P3-NS1/1-Ag-4, and splenocytes from a BALB/c mouse immunized with CaCL 78-1 melanoma cells as described previously [10, 15]. The serological specificity, structural characterization and biosynthesis of the antigen (gp87) bearing the epitope identified by this monoclonal antibody have been described elsewhere [11, 12]. This antibody also reacted with 10–30% cultured normal melanocytes.

Serological assay

The mixed hemadsorption assay was performed as described elsewhere [10, 16]. Sufficient target cells (CaCL 78-1) were plated to yield 100 cells/well after overnight incubation in 60-well microtiter plates. Ten microliters of hybridoma supernatant was added to each well and the plates incubated for 2 hr at room temperature. After three washes with phosphate-buffered saline (PBS), pH 7.4, containing 0.2% gelatin, 10 μl of indicator sheep red blood cells (SRBC) were added and the plate was incubated for 1 hr at room temperature. Indicator SRBC were prepared using appropriate concentrations of mouse anti-SRBC followed by goat anti-mouse IgG antiserum. After careful washing the plates were flooded with 0.25% glutaraldehyde to stabilize the rosettes for subsequent scoring. Target cells were registered as positive if five or more SRBC were adherent to the test target cell surface. The highest dilution at which at least 10% of the target cells were positive was taken as the endpoint titer.

Preparation of shed material from spent medium

Monolayer culture cells were prepared by seeding approximately 5×10^6 cells in 40 ml of minimum essential medium plus 10% FCS in each of the 600-ml culture flasks, each with a culture surface area of 175 cm^2 (A/S NUNC, Roskile, Denmark). When cultures reached subconfluency the medium was discarded and cells were washed twice with sterile PBS (pH 7.4). Thirty-five milliliters of fresh medium without FCS was then added to each flask and incubated at 37°C for 72 hr. The spent medium was then collected and centrifuged at 10,000 *g* for 20 min. The cell-free supernatant (7000 ml) was concentrated over 80-fold in an Amicon ultrafiltration (Amicon Corp., Lexington, MA.) using an XM50 membrane (50,000 molecular-weight cutoff) and a stainless steel reservoir model RS4. The concentrate was centrifuged in a B60 International Preparative Ultracentrifuge (IEC, Needham HTS, MASS.) at 136,000 *g* for 1 hr. The clear supernatant was collected and dialyzed against PBS containing 0.02% sodium azide.

Quantitative absorption test

Fractions were tested for antigenic activity by quantitative absorption tests using MoAb 140.240. The working dilution of the antibody was the concentration two doubling dilutions above 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 was brought up to 100 μl with PBS containing 0.2% gelatin, and the mixtures incubated for 1 hr at room temperature and overnight at 4°C. Ten microliters of each mixture was then transferred into duplicate wells of a prewashed plate of CaCL 78-1 target cells and the residual antibody activity was measured by the mixed hemadsorption assay. The percentage inhibition of antibody reactivity relative to the unabsorbed control was plotted on an arithmetic scale against the amount of material used for absorption on a logarithmic scale. The amount of

absorbing material required to reduce 50% of antibody reactivity was calculated from the inhibition curve and was termed the absorption dose 50 (AD_{50}) [10].

Solubilization of melanoma cell membrane

About 1×10^9 cells were dispersed from confluent monolayer cultures grown in 20 flasks (175 cm^2 culture surface area/flask) by trypsin (0.025%). The cells were washed in PBS and resuspended to a concentration of 5×10^6 cells/ml in 200 ml of PBS (pH 7.4) containing 0.25 M sucrose and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and homogenized as described previously [17]. The cell homogenate was centrifuged at 20,000 *g* for 20 min to remove cell organelles, and to the supernatant was added Nonident P-40 (NP-40) with constant stirring to a final concentration of 0.5%. The mixture was stirred for 30 min at 4°C and centrifuged at 136,000 *g* for 1 hr, and the supernatant was subjected to ammonium sulfate precipitation.

Ammonium sulfate precipitation

Sufficient quantity of saturated ammonium sulfate solution was slowly added to the supernatant with constant stirring until a concentration of 50% was reached. Stirring, while in an ice bucket, was continued for 10 min, and the mixture was allowed to stand for 30 min. The precipitated proteins were recovered by centrifugation at 20,000 *g* for 15 min and dissolved in 45 ml of 0.1 M Tris–HCl buffer (pH 7.4) containing 0.005% NP-40 and 0.02% sodium azide. The suspension was then dialyzed against several changes of the same buffer and stored at −70°C before use.

DEAE-ion exhange chromatography

Concentrated shed material and the ammonium sulfate precipitated protein fraction of the cell membrane preparation were separately applied to DEAE–Sephadex A-25 columns (2.6 × 35 cm) previously equilibrated with the Tris–HCl buffer. The columns were washed with Tris–HCl buffer. Unbound materials were collected and bound components were eluted in fractions of 4–5 ml with the use of a continuous gradient of 0.005–0.5 M NaCl made in the same buffer at a flow rate of 15 ml/hr. Protein profiles were monitored by optical density measurement at 280 nm in a Beckman DB-G spectrophotometer. Fractions comprising each protein peak were pooled, concentrated by Amicon ultrafiltration and dialyzed against PBS (pH 7.4) containing 0.02% sodium azide. Each pooled fraction was then tested for antigenic activity by quantitative absorption analysis using MoAb 140.240.

Gel-filtration chromatography

The antigen-containing fractions resulting from DEAE–Sephadex A-25 columns were dialyzed against PBS (pH 7.4) containing 0.005% NP-40 and 0.02% sodium azide and then applied to Sephacryl S-200 columns previously equilibrated with the PBS. Elution was carried out with the same buffer at a downward flow rate of about 20 ml/hr, and 4- and 5-ml fractions were collected for shed and cellular samples respectively. Blue dextran 2000 was used for determination of void volume (VO), and Pharmacia gel filtration molecular weight standards were used to calibrate the columns. Antigen-containing fractions were identified by qualitative absorption analysis using MoAb 140.240.

Antibody-affinity chromatography

The IgG2a [18] of spent medium of a cultured hybridoma clone (140.240 6P1-4P1) producing MoAb 140.240 was prepared by fractionation on protein A–Sepharose CL-4B as described by Ey *et al.* [19]. Briefly, 500 ml of the spent medium 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% 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 profiles were determined by optical density measurement at 280 nm. Fractions pooled from each peak were concentrated and analyzed for binding activity to CaCL 78-1 target cells by the mixed hemadsorption assay. The IgG2a fraction was concentrated in the immunoglobulin peak eluted with citrate buffer, pH 5.0. The immunoglobulin fraction (25 mg protein) was subsequently coupled to CNBr-activated Sepharose 4B as described elsewhere [20]. The Sepharose 4B–IgG2a beads were then packed into a column (5 × 1.6 cm) and equilibrated with PBS at 4°C. Fractions containing gp87 antigenic activity recovered from the Sephacryl S-200 columns were pooled, concentrated by Amicon ultrafiltration using an XM50 membrane, and applied to the immunoadsorbent column. The column was allowed to stand for 1 hr at room temperature followed by 1 hr at 4°C. Unbound material was collected and the column was washed extensively with 50 column-volumes of PBS (pH 7.4) containing 0.005% NP-40 and 0.02% sodium azide. The bound material was eluted from the column with 3M KSCN, dialyzed immediately against three changes of PBS, and concentrated by Amicon ultrafiltration using XM50 membrane. The unbound fraction was treated in a similar manner.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide slab gels (1.5-mm thick) were prepared according to the method of Laemmli [21]. Samples were analyzed under reducing condition by electrophoresis on gradient gels, 7.5–15% (w/v) polyacrylamide, at a current of 10 mA per slab for 18 hr. After electrophoresis, 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 25°C overnight. The stained gels were destained with 37% methanol in 10% acetic acid for 6 hr and then incubated in acetic acid:methanol:water (1:1:8) until the background became clear. For molecular weight determination, Pharmacia low molecular weight standards were used.

Protein concentration

Protein content was determined by the BIO-RAD assay using bovine serum albumin as a standard.

RESULTS

For purification of cellular and shed gp87, CaCL 78-1 melanoma cells and the spent culture medium were used as two separate sources of starting material. Purifications were performed according to the protocols outlined in Fig. 1. The spent medium concentrate (Table 1) and the melanoma cell membrane lysate (Table 2) were found to contain the antigen activity as determined by their ability to inhibit binding of MoAb 140.240 to CaCL 78-1 melanoma target cells. The amounts of protein required to reduce

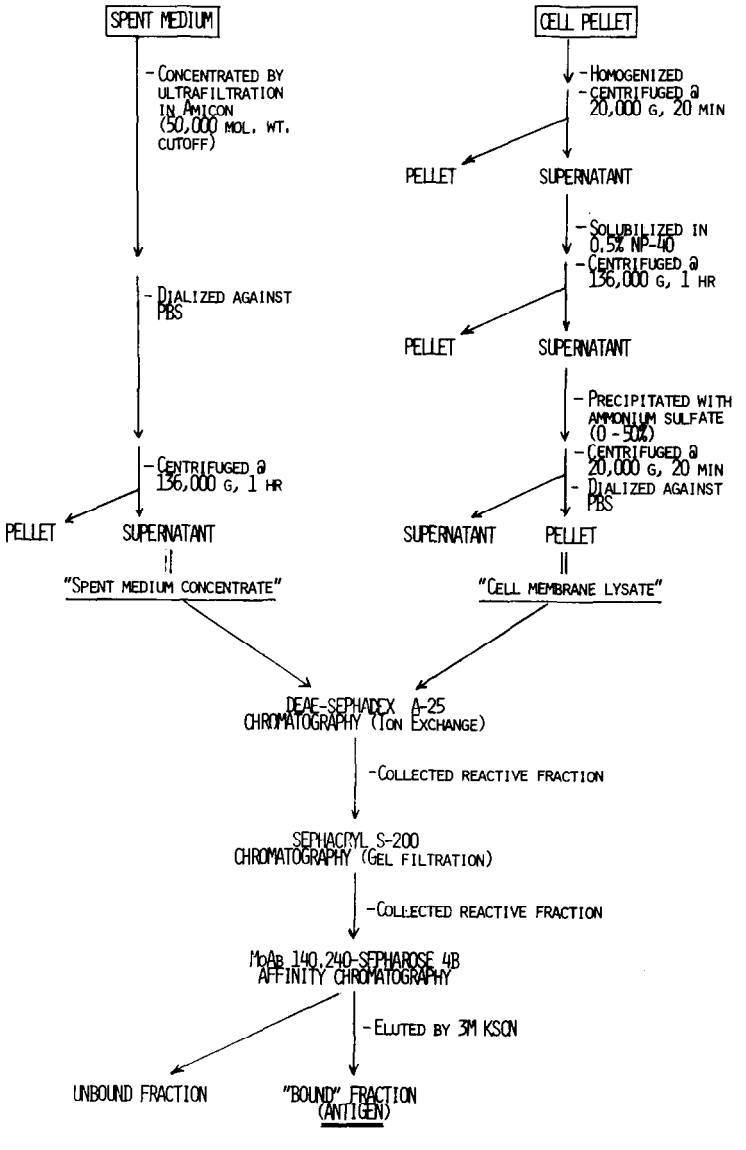

Fig. 1. Flow chart of antigen purification steps used in this study.

Table 1. Distribution of gp87 antigenic activity following DEAE-Sephadex A-25 ion-exchange chromatography of spent medium concentrate of CaCL 78-1 melanoma cell culture

Fraction	% of inhibition of MoAb 140.240 reactivity* by material in fractions tested in following amounts (μg)†						
	14	28	56	155	270	306	AD_{50} (μg)‡
Melanoma cells							
CaCL 78-1							
Spent medium concentrate	NT§	10	47	100	NT	NT	60
DEAE-Sephadex							
Peak 1	NT	0	0	0	0	NT	⩾270
Peak 2	32	95	99	100	NT	NT	17
Peak 3	0	2	13	44	85	NT	158
Peak 4	NT	0	0	34	NT	84	190
Peak 5	0	0	0	NT	NT	NT	⩾56
Carcinoma cells							
HT-29							
Spent medium concentrate	0	0	1	0	0	1	⩾306

*Tested against CaCL 78-1 (immunizing line) in the mixed hemadsorption assay.
†Absorption was carried out with 10 μl of antibody (1:16) and 90 μl of serially diluted absorbing material, giving a final antibody dilution of 1:160.
‡Amount of protein required to inhibit 50% of antibody (100 μl, 1:160 dilution) binding activity.
§Not tested.

the reactivity of 100 μl of MoAb 140.240 by 50% (AD_{50}) were found to be 60 μg for the spent medium concentrate and 168 μg for the cell membrane lysate. Absorptions with the spent medium or the cell homogenate prepared from the colon carcinoma cells (HT-29) failed to reduce antibody reactivity to the same melanoma target cells as observed previously [10].

DEAE-Sephadex A-25 chromatography

The spent medium concentrate and the melanoma cell membrane lysate were applied to DEAE-Sephadex A-25 columns. Unretarded material was collected and the bound components were eluted with a continuous gradient of 0.005–0.5 M NaCl made in the Tris-HCl buffer (pH 7.4). Figure 2A illustrates the elution profile

Table 2. Distribution of gp87 antigenic activity following DEAE-Sephadex A-25 ion-exchange chromatography of ammonium sulfate precipitate of CaCL 78-1 melanoma cell membrane detergent lysate

Fraction	% of inhibition of MoAb 140.240 reactivity* by material in fractions tested in following amounts (μg)†						
	7	14	28	56	168	336	AD_{50} (μg)‡
Melanoma cells							
CaCL 78-1							
Homogenate	0	0	0	0	20	55.9	300
Ammonium sulphate (0–50%) precipitate	0	0	3	12	50	81	168
DEAE-Sephadex							
Peak 1	NT§	NT	0	0	0	0	⩾336
Peak 2	10.2	29	55	83	100	NT	25
Peak 3	NT	0	0	0	0	NT	⩾168
Peak 4	NT	0	0	0	0	NT	⩾168
Peak 5	0	0	0	0	NT	NT	⩾56
Peak 6	0	0	0	0	0	NT	⩾168
Carcinoma cells							
HT-29							
Homogenate	0	1	0	0	2	0	⩾336
Ammonium sulfate (0–50%) precipitate	0	0	1	2	1	1	⩾336

*†‡§Detailed as in Table 1.

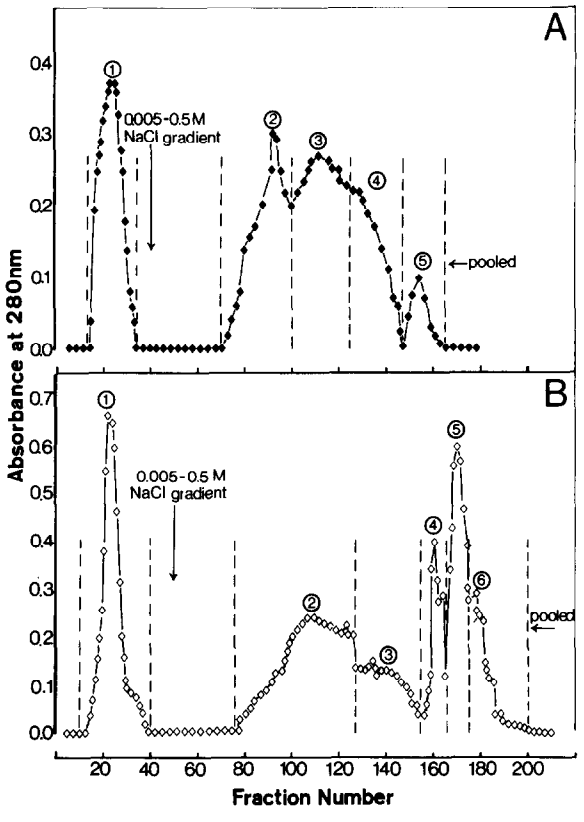

Fig. 2. Ion-exchange chromatography of shed (A) and cellular (B) gp87 on a DEAE-Sephadex A-25 column. Spent medium concentrate of the cells and ammonium sulfate-precipitated protein fraction from NP-40 (0.5%) lysate of crude CaCL 78-1 melanoma cell membrane preparations were separately applied onto columns (2.6 × 35 cm) in 0.1 M Tris-HCl buffer (pH 7.4) containing 0.02% sodium azide and 0.005% NP-40. The fraction numbers at which 0.005–0.5 M NaCl was applied are where the arrows indicate. Fractions comprising each protein peak were pooled as indicated and concentrated to approximately 5–10 ml. Following dialysis against PBS (pH 7.4) containing 0.02% sodium azide and 0.005% NP-40, peak fractions were tested for antigenic activity by quantitative absorption analysis of MoAb 140.240 to CaCL 78-1 melanoma target cells. Antigenic activity was predominantly concentrated in peak 2 for both (A) and (B).

of the spent medium concentrate. Five distinct peaks of protein were detected. Fractions comprising these peaks were pooled, concentrated by Amicon ultrafiltration and dialyzed against PBS (pH 7.4). Each peak fraction was tested for antigenic activity by quantitative absorption of MoAb 140.240. As shown in Table 1, the antigenic activity was predominantly concentrated in peak 2 (AD_{50}, 17 vs 60 μg unfractionated), which was the protein eluted immediately after the application of the salt gradient (Fig. 2A). The AD_{50} of the material in peaks 1 (unbound material), 3, 4 and 5 were found to be in the order of ⩾270 μg, 158 μg, 190 μg and ⩾56 μg respectively.

Ion-exchange chromatography of the cell membrane lysate resulted in the separation of the material into six distinct protein peaks (Fig. 2B). The antigenic activity was concentrated exclusively in peak 2 (AD_{50}, 25 vs 168 μg unfractionated), which was eluted after the application of the salt gradient (Table 2). No appreciable antigenic activity could be detected in the unbound material or in any of the remaining five peaks.

Sephacryl S-200 gel filtration

The antigenic material recovered in peak 2 following DEAE-Sephadex ion-exchange chromatography of each preparation was concentrated, dialyzed and then applied to a precalibrated S-200 column. Figure 3A shows

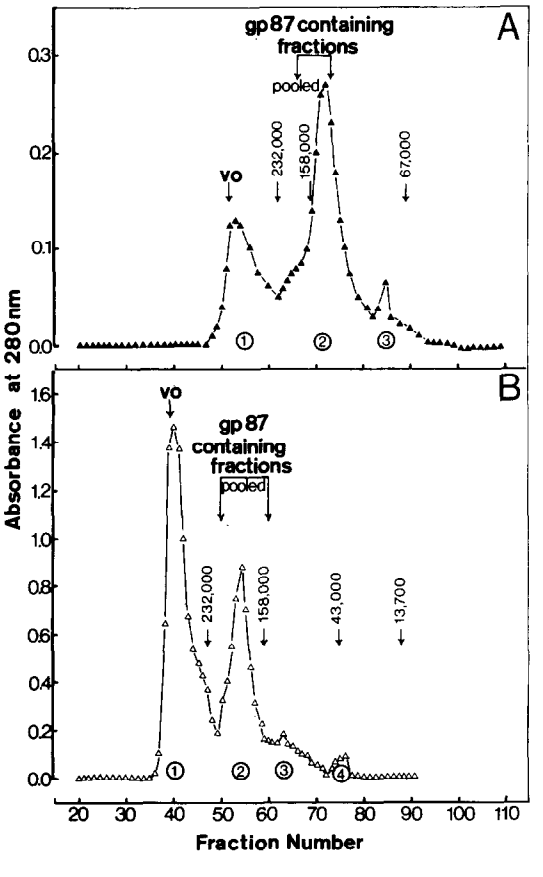

Fig. 3. Gel-filtration chromatography of shed (A) and cellular (B) gp87 on separate Sephacryl S-200 columns (2.6 × 86 cm for A and 2.6 × 89 cm for B). The antigenic material recovered in peak 2 (5 ml of shed sample and 10 ml of cellular sample) of the DEAE-Sephadex columns were passed through Sephacryl S-200 equilibrated with PBS (pH 7.4) containing 0.02% sodium azide and 0.005% NP-40. The gel filtration conditions are detailed in Materials and Methods. Fractions were tested for antigenic activity by quantitative absorption analysis of MoAb 140.240 to CaCL 78-1 melanoma target cells. Shed and cellular gp87 were eluted at a region corresponding to an approximate molecular weight of 158,000 daltons as indicated. Molecular weight standards include catalase (232,000 daltons), aldolase (158,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons) and ribonuclease A (13,700 daltons). VO = void volume, determined by using blue dextran 2000.

the elution profile of Sephacryl S-200 column through which the spent medium concentrate recovered from the DEAE-Sephadex A-25 column was passed. In addition to the material peaked at the void volume (VO) (peak 1), two distinct protein peaks were also found in the region corresponding to molecular weights of approximately 158 (peak 2) and 67 kd (peak 3) respectively. Fractions comprising these peaks were tested for antigenic activity by their ability to inhibit binding of MoAb 140.240 to CaCL 78-1 melanoma cells in a quantitative fashion. The antigenic material in peak 2 (fractions 66–73) eluted in the region of the aldolase marker protein (molecular weight 158 kd).

In the elution profile of Sephacryl S-200 gel to which melanoma cell membrane lysate recovered in peak 2 of the DEAE-Sephadex column was applied (Fig. 3B), two major (peaks 1 and 2) and two minor (peaks 3 and 4) protein peaks were observed. The antigenic activity was also localized in peak 2 (fractions 50–60) eluted in the region of the 158-kd marker protein.

Antibody-affinity chromatography

The antibody-reactive fractions of spent medium concentrate eluted from the Sephacryl S-200 column were pooled and applied to an immunoadsorbent column constructed by coupling the purified IgG2a of MoAb 140.240 to CNBr-activated Sepharose 4B. Unbound material was removed by washing the column with PBS, and bound material was eluted with 3 M KSCN and dialyzed against PBS (pH 7.4). The antigenic activity was found exclusively in the bound material (AD_{50}, 0.05 vs $\geqslant$155 μg unbound) as determined by quantitative absorption analysis using MoAb 140.240.

When antigenic material recovered in Sephacryl S-200 fractions 50–60 from the cell membrane lysate (i.e. DEAE-Sephadex peak 2) was chromatographed on the immunoadsorbent column the activity was found only in the bound material (AD_{50} of 0.09 μg for bound material vs AD_{50} of $\geqslant$56 μg for the unbound).

The results of sequential purification of shed and cellular gp87 are given in Tables 3 and 4 respectively. The antigenic activity of the spent medium concentrate was enriched by a factor of 3.5 with 49% recovery in material eluted in peak 2 of the DEAE-Sephaex column (Table 3). The further fractionation of this material by Sephacryl S-200 gel filtration resulted in a 19.4-fold increase in specific activity with 48% recovery of the antigen in peak 2 (fractions 66–73) as compared with the original spent medium concentrate. Affinity chromatography of the antigen-containing fractions 66 to 73 from the Sephacryl S-200 column on the MoAb 140.240-immunoadsorbent enriched the antigen by 1205-fold in the bound material with 42% recovery of the original antigenic activity. Similarly, the antigenic activity of melanoma cell homogenate was enriched by 3366-fold with 29% recovery in the material bound to the immunoadsorbent column (Table 4).

The analytical SDS-polyacrylamide gel electrophoretic patterns of the various antigen-containing fractions obtained at different stages of purification of shed and cellular gp87 are shown in Figs 4(B) and 4(A) respectively. While the spent medium concentrate, DEAE-Sephadex A-25 peak 2 and the Sephacryl S-200 fraction (fractions 66–73) contained a variety of Coomassie brilliant blue-stained bands, the purified gp87 band was evident in the bound fraction eluted

Table 3. Summarized results on purification of gp87 from spent medium concentrate of CaCL 78-1 melanoma cell culture by three-step procedure

Isolation step	Volume (ml)	Total protein recovered (mg)	AD_{50} (μg)*	Specific activity (units/mg)†	Purification factor	% yield
Spent medium concentrate	85	289	60	16.6	1.0	100
DEAE-Sephadex chromatography (peak 2)	5	40	17	58.8	3.5	49
Sephacryl S-200 chromatography (fractions 66–73)	24	7.2	3.1	322.5	19.4	48
MoAb 140.240 affinity chromatography (eluted material)	0.2	0.1	0.05	20,000	1204.8	42

*Amount of protein required to inhibit 50% of antibody (100 μl, 1:160 dilution) binding activity.

†The AD_{50} value is taken to represent one unit of antigenic activity. The number of units in 1 mg protein (specific activity) can be calculated, e.g. if AD_{50} is 60 μg/100 μl antibody, the specific activity will be 1000/60 = 16.6 units/mg.

Table 4. Summarized results on purification of gp87 from CaCL 78-1 melanoma cell homogenate by four sequential procedures

Isolation step	Volume (ml)	Total protein recovered (mg)	AD_{50} (μg)*	Specific activity (units/mg)†	Purification factor	% yield
Homogenate	200	1280	300	3.3	1	100
Ammonium sulfate fraction (0–50% precipitates)	45	648	168	5.9	1.8	90
DEAE-Sephadex chromatography (peak 2)	10	70	25	40.0	12	65
Sephacryl S-200 chromatography (fractions 50–60)	11.5	23	15	66.6	20.1	39
MoAb 140.240 affinity chromatography (eluted material)	0.22	0.11	0.09	11,110.9	3366	29

*†Detailed in Table 3.

from the immunoabsorbent column. The purification procedure also allowed a relatively efficient recovery of the antigen from the cell homogenate which contained considerably more protein bands as compared to the spent medium concentrate. Only the gp87 band in the material bound to the immunoadsorbent column was noted.

DISCUSSION

The isolation of highly pure cellular and shed gp87 has been achieved by a combination of ion-exchange, gel-filtration and antibody-affinity chromatography. Essentially the same strategy was used for antigen purification from cultured melanoma cells and from shed material contained in the spent culture medium, except that crude membrane fraction of the cell homogenate was solubilized by the detergent NP-40 and then saturated with ammonium sulfate for selective precipitation of proteins, thereby eliminating the high quantity of lipid present in this preparation. The purification procedure was monitored by quantitative absorption of MoAb 140.240 in the mixed hemadsorption assay.

Shed gp87 activity was predominantly concentrated in the first peak (peak 2) of DEAE-Sephadex A-25; this peak was obtained immediately after the application of the salt gradient (Fig. 2A). The further fractionation of this antigenic material by S-200 gel filtration resulted in the elution of gp87 activity in fractions 66–73, corresponding to the region of the 158-kd marker protein (Fig. 3A). The final antibody-affinity chromatography step gave a 88% recovery of the antigenic activity contained in material eluted from the immunoabsorbent column from Sephacryl antigen-containing fractions 66–73 (Table 3). The specific activity of the purified gp87 was 20,000 units/mg of protein (Table 3). As compared with the specific activity of the initial spent medium concentrate, the shed gp87 preparation represented a purification factor of approximately 1205 with a 42% recovery.

Similarly, ion-exchange chromatography of the ammonium sulfate-precipitated fraction of the melanoma crude cell membrane lysate resulted in the elution of gp87 in peak 2 that was also the first peak eluted from the column after the application of the salt gradient (Fig. 2B). The antigenic activity contained in this peak, subsequently eluted from S-200 column in fractions 50–60, was also found in the region of the 158-kd marker protein (Fig. 3B). The specific activity of purified cellular gp87 preparation recovered from S-200 fractions 50–60 in material bound to the immunoadsorbent column was 11,110 units/mg of protein (Table 4). Based on the specific activity of the initial cell homogenate, the isolation procedure achieved a purification factor of over 3300-fold with 29% recovery.

The purified cellular and shed gp87 were electrophoresed as a single narrow band on analytical SDS-polyacrylamide gels, having the usual molecular weight of approximately 87 kd (Fig. 4). These results indicate that the three-step procedure permits efficient purification of the antigen from both cultured melanoma cells and from spent medium concentrate. Because a band in the staining gels corresponding to gp87 could not be detected in the cell homogenate as well as in the concentrated shed material, or in any of the antigen-containing fractions recovered from DEAE-Sephadex and S-200 columns, it may be concluded that gp87 comprises a very small percentage of protein in the initial unfractionated materials. In this regard, gp87 is similar to a 97-kd melanoma-associated surface glycoprotein (p97), which has been reported by Brown *et al.* [3, 22] to represent less than 0.1% of protein contained in a

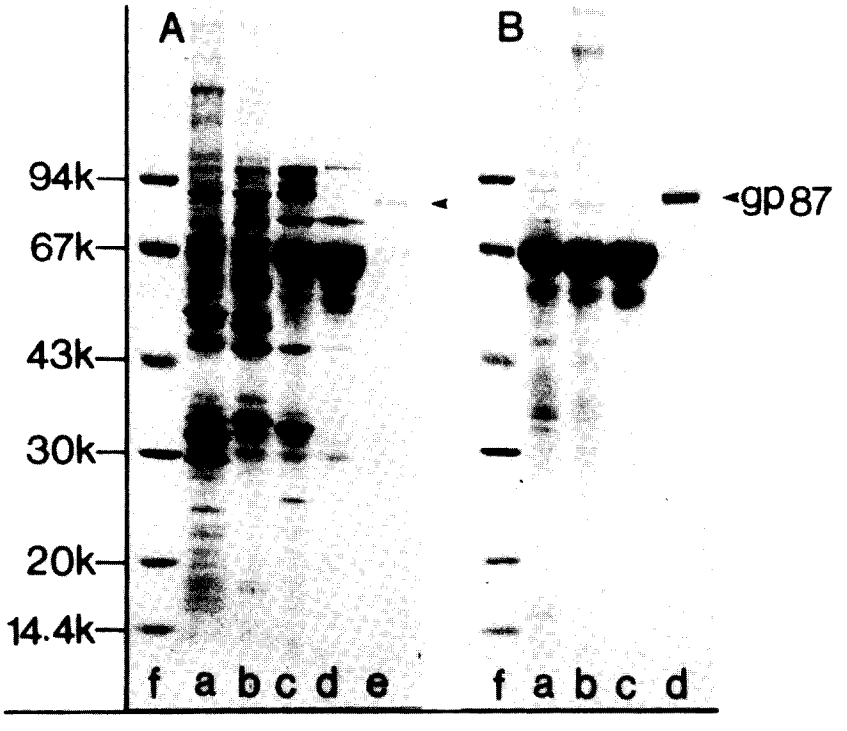

Fig. 4. Analytical SDS–polyacrylamide electrophoretic pattern of the various antigen-containing fractions at different stages of purification of cellular and shed gp87. Sample loads were approximately 200 μg, *except for the purified preparations that contained* 30 μg *of protein. Gels were* 7.5–15% *gradient slab and were stained with Coomassie brilliant blue. Samples in gel A include: CaCL* 78-1 *cell homogenate (a), ammonium sulfate-precipitated fraction of the cell membrane detergent lysate (b), material recovered in DEAE-Sephadex A25 peak* 2 *(c) and in Sephacryl S-200 fractions* 50–60 *(d), and purified cellular gp87 eluted from the antibody-affinity column (e). In gel B samples include: CaCL* 78-1 *spent medium concentrate (a), material recovered in DEAE-Sephadex peak* 2 *(b) and in Sephacryl S-200 fractions* 66–73 *(c), and purified shed gp87 eluted from the antibody-affinity column (d). Molecular weight standards (f) were: phosphorylase b* (94,000 daltons), *bovine serum albumin* (67,000 daltons), *ovalbumin* (43,000 daltons), *carbonic anhydrase* (30,000 daltons), *trypsin inhibitor* (20,000 daltons) *and alpha-lactalbumin* (14,400 daltons).

detergent lysate of a melanoma cell line. These investigators also achieved a purification factor of over 1000-fold during isolation of p97 by a two-step procedure involving antibody-affinity chromatography and preparative SDS–polyacrylamide gel electrophoresis. Several investigators have also described approaches involving a combination of gel-filtration, ion-exchange, lectin and antibody-affinity chromatography for isolation of a variety of MAAs defined by melanoma patients' sera [23–28] and by conventional xenoantisera [29–32]. Because of the great heterogeneity of melanoma-associated antigens and the fact that these MAAs have been only partially purified, it is impossible to make any conclusion with regard to the overall efficiency of the strategy reported here and those reported by other investigators. However, the main advantage of the present isolation procedure is that its application is not limited by the volume or the complexity of the initial material, and it could readily be used for antigen purification from fresh tumor tissues as well as biological fluids of melanoma patients.

The elution of cellular and shed gp87 from Sephacryl S-200 in a region of molecular weights in the approximate region of the aldolase marker protein (158 kd) suggests either that in a soluble state the gp87 molecules are linked non-covalently to form dimers, or that its apparently higher molecular weight is a reflection of the gel filtration procedure which assumes the same relationship in terms of three-dimensional configuration between the unknown and the molecular weight standards, which are globular in shape. The former possibility seems unlikely because as a glycosylated protein gp87 may be expected to migrate differently from the globular proteins used to calibrate the column.

The detailed chemical structure of gp87 remains unknown at this time. We have previously noted that shed gp87 has a slightly higher molecular weight than its cellular counterpart and that the tryptic peptide map of the shed gp87 is essentially similar to that of the cellular gp87 except that two additional methionine-containing peptides are noted in the shed gp87 [11]. These results suggest that certain biochemical modifications must occur before or during shedding. It is not known, however, whether the two additional methionine-containing peptides could account for the slight molecular weight increase in the shed form of gp87. The availability of a sufficient amount of highly purified cellular and shed gp87 molecules should aid in the determination of the possible molecular microheterogeneity that may exist between these two forms of gp87 [11] and between possibly identical antigen molecules identified by different monoclonal antibodies, and in the elucidation of the mechanism involved in the shedding of this molecule. Information on the detailed structure of gp87 should ultimately permit a comparison of gp87 expressed by melanoma cells with that found in normal melanocytes and fetal tissues, and may aid in the delineation of malignant transformation of normal melanocytes.

Acknowledgements—We would like to thank Mr Pak C. Kwong and Mrs Anna Fenyes for expert technical assistance.

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