Amphipathic Lipid-Bound Protein Antigens in Mouse Bladder Carcinomas Detected by a Monoclonal Antibody†

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ABSTRACT: We have found organic solvent soluble proteins that contain tightly bound fatty acids. They were demonstrated in mouse bladder carcinomas by a monoclonal rat antibody, 33E7C. The antigens recognized by antibody 33E7C copurified with lipids when extracted from bladder carcinomas with a mixture of chloroform–methanol. The antigens remained in the chloroform phase after solvent partition with water. Chromatographic analysis using an α-hydroxypropylated Sephadex G-50 (LH60) column led to the isolation of a group of proteins binding to antibody 33E7C. Four proteins (10K, 18K, 25K, and 40K daltons) were found on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) after radioimmunoprecipitation with antibody 33E7C. After preparative SDS–PAGE, the purified antigens were soluble in both water and a mixture of chloroform–methanol (2:1) but were freed from phospholipids and glycolipids. The antigens have been characterized by the presence of tightly bound fatty acids with the following findings: (1) They were metabolically colabeled by incubating bladder carcinoma cells with [9,10-^{3}H]palmitic acid and [^{14}C]leucine, followed by radioimmunoprecipitation assay and by SDS–PAGE. (2) After hydrolysis of proteins in NaOH–methanol, [^{3}H]palmitic acid methyl ester was separated from the 40K and 18K proteins by thin-layer chromatography.

Animal models are useful for studying immunological responses to cancer and developing new techniques for tumor diagnosis and therapy. The characterization of tumor cell surface antigens is an essential part of such studies (Old, 1981). Many cell surface antigens have been reported to undergo dramatic changes during cellular differentiation (Gooi et al., 1981; Kannagi et al., 1982, 1983; Solter & Knowles, 1978) or neoplastic transformation (Hakomori & Kannagi, 1983). The monoclonal antibody technique (Köhler & Milstein, 1975; Koprowski et al., 1978) has been used for many types of studies and has revealed proteins (Brown et al., 1980; Woodbury et al., 1980) and glycolipids (Nudelman et al., 1982, 1983; Pukel et al., 1982; Young et al., 1979) that are more strongly expressed by tumors than by normal tissues, even if small amounts of most tumor-associated antigens can be detected also in normal tissues.

Glycolipid antigens have been generally identified after extraction of cells with organic solvents. Since most proteins are insoluble in such solvents, protein antigens have generally been identified in aqueous buffers. As a result of this, little attention has been paid to hydrophobic proteins that are extractable into organic solvents in the form of chloroform–methanol mixtures. Such proteins were first described by Folch & Lees (1951). They were named proteolipids and were believed to combine with lipids in the form of a complexes with lipid-like solubility (Lees et al., 1979). Similar lipophilic or amphipathic proteins were also found to be closely associated with gangliosides (Folch et al., 1951; Rosenberg & Chargett, 1958) and the heterophile Paul–Bunnell antigen was identified as this class of protein (Watanabe et al., 1980). In view of their unusual characteristics, proteolipids may be easily missed during the conventional analysis for proteins and glycolipids. If present as tumor markers, much of their tumor-associated antigenicity may be masked by the surrounding lipids.

In this paper, we report proteins that are soluble in both organic solvents and aqueous solvents and were detected in mouse bladder tumor extracts by using a monoclonal rat antibody. These proteins were found to be tightly bound to fatty acids.

Materials and Methods

Tumors. Transitional cell bladder carcinomas induced in BALB/c mice by 3-methylcholanthrene (MCA, 1656, 1657, 1660, 1670, 1672, 1674) and by [(5-nitrofur-4-yl)-2-thiazolyl]formamide (FANFT, 1682) were used for all experiments (Hellström et al., 1982). The tumors were transplanted into syngeneic BALB/c mice. Six MCA-induced fibrosarcomas in BALB/c mice, the B16 melanoma in C57 BL/6 mouse, a spontaneous C3H mammary carcinoma, three transplanted lymphomas, a transplanted lymphosarcoma, two cultured lymphoma lines (Abelson and LSTRA), and two myelomas (SP-2/0 and NS-1) were also studied.

Membrane Preparations. Tissues were washed with phosphate-buffered saline (PBS) and homogenized in lysing solution [1 mM $NaHCO_3$ and 1 mM phenylmethanesulfonyl fluoride (PMSF); Calbiochem-Behring Corp., San Diego, CA) in deionized water] with a Dounce homogenizer. The homogenates were centrifuged for 2 min at 300*g*, and the supernatant was further centrifuged for 15 min at 20000*g*. The pellet was resuspended in 1 mM PMSF–PBS and sonicated. The protein concentration was determined.

Establishment of Hybridoma 33E7C. NS-1 mouse myeloma cells (Köhler & Milstein, 1975) were fused with spleen cells of a 1-month-old Wistar Furth rat that had been immunized by three intraperitoneal injections of 10^7 cells from a transplantable FANFT-induced bladder carcinoma, 1682, and boosted 2 weeks later by injection of 20 μg of membrane preparations from carcinoma 1682. Hybrid cells were grown in HAT medium, and cloning was performed by limiting dilution. The supernatants from hybridoma cultures were screened by binding assays. One hybridoma, 33E7C, was selected, and the specificity of the antibody made by this

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hybridoma was further tested in binding assays against preparation of membranes from various cell types as well as chloroform–methanol- (C–M-)[1] soluble fractions. Antibody 33E7C was shown to be an IgG2a by double immunodiffusion.

Antibody Binding Assays. Four micrograms of membrane preparations in PBS was plated into Costar 96-well tissue culture cluster 3596 (Costar, Cambridge, MA). After treatment with PBS containing 5% bovine serum albumin (BSA), 50-μL aliquots of culture media and hybridoma cells or 5 μg/mL of purified antibody were added. After incubation at room temperature for 1 h, the plates were washed, and 50-μL aliquots of ^{125}I-labeled rabbit anti-rat IgG (affinity purified; Zymed Laboratory, Burlingame, CA; 1×10^5 cpm) were then added to each well. After being incubated and washed, bound radioisotope was removed with 2 M NaOH for counting in a gamma counter. When the C–M fractions were tested, 5–0.05 μL of C–M was added to 75 μL of ethanol, and the ethanol solutions were plated, dried, and followed by the procedure described above. Normal rat serum (100 times dilution) and culture supernatants of NS-1 cells and rat antibody nonproducing hybridoma cells were used as negative controls.

Extraction and Preparation of Organic Solvent Soluble Antigen. Tissues were homogenized in chloroform–methanol (C–M), 2:1 v/v, followed by reextraction with C–M (1:1 and 1:2). The extracts were evaporated, redissolved in C–M (2:1), and partitioned with water according to Folch's procedure (Folch & Lees, 1951). An aqueous upper phase and a chloroform lower phase were obtained. The chloroform lower phase was evaporated and dissolved in C–M (2:1). The aqueous upper phase was evaporated, dialyzed against water, evaporated again, dissolved in C–M–water (30:60:8), and applied onto a DEAE-Sephadex column (Kannagi et al., 1982). The aqueous upper neutral fraction was collected as DEAE-Sephadex column pass through. The aqueous upper acidic fraction was eluted from the DEAE-Sephadex column with C–M–0.8 mM sodium acetate (30:60:8). These two fractions were evaporated and dissolved in C–M (2:1). Since the major antigen activity was associated with the chloroform phase, this phase was subjected to fractionation through a column (0.9 × 55 cm) of α-hydroxypropylated Sephadex G-50 (LH60) in C–M (2:1) (Watanabe et al., 1980). The pooled fraction binding to antibody 33E7C (LH60-purified sample) was separated by 0.1% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) on 15% acrylamide gel. The gel was sliced into 0.5-cm sections after electrophoresis, and the antigen was extracted from each section and precipitated with acetone (Hager & Burgess, 1980). The precipitates were dissolved in PBS or C–M (2:1) and tested for binding to antibody 33E7C.

Radioimmunoprecipitation of Antigens. LH60 column purified samples suspended in 0.5% Nonidet P-40 in PBS were labeled with Na^{125}I by the chloramine T method (Brown et al., 1980). The labeled proteins were analyzed by immunoprecipitation with antibody followed by SDS–PAGE.

Enzymatic and Chemical Treatment of Antigen. Membrane preparations or LH60 column purified fractions dissolved in PBS were treated with proteinase K (MCB Manufacturing Chemists, Inc. Darmstadt, West Germany) at a final concentration of 0.1 mg/mL, trypsin (Sigma Chemical Co., Saint Louis, MO) at a final concentration 0.05%, or mixed glucosidases from *Charonia lampus* (Miles Biochemicals, Elkhart, IN) at a final concentration of 0.2 mg/mL; the same preparation of mixed glucosidases had been used to degrade various glycolipds and glycoproteins in one of the authors' laboratories (Kannagi et al., 1982). The LH60 column purified samples were also treated with 10% formic acid for 15 min in boiling water. The samples were assayed for inhibition of binding of antibody 33E7C (see below).

Antigen Inhibition Assay. Samples (100 μg) to be assayed for antigenicity were incubated with ^{125}I-labeled antibody 33E7C (4×10^5 cpm, 100 μL) for 30 min at room temperature. The mixtures were centrifuged for 10 min at 300*g*, and antibody remaining in the supernatant was assayed in binding assay. The percentage of specific inhibition was calculated as [1 − (sample cpm)/(maximum binding cpm)] × 100. Unabsorbed antibody was used as a control to establish maximum binding.

Cell Labeling and Analysis of Antigen. [9,10-^{3}H]Palmitic acid (15.2 Ci/mmol) and [^{14}C]leucine (>500 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Mouse bladder carcinoma 1660 cells were seeded in a 75-cm^2 flask and cultured for 4 h in 5 mL of leucine-free RPMI medium supplemented with 10% dialyzed heat-inactivated fetal calf serum and containing 25 μCi of [^{14}C]leucine. Cells were further incubated for 6 h after addition of 5 mL of normal RPMI medium containing 1 mCi of [9,10-^{3}H]palmitic acid. The labeled cells were collected with PBS by scraping with a rubber policeman. The cell pellets were immersed in C–M (2:1) and sonicated. The C–M solvents were evaporated to dryness, and the residues were dissolved with lysis buffer (10 mM Tris-HCl, pH 7.5/50 mM NaCl/1.0% NP-40/1.0% deoxycholate/0.1% SDS). The labeled proteins in the soluble lysate were analyzed by radioimmunoprecipitation followed by SDS–PAGE. The radioactive counts in the gel were extracted as described above and monitored by liquid scintillation counting with a Beckman LS1800 counter.

Hydrolysis of [^{3}H]Palmitic Acid Labeled Proteins with Methanolic NaOH. The 40K and 18K proteins separated by SDS–PAGE after immunoprecipitation were extracted from the excised gel with 0.01 M $NaHCO_3$. The extracts were lyophilized and hydrolyzed by 1 M NaOH in methanol for 1 h at 50 °C. The hydrolysates were extracted with hexane after evaporation of methanol, and the hexane extracts were dried with a N_2 stream. The extracts were dissolved in 20–40 μL of hexane and analyzed by thin-layer chromatography (TLC) on silica gel (5 × 20 cm, 0.24 mm thick; Baker) in petroleum ether–ethyl ether (4:1). To determine the migrated position of methyl palmitate and sodium palmitate, authentic standards were analyzed in parallel, and the silica gel was scraped from the plate and analyzed by scintillation counting.

Results

Specificity of Monoclonal Antibody 33E7C As Analyzed by Binding to Extracts from Tumors and Normal Tissues. Culture medium of hybridoma 33E7C was tested for binding to membrane fractions immobilized on plastic plates as indicated under Materials and Methods. Both transplanted tumors from mice (seven transitional-cell bladder carcinomas, one melanoma, one lymphoma, six sarcomas, and one spontaneous mammary carcinoma) and cultured mouse cell lines (two lymphomas and two myelomas) were used as targets. Membrane fractions from different adult mouse tissues (bladder, kidney, spleen, thymus, lung, brain, heart, liver) and from whole 18-day mouse embryos were also included.

As shown in Figure 1, the supernatant from hybridoma 33E7C gave more than 700 counts over background with membrane fractions from all bladder carcinomas tested ($p <$

[1] Abbreviations: C–M, chloroform–methanol; NP-40, Nonidet P-40; TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

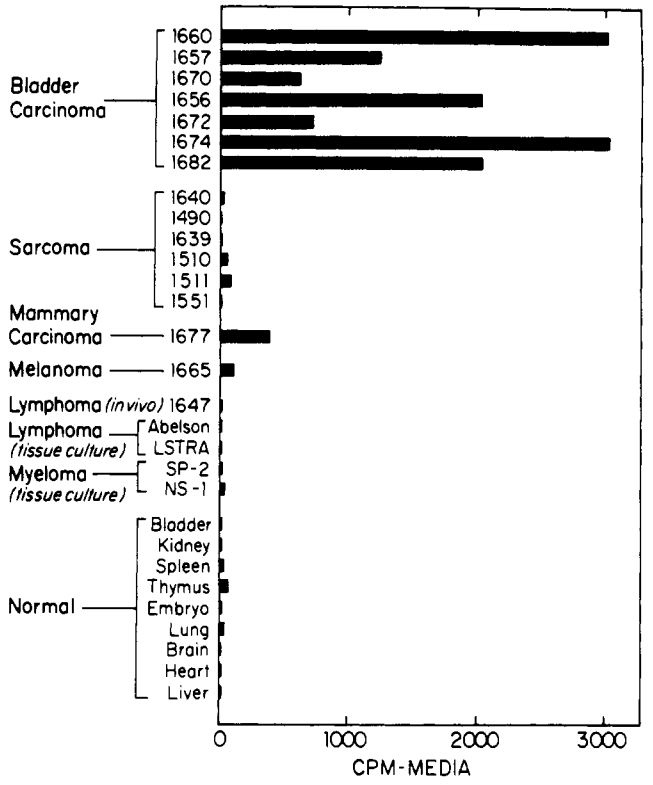

FIGURE 1: Summary of data in which supernatants from hybridoma 33E7C were tested for binding to cell extracts from a variety of tissues. The number of counts in controls with culture medium alone was substracted.

0.01), while the degree of binding to membrane fractions from all the other tissues was approximately the same as the background except for the one mammary carcinoma tested, which gave about 400 counts over background. Five of the seven bladder carcinomas showed more than 1200 counts over background. Antibody 33E7C bound very weakly to intact cultured bladder carcinoma cells (200–300 counts over background). It did not bind to intact control cells.

We conclude from this that the antigen(s) defined by antibody 33E7C is (are) present in extracts of most mouse bladder carcinomas but make no claim that it is (they are) entirely specific for such tumors, since one mammary carcinoma was (weakly) positive and since isolated normal urothelium was not studied.

Characterization and Isolation of Antigen(s) Recognized by Antibody 33E7C. For an initial characterization of the antigenic target of antibody 33E7C, we employed an antigen inhibition assay; that is, we tested the binding of ^{125}I-labeled antibody 33E7C to membrane fractions prepared from bladder carcinoma after the antibody had been preadsorbed by antigens treated in various ways as described under Materials and Methods. As shown in Table I, the antigen identified by antibody 33E7C was heat resistant (15 min in boiling water), and antigen activity was lost after 30 min of treatment with proteinase K. On the other hand, mixed glycosidases from *C. lampus* had no effect on the binding of antibody 33E7C to cell membrane preparations from bladder carcinoma 1660 when added under the same conditions, which could abolish blood group ABH activity (Kannagi et al., 1982). These data indicated that the antigen is a protein. However, trypsin digestion had no effect on its antigenicity.

After the partition of C–M extracts with water by Folch's procedure, we tested the ability of the water and chloroform phases to bind to antibody 33E7C. There was about 10% antibody binding to the aqueous upper phase and about 90%

Table I: Effects of Various Treatments of Antigens Defined by Antibody 33E7C As Assessed by Binding Assays with Bladder Carcinoma 1660 Cell Membranes or LH60 Column Purified Antigen from Carcinoma 1660

treatment of antigen[a]	degree of inhibition by antigen in the form of[b]: cell membranes	LH60 purified antigen
none	100	100
proteinase K	0	0
trypsin	73	93
mixed glucosidases	100	NT[c]
10% formic acid (100 °C, 15 min)	NT	100
acetylation–deacetylation	NT	0
heat (100 °C, 15 min)	93	100

[a] Antigen preparations tested as described under Materials and Methods. [b] [1 − (33E7C absorbed with treated antigen)/(nonabsorbed 33E7C binding)] × 100. [c] NT, not tested.

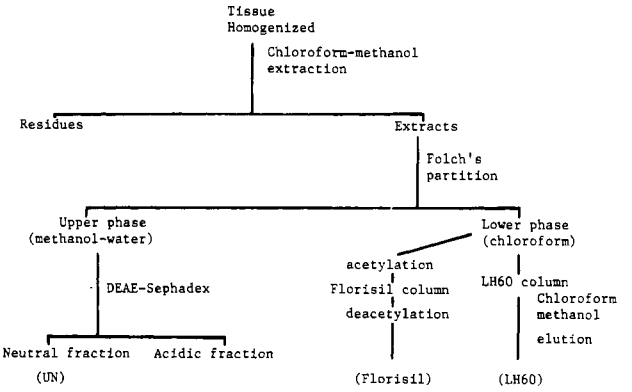

FIGURE 2: Outline of procedure for extraction and fractionation of antigen from tissues.

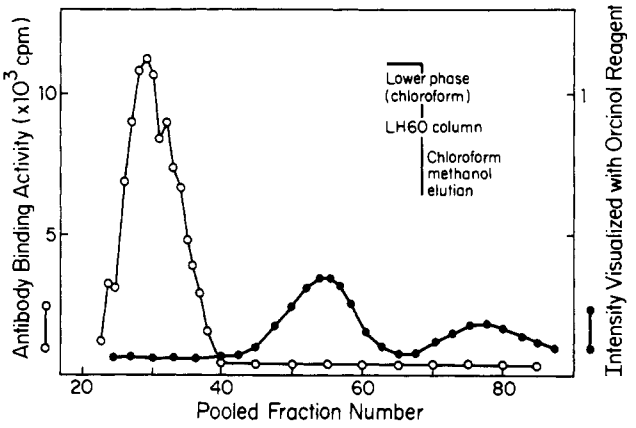

FIGURE 3: Antibody binding activity of a fraction eluted from α-hydroxypropylated Sephadex G-50 (LH60) in chloroform–methanol (2:1). The chloroform lower phase after partition with water was applied onto a LH60 column (0.9 × 55 cm) and eluted with C–M (2:1). Each fraction (0.5 mL) was tested for antibody binding activity.

binding to the chloroform lower phase. The (lower) chloroform phase was applied onto a LH60 column in order to purify the antigen from glycolipids and phospholipides as outlined in Figure 2. Fractions with antigen activity were separated from the major glycolipid fractions (Figure 3). The fraction reacting with fluorescamine–triethanolamine (Sherman & Touchstone, 1974) and phosphate reagent (Dittmer & Lester, 1964) was the same as that identified with orcinol–sulfuric acid. The antigen in the chloroform phase was inactivated by acetylation and deacetylation (Figure 4) when these procedures were performed as part of the usual method for preparing

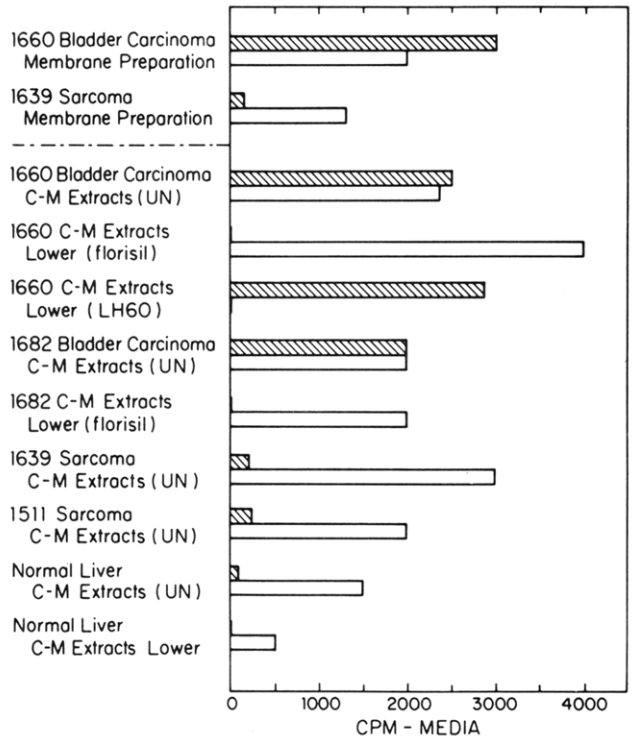

FIGURE 4: Chloroform–methanol extracts from various tissues were separated with water into chloroform lower phase and methanol–water upper phase. These two phases were further purified as described under Materials and Methods. Various fractions were tested for the binding of antibody 33E7C and of anti-Forssman rat monoclonal antibody 9.1. Antibody binding assays were performed as described under Materials and Methods by using serial dilution of C–M antigen preparation. The data were shown at the same amount of C–M extracts calculated from the starting materials (equivalent ω 0.2 μg of glycolipids). (Hatched bars) Binding of antibody 33E7C; (open bars) binding of antibody 9.1.

glycolipids (Saito & Hakomori, 1971). This suggested that the antigen was not a glycolipid. The binding of antibody 33E7C to the LH60 column purified fraction of mouse bladder carcinoma was more than 4000 cpm over background, while the degree of binding to C–M extracts of mouse sarcoma was only 100–200 counts above background. Monoclonal rat antibody 9.1, which was isolated in our laboratory and can detect the Forssman antigen, gave only background binding to the LH60 column purified fraction of mouse bladder carcinomas (Figure 4). The LH60 column purified antigen was still heat resistant, proteinase K sensitive, and insensitive to mixed glucosidases from *C. lampus* (Table I). It was also resistant to 1 and 10% formic acid, a treatment capable of degrading sialic acid.

The LH60 column purified fraction of mouse bladder extracts was further separated by SDS–PAGE to exclude the possibility of lipid contamination. After electrophoresis, the gel was cut into 0.5-cm sections, and proteins were extracted and tested for binding to antibody 33E7C. As shown in Figure 5, antigen binding to antibody 33E7C was detected in fractions recovered from the SDS–polyacrylamide gel dissolved in either PBS or C–M (2:1). The majority of antigen activity was recovered in fractions corresponding to a molecular weight (M_r) range of 17 000–20 000. The antigen activities were also detected in M_r 40K, 25K, and 10K fractions.

Autoradiograph of Immunoprecipitation of Organic Solvent Soluble Proteins. The LH60 column purified fraction was labeled with $Na^{125}I$ in the presence of NP-40 detergent by the chloramine T method. The labeled proteins were precipitated with affinity column purified antibody 33E7C, as described

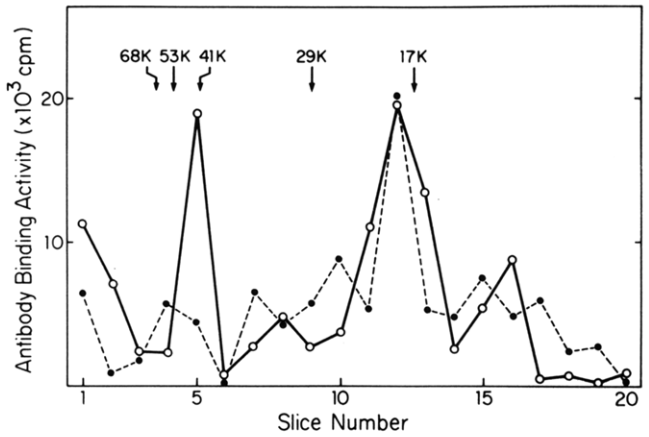

FIGURE 5: Antibody binding activity of fractions extracted from SDS–polyacrylamide gel. LH60 column purified samples were separated on a 0.2% SDS–15% polyacrylamide gel, dissolved in PBS (○) or in C–M (2:1) (●), and tested for antibody binding activity.

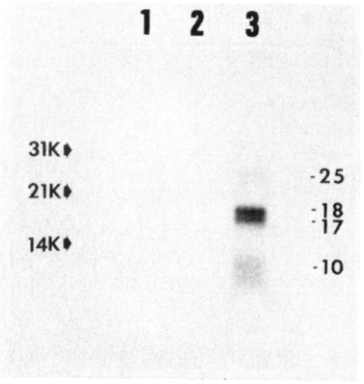

FIGURE 6: SDS–PAGE analysis of immunoprecipitates from ^{125}I-labeled LH60 column purified samples, as assayed by autoradiography for 24 h: (track 1) precipitation with culture medium (control); (track 2) precipitation with normal rat serum (control); (track 3) precipitation with antibody 33E7C. The numbers at the side of the autoradiograph indicate the molecular masses of marker proteins in kilodaltons.

under Materials and Methods, and electrophoresed on a 15% SDS–polyacrylamide gel.

As shown in Figure 6, antibody 33E7C precipitated antigens with M_r 18K as a doublet as well as M_r 25K and 10K. A control with normal rat serum gave no precipitation.

Analysis of [^{3}H]Palmitic Acid and [^{14}C]Leucine Double-Labeled Antigen. Mouse bladder carcinoma 1660 cells were labeled with [^{3}H]palmitic acid as a marker for the lipid moiety and [^{14}C]leucine as a marker for protein, and C–M extracts dissolved in lysis buffer were precipitated by antibody 33E7C followed by SDS–PAGE. After electrophoresis, proteins were extracted from the gel with buffer containing SDS. ^{3}H counts and ^{14}C counts in the gel extracts were monitored by liquid scintillation counting. As shown in Figure 7, [^{3}H]palmitic acid and [^{14}C]leucine were incorporated into 40K and 18K proteins by 10-h incubation. In order to confirm the ^{3}H counts to be from palmitic acid, 40K and 18K proteins were extracted from the polyacrylamide gel after electrophoresis and hydrolyzed with 1 M NaOH in methanol for 1 h at 50 °C. Extraction of the hydrolysates with hexane and analysis of the extracts by TLC revealed that the ^{3}H-labeled compounds migrated to the position of methyl palmitate and sodium palmitate (Figure 8). These data revealed that the antigens defined by antibody 33E7C were all acylated proteins.

Discussion

We have isolated a monoclonal rat antibody, 33E7C, that

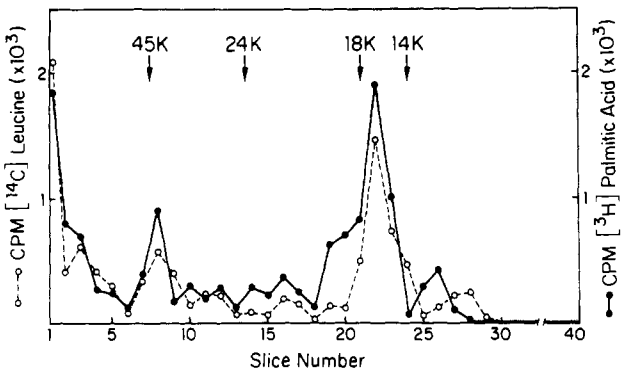

FIGURE 7: SDS–polyacrylamide gel electrophoresis of isolated proteins following incubation of mouse bladder carcinoma 1660 cells with [^{3}H]palmitic acid and [^{14}C]leucine. The cells were labeled with [^{3}H]palmitic acid (1 mCi) and [^{14}C]leucine (25 μCi) as described under Materials and Methods. The CM extracts were analyzed by immunoprecipitation followed by SDS–PAGE. The radioactivities were extracted from the gel and monitored by liquid scintillation counting. Vertical cpm = cpm(antibody 33E7C) − cpm(medium control).

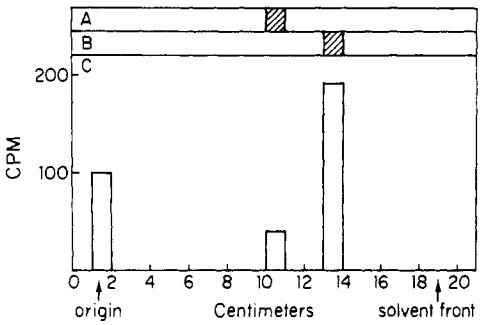

FIGURE 8: Hydrolysis of 18K and 40K proteins by methanolic NaOH. The 18K protein was purified by SDS–PAGE from cells labeled with [^{3}H]palmitic acid and hydrolyzed in methanolic NaOH for 1 h at 50 °C as described under Materials and Methods. The released materials were analyzed in parallel with [^{3}H]methyl palmitate and sodium [^{3}H]palmitate by TLC on silica gel. The experimental samples were scraped from the plate and analyzed by scintillation counting. The scales of the panels are identical: (A) tracing of the sodium palmitate marker; (B) tracing of the methyl palmitate marker; (C) material released from the 18K protein. The material released from the 40K protein gave the same result.

shows a high degree of binding to mouse bladder carcinoma extracts and recognizes proteins that are soluble in both organic solvents and aqueous solvents and are tightly bound to lipid. The physical properties of these proteins are similar to those of proteolipids as first described by Folch & Lees (1951) in the central nervous system. Although proteolipids with such an unusual solubility are not common (Lees et al., 1979), they have been found in a variety of animal and plant tissues (Folch-Pi & Stoffyn, 1972), especially in mitochondrial (Burke & Beattie, 1973) and bacterial (Guerin & Napias, 1978) membranes where they play a role in energy transport. In the absence of functional criteria, proteolipids have been classified as proteins on the basis of the procedure used for their isolation (Lees et al., 1979).

In this paper, we have found amphipathic protein antigens in C–M extracts of mouse bladder carcinomas. They are hydrophobic and heat-resistant proteins with an M_r of 40K, 25K, 18K, and 10K in the chloroform phase after the partition with water according to Folch's procedure (Folch & Lees, 1951).

The antigens defined by antibody 33E7C in bladder carcinoma extracts were present only in small amounts in other tumors or normal tissues, except for the one mammary carcinoma tested; however, we make no claims as to bladder carcinoma specificity beyond concluding that the antigens (as tested) were the strongest expressed in such tumors. The antigens were hardly detected and hardly labeled by cell surface radioiodination on intact cells, which implies that they are not located in the cell surface, and they were found in the cytoskeleton by immunofluorescence tests (data not shown). The precise investigation of antigen localization is under study.

We were not able to directly label with ^{125}I the proteins extracted into C–M, perhaps because the proteins are highly hydrophobic in C–M and form complexes with lipids or glycolipids. This led us to apply the C–M extracts onto a LH60 column to separate proteins from lipids and glycolipids, after which the LH60 column purified samples were separated by SDS–PAGE. The antigens could then be recovered in extracts of the polyacrylamide gel and were soluble in C–M (2:1) as well as in PBS. This revealed that the proteins were amphipathic.

The LH60 column purified samples were labeled with Na^{125}I in the presence of detergent and were precipitated with purified antibody 33E7C. The iodination of the C–M extracted antigens revealed a doublet at M_r 18K and 25K after immunoprecipitation, but no M_r 40K protein. However, our data show that the M_r 40K protein is lipid bound and is a major protein that binds to antibody 33E7C after aqueous buffer extraction. The M_r 40K protein seems to behave more like an ordinary protein that is soluble in aqueous buffer, even after it is bound to lipid.

Proteolipids have been defined as proteins that have a lipid moiety as part of their primary structure (Schlesinger, 1981). Some proteolipids (Townsend et al., 1982) and a number of virus glycoproteins (Schmidt et al., 1979) have been found to contain covalently bound fatty acids as part of their primary structures. The transforming proteins of Rous sarcoma virus (p60^{src}), Harvey sarcoma virus (p21), and Abelson leukemia virus (p120) have been also reported to contain tightly bound lipids (Sefton et al., 1982).

In a double-labeling experiment with mouse bladder carcinoma cells, [^{3}H]palmitic acid labeled cell lysates were precipitated by antibody 33E7C and found also to be labeled with [^{14}C]leucine. It is unlikely that [^{3}H]palmitic acid was converted into ^{3}H-labeled amino acid during the experiment, since many other proteins labeled by [^{14}C]leucine were found without ^{3}H counts when the double-labeled cell lysates were separated by SDS–PAGE without immunoprecipitation (data not shown). Furthermore, the ^{3}H counts were isolated from the proteins as palmitic acid after hydrolysis with 1 M NaOH in methanol. We conclude that the antigens defined by antibody 33E7C comprise a group of proteins containing tightly bound lipids. In preliminary experiments with double labeling with [^{3}H]palmitic acid and [^{14}C]leucine, the 18K protein or the 25K protein seems to be a precursor of the 40K protein by pulse–chase experiments. The kinetics of the formation of these proteins and its regulation by lipids are under investigation.

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Effect of Skeletal Muscle Myosin Light Chain 2 on the Ca^{2+}-Sensitive Interaction of Myosin and Heavy Meromyosin with Regulated Actin†

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ABSTRACT: A low-speed centrifugation assay has been used to examine the binding of myosin filaments to F-actin and to regulated actin in the presence of MgATP. While the cross-linking of F-actin by myosin was Ca^{2+} insensitive, much less regulated actin was cross-linked by myosin in the absence of Ca^{2+} than in its presence. Removal of the 19 000-dalton, phosphorylatable light chain from myosin resulted in the loss of this Ca^{2+} sensitivity. Readdition of this light chain partially restored the Ca^{2+}-sensitive cross-linking of regulated actin by myosin. Urea gel electrophoresis has been used to distinguish that fraction of heavy meromyosin which contains intact phosphorylatable light chain from that which contains a 17 000-dalton fragment of this light chain. In the absence of Ca^{2+}, heavy meromyosin which contained digested light chain bound to regulated actin in MgATP about 10-fold more tightly than did heavy meromyosin which contained intact light chain. The regulated actin-activated ATPases of heavy meromyosin also showed that cleavage of this light chain causes a substantial increase in the affinity of heavy meromyosin for regulated actin in the absence of Ca^{2+}. Thus, the binding of both myosin and heavy meromyosin to regulated actin is Ca^{2+} sensitive, and this sensitivity is dependent on the phosphorylatable light chain.

In vertebrate striated muscles, contraction is regulated by Ca^{2+} binding to troponin on the thin filament. In the absence of Ca^{2+}, troponin–tropomyosin inhibits the cyclic interaction of actin with myosin. Ca^{2+} binding to troponin causes a shift in the position of tropomyosin on the thin filament (Huxley, 1972; Haselgrove, 1972; Parry & Squire, 1973), allowing for myosin to interact with actin and force to be developed. An in vitro model of the thin filament is regulated actin, F-actin, and troponin–tropomyosin. In the presence but not in the absence of Ca^{2+}, regulated actin activates the MgATPase of myosin, heavy meromyosin (HMM),[1] and myosin subfragment

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