Monoclonal Antibodies As Probes of Epithelial Membrane Polarization

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ABSTRACT Monoclonal antibodies directed against antigens in the apical plasma membrane of the toad kidney epithelial cell line A6 were produced to probe the phenomena that underlie the genesis and maintenance of epithelial polarity. Two of these antibodies, 17D7 and 18C3, were selected for detailed study here. 17D7 is directed against a 23-kD peptide found on both the apical and basolateral surfaces of the A6 epithelium whereas 18C3 recognizes a lipid localized to the apical membrane only. This novel observation of an apically localized epithelial lipid species indicates the existence of a specific sorting and insertion process for this, and perhaps other, epithelial plasma membrane lipids. The antibody-antigen complexes formed by both these monoclonal antibodies are rapidly internalized by the A6 cells, but only the 18C3-antigen complex is recycled to the plasma membrane. In contrast to the apical localization of the free antigen, however, the 18C3-antigen complex is recycled to both the apical and basolateral surface of the epithelium, which indicates that monoclonal antibody binding interferes in some way with the normal sorting process for this apical lipid antigen.

The plasma membrane of epithelial cells shows a striking polarization into two morphologically and functionally distinct domains: the apical and basolateral membranes which face the external and internal milieu, respectively (1). The functional polarity of an epithelium results from the specific localization of various membrane components (enzymes, receptors, transport systems, etc.) to one or the other of these cell surfaces. The apical and basolateral membranes are separated from one another by tight junctions where neighboring cells attach to each other (2). The tight junction is thought to contribute to the maintenance of epithelial polarity by preventing the lateral diffusion of at least some proteins and lipids between these two membranes (3–5). Little is known about the basic question of how this polarity in membrane structure is generated and, in particular, at which intracellular step(s) membrane components destined for the apical or basolateral surface are sorted. Recent observations have also shown that many plasma membrane components in a variety of cell types, including epithelia, undergo or can be induced to undergo some form of internalization (endocytosis) and that at least some of these components are subsequently recycled back to the cell surface (4, 6). Clearly, in epithelia such recycling events must be integrated into the sorting mechanism for apical and basolateral membrane components.

Continuous cultured epithelial cell lines provide particularly convenient and powerful tools for the study of epithelial biogenesis and function (1, 7). A number of these lines have now been extensively characterized and shown to retain many of the differentiated properties associated with naturally occurring epithelia, including morphological and functional polarity (1, 7). The A6 cell line is derived from the kidney of the aquatic toad *Xenopus laevis* (8). In culture these cells form confluent polarized monolayers oriented with their basal surface against the supporting substrate. Morphologically they exhibit apical microvilli, occasional apical cilia, and tight junctions (9). When grown on a solid support they form domes or hemicysts, structures thought to be due to transepithelial transport of solute and water. When grown on a permeable support confluent A6 monolayers exhibit a high electrical resistance (5,000 $\Omega \cdot cm^2$), a mean transepithelial potential difference of 10 mV (apical side negative), and short circuit currents of 2 $\mu A/cm^2$, which are equivalent to their net sodium transport (9). This net transepithelial sodium flux

is competitively inhibited by apical amiloride and reversibly stimulated by cAMP and adrenal steroid hormones (9, 10). These properties of the A6 cells, together with their ease of handling in culture, have made them a particularly valuable system for studying salt transport in high resistance epithelia (7, 9–11).

The present study focuses on the behavior of epithelial cell surface components using the A6 cells as a model epithelium. We have employed monoclonal antibodies (MAb's)[1] directed against specific membrane components of these cells as probes of the phenomena that underlie the genesis and maintenance of epithelial polarity. A number of MAb's directed against the A6 cell surface were generated using the hybridoma technique developed by Kohler and Milstein (12). Two MAb's have been selected for detailed study here. We show that one of these is directed against a 23-kD peptide found on both surfaces of the A6 cells and the other recognizes a lipid localized to the apical membrane only. This is, to our knowledge, the first demonstration of the existence of a lipid that is localized to one side of an epithelium. We report the results of a series of experiments that characterize the internalization and recycling properties of the antibody–antigen complexes formed by these MAb's. Our data show that although both MAb–antigen complexes are internalized by the A6 cells, only the MAb–lipid complex is recycled. Surprisingly, however, this complex is recycled to both the apical and basolateral surface, which indicates that MAb binding interferes in some way with the normal sorting process for this apical lipid antigen. The mechanism of this interference remains to be determined.

MATERIALS AND METHODS

Cell Culture

A6 cells were purchased from American Type Culture Collection (Rockville, MD) in the 68th plating. All experiments described here were carried out on platings 72–79. Stock cells were grown in 10-cm plastic tissue culture dishes (Nunc, Roskilde, Denmark) and subcultured as previously described (10). Cells were maintained at 28°C in a humidified incubator, gassed with 1% CO_2 in air. The growth medium was CL-2 (seven parts Coon's modification of Ham's F12 [13] and three parts Leibovitz's L-15 [14] modified for amphibian cells to contain 90 mM NaCl and 8 mM $NaHCO_3$) supplemented with 5% fetal bovine serum (Gibco Laboratories, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells growing in 10-cm tissue culture dishes were fed (7 ml) three times per week and cells in "cluster 24's" (24-well tissue culture plates; Falcon 3047, Falcon Labware, Oxnard, CA) were fed (1 ml/well) once per week.

Sp2/0 myeloma cells and rat fibroblasts, both obtained from Dr. K. Ozato (National Cancer Institute, Bethesda, MD), were grown and maintained as described in reference 15 with calf serum (Gibco Laboratories) replacing fetal bovine serum. Hybridoma cell lines were grown at 37°C and 10% CO_2 in HAT medium composed of DME (Dulbecco's modified Eagle's medium, Gibco Laboratories) plus 15% calf serum, 0.03% glutamine, 10% NCTC 109 (M.A. Bioproducts, Bethesda, MD), 1% nonessential amino acid solution 100 × (Gibco Laboratories), 0.25 U/ml insulin (I-5500, Sigma Chemical Co., St. Louis, MO), 1 mM oxalacetic acid (Sigma 0-4126), 0.5 mM sodium pyruvate (Gibco Laboratories), 100 μM hypoxanthine (Sigma H-9377), 1 μM aminopterin (Sigma A-2255), 30 μM thymidine (Sigma T-9250), and 50 μg/ml gentamicin. Hybridomas were later switched (see below) to HT medium (HAT medium without aminopterin).

[1] *Abbreviations used in this paper:* MAb, monoclonal antibody; MDCK, Madin-Darby canine kidney; DME, Dulbecco's modified Eagle's medium; HAT medium, medium that includes hypoxanthine, aminopterin, and thymidine; HT medium, HAT medium without aminopterin.

Generation of Hybridoma Cell Lines

Plasma membranes were prepared from A6 cells growing in 10-cm plastic tissue culture dishes using the procedure described in reference 11. BALB/c mice were immunized intraperitoneally approximately once a month for 4 mo with A6 plasma membranes (~120 μg protein per immunization) suspended in Dulbecco's phosphate-buffered saline (PBS; Gibco Laboratories). 3 d after the last injection, the spleen from one mouse was removed and teased into a cell suspension in 5 ml PBS. The cell suspension was transferred to a 15 ml centrifuge tube, diluted to 10 ml with DME, and allowed to stand for 10 min. The upper 9 ml of this suspension was diluted to 50 ml with DME and spun at 200 *g* for 10 min. This pellet was taken up in 20 ml DME (~10^7 lymphocytes/ml).

For cell fusion 3 ml of lymphocytes was mixed with 9 ml of Sp2/0 myeloma cells in DME (10^7/ml). The mixed cells were pelleted (200 *g* for 10 min) and fusion was carried out according to the final procedure of reference 16, except that the fusing solution was 1 ml of a mixture of 5 g polyethylene glycol 1500 (BDH Chemicals, Poole, U.K.), 5 ml DME, plus 0.5 ml dimethylsulfoxide. The final fusion partners were diluted to 300 ml in HAT medium and seeded (80 μl/well) into the central 60 wells of sixteen 96-well tissue culture plates (Costar 3596, Costar, Cambridge, MA); each of these wells also contained a feeder layer of 10^4 irradiated (2500 R; in a Gammator M, Isomedic, Parsippany, NJ) rat fibroblasts in 160 μl HAT medium. The unused fusion partners (~225 ml) were discarded.

The fusion partners in 96-well plates were fed once a week by replacing 80 μl of supernate with fresh HAT medium. At the end of 3 wk, culture supernates were screened for anti-A6 apical membrane activity (see below). Cells that screened positive were transferred to the wells of cluster-24 plates containing 1 ml HAT medium and 6 × 10^4 irradiated rat fibroblasts. 3 d later selected lines were cloned (see below), and aliquots of all positive lines were frozen (1.5 × 10^5 cells/ml) in a 1:1 mixture of HAT medium and cyroprotective medium (M.A. Bioproducts).

Establishment of Hybridoma Cell Lines and MAb Production

Positive lines selected for further study were cloned by limiting dilution in 96-well plates (0.3 cells/well with each well containing 240 μl HAT medium and 10^4 irradiated rat fibroblasts). Clones were carried in culture using the same procedures described above for fusion partners (with HT medium replacing HAT). Selected positive clones were recloned. Selected lines from the second cloning were slowly expanded (in the absence of irradiated fibroblasts) into successively larger tissue culture flasks while a cell density of 10^5–10^6/ml was maintained. When enough cells were available, these were injected intraperitoneally into pristane-primed BALB/c mice (2 × 10^6 cells in DME/mouse). Ascites fluid typically containing titers of MAb several orders of magnitude higher than those of culture supernates was collected 1–2 wk later, filtered, and stored in aliquots at −20°C.

MAb Screening Assay for Hybridomas Secreting Anti-A6 Apical Membrane Antibody

In this assay hybridoma supernates were incubated with intact A6 epithelia growing on a plastic surface (cluster-24 wells) to test for the presence (binding) of anti-A6 plasma membrane antibody. Since confluent epithelia with intact tight junctions were used in the assay, only the apical membrane of the A6 cells was exposed to the hybridoma supernates. Thus any antibodies detected must bind to antigens present on the extracellular surface of the apical membrane of the A6 cells. The protocol for the assay was as follows.

100-μl aliquots of hybridoma supernate were transferred to the culture medium over confluent A6 monolayers growing in cluster-24 wells. After 2 h of incubation at room temperature on an orbital shaker set at 2 cycles/s, the wells were emptied and rinsed three times by successive immersion of the entire cluster-24 plate in A6 Ringer's solution (110 mM NaCl, 2.5 mM $NaHCO_3$, 3 mM KCl, 1 mM KH_2PO_4, 1 mM CaCl, 0.5 mM $MgSO_4$, and 5 mM glucose). 500 μl of A6 Ringer's solution containing 50,000 dpm of high specific activity (70–100 μCi/μg) ^{125}I-protein A (NEX-146, New England Nuclear, Boston, MA) were then added to each well. After 30 min of incubation as above, the wells were emptied, rinsed four times with A6 Ringer's solution, and solubilized in 1 ml of 1% Triton X-100 in water. The solubilized cells were transferred to a scintillation vial and counted for radioactivity. ^{125}I-Protein A binding three or more times background (250 dpm, measured using HAT medium in place of hybridoma supernate) was taken as indication of the presence of anti-A6 apical membrane antibody in the hybridoma supernate. Binding as high as 100 times background was often observed.

Identification of Protein Antigens

A6 proteins were labeled with ^{35}S by growing cells overnight in methionine-free CL-2 containing 40 μCi/ml [^{35}S]methionine (NEG-009A, New England Nuclear), 10% dialyzed fetal bovine serum, penicillin, and streptomycin (5 ml medium/10-cm dish). Cells labeled in this way were rinsed in A6 Ringer's solution, incubated in A6 Ringer's solution for 1 h at room temperature, rinsed again, and then solubilized in 5 ml of buffer SB (50 mM Tris HCl, pH 7.4, containing 110 mM NaCl, 2 mM Na_2EDTA, 0.02% NaN_3, 10 mM methionine, 0.5% Nonidet P-40 [Bethesda Research Laboratories, Bethesda, MD] and 0.2 mM phenylmethylsulfonyl fluoride). The solubilized cells were spun at 48,000 *g* for 20 min, and the pellet was discarded.

Immunoprecipitation of MAb antigens was carried out as follows. 500 μl of the above 48,000-*g* supernate was combined with 1 μl of ascites fluid in a 1.5 ml Eppendorf microcentrifuge tube and shaken for 6 h at 4°C in a Brinkmann Vortex Shaker (Brinkmann Instruments, Inc., Westbury, NY). 1 mg prewashed (see below) Pansorbin (Calbiochem-Behring Corp., La Jolla, CA) was then added and shaking continued for 30 min. The Pansorbin was then spun out of the solubilized extract (all spins were done in an Eppendorf microcentrifuge model 3200 for 1 min) and washed six times with buffer SB/SDS (buffer SB titrated to pH 8.6 with Tris and containing 0.1% SDS and an additional 200 mM NaCl), changing tubes for the last spin. The final pellet was suspended in 200 μl of low pH buffer (50 mM glycine-HCl, pH 2.3, containing 150 mM NaCl and 0.5% Nonidet P-40) left at room temperature for 10 min and centrifuged as above. The supernate from this spin was combined with 150 μl of 30% trichloroacetic acid, left on ice for 30 min and recentrifuged. The resulting pellet was taken up in 100 μl of 80 mM Tris-HCl, pH 6.8, containing 10% glycerol, for SDS PAGE (see below).

Pansorbin was prewashed twice in buffer SB/SDS, once in low pH buffer, once in buffer SB, and suspended in buffer SB containing 1% ovalbumin (100 mg Pansorbin/ml) before use in the above immunoprecipitation procedure.

SDS PAGE and autoradiography were carried out according to published procedures (17). The low molecular weight standards from Bio-Rad Laboratories were used to calibrate the gels. Gels were treated with Enlightning (New England Nuclear) according to the manufacturers instructions before drying.

Identification of Anti-A6 Lipid Antibodies and Their Antigens

A total lipid extract from A6 cells was obtained using the procedures of Magnani et al. (18) as follows. Two confluent 10-cm plates of A6 cells (total cell wet weight, 160 mg) were rinsed in A6 Ringer's solution, scraped (5 ml A6 Ringer's/plate) and spun at 300 *g* for 10 min. The pellet was taken up in 450 μl of double-distilled water and homogenized on ice with a tight-fitting Dounce homogenizer (Arthur H. Thomas Co., Philadelphia, PA). After 1.62 ml methanol was added to the homogenate, 0.81 ml chloroform was added with constant stirring. This mixture was left at room temperature for 30 min with occasional mixing then spun at 15,000 *g* for 10 min. The supernatant was saved and the pellet was re-extracted as above in 300 μl water, 1.08 ml methanol, and 540 μl chloroform. The combined supernates were evaporated under nitrogen and taken up in 3.2 ml methanol. The resulting cloudy suspension was sonicated for 2 min in an ultrasonic water bath, then spun at 15,000 *g* for 10 min. The supernate from this spin was saved and the pellet was discarded.

Hybridoma supernates were tested for anti-A6 lipid activity using a solid-phase radioimmunoassay (19, 20) as follows. 20-μl aliquots of a 1:30 dilution of the above lipid extract (in methanol) were evaporated in the wells of a round-bottom polyvinylchloride microtiter plate (Dynatech Laboratories, Inc., Alexandria, VA). The dry wells were rinsed by immersing the plate six times in PBS containing 1% ovalbumin (Sigma A-5503). After another rinse in PBS, 20 μl of hybridoma supernate was added to each well and the plate was covered with parafilm and left at room temperature for 3 h. The hybridoma supernate was then aspirated, the plate was washed three times in PBS, and 20 μl of PBS containing 1% ovalbumin and 15,000 dpm of high specific activity ^{125}I-protein A was added to each well. After 15 min the ^{125}I-protein A solution was aspirated and the plate was rinsed six times in PBS. Individual wells were cut from the plate and counted for radioactivity. ^{125}I-Protein A binding three or more times background (125 dpm) was taken as an indication of the presence of anti-A6 lipid antibody in the hybridoma supernate. All but one positive supernate showed binding 10 or more times greater than background.

Autoradiography of glycolipid antigens was carried out on thin layer chromatograms of A6 lipid extract as described in references 19 and 20. In brief, 10-μl aliquots of lipid extract (see above) were chromatographed on aluminum-backed thin layer silica gel chromatography plates (Silica Gel 60, E. Merck, Darmstadt, Federal Republic of Germany). The plate was then overlaid with a 1:1,000 dilution of ascites fluid and left overnight at 4°C. After washing, the plate was overlaid with ^{125}I-labeled rabbit anti-mouse $F(ab)_2$ fragments (2×10^6 dpm/ml) and left again overnight. The plate was then washed, dried, and exposed to x-ray film.

Determination of Fluid-Phase MAb Concentration

Fluid-phase MAb concentrations were determined by the method of Langone (21) using mouse IgG (Sigma I-5381) as a standard.

General Procedure for Assaying MAb Binding Using ^{125}I-Protein A

MAb binding to the A6 cells was determined using "low specific activity" ^{125}I-protein A (2–10 μCi/μg) as a second antibody (compare the screening assay described above). In control experiments (not shown) we have established that ^{125}I-protein A binding is a linear function of MAb binding over the range of experimental conditions employed here. Since protocols varied from experiment to experiment the details of each are given in the figure and table legends. Unless otherwise noted the following general procedures apply to all assays of MAb binding presented here.

EPITHELIA: Confluent A6 epithelia 9–14 d old in cluster-24 culture plates (2 cm^2/well) were used in all experiments. All cells were preincubated at room temperature (23°C) in CL-2/OV (CL-2 containing 1% ovalbumin and equilibrated with room air) for 1 h before the experiment. When appropriate, epithelia were scraped from the plates into suspension as epithelial sheets (~2–4 mm^2) using a Teflon policeman (Arthur H. Thomas Co.).

INCUBATIONS: Incubations with MAb and ^{125}I-protein A were carried out in CL-2/OV (500 μl/well) at 4 or 28°C as appropriate (see below). MAb's were typically used as a 1:2,000 dilution of ascites fluid. ^{125}I-Protein A was used at a concentration sufficient to yield 200,000 dpm/500 μl. Typically, <5% of these counts were bound after epithelia were incubated with MAb. Incubation with intact attached monolayers in cluster-24 plates were carried out on an orbital shaker (Vibrax VXR7, Terochem Laboratories, Ltd., Rexdale, Ontario) set at 2 cycles/s. Incubations with suspended epithelial fragments were carried out in disposable 1.5-ml polypropylene microcentrifuge tubes on the Vibrax shaker at setting 1000. This speed was sufficient to keep the epithelial fragments in suspension. In some cases it was desirable to carry out incubations on attached and suspended cells under similar experimental conditions. In these situations incubations were carried out in cluster-24 plates with wells containing attached epithelia and suspended epithelial fragments incubating side by side. These plates were placed on the Vibrax shaker which was set at a speed sufficient to keep the monolayer fragments in suspension (~4 cycles/s). In preliminary studies we have verified that both MAb and ^{125}I-protein binding are complete after 1 h of incubation under the experimental conditions employed here. In those situations where it was important to ensure that no internalization, recycling, or other rearrangement of membrane components took place, incubations were carried out at 4°C.

RINSES: Between all incubation steps intact monolayers in cluster-24 plates were rinsed three times by successive immersion of the entire plate in a beaker of A6 Ringer's solution (the temperature of the A6 Ringer's solution corresponded to that of the incubation step that followed). Cells in suspension were washed three times with 1 ml A6 Ringer's solution by successive centrifugations (1 min in an Eppendorf microcentrifuge model 3200).

^{125}I BINDING AND PROTEIN DETERMINATION: Attached monolayers in 24-well plates were solubilized in 1 ml of 1% Triton X-100 and counted for radioactivity. Suspended monolayer fragments were solubilized in 400 μl 1 N NaOH. A 200-μl aliquot of this extract was counted for radioactivity, and two 50-μl aliquots were neutralized with 1 N HCl and assayed for protein using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Protein was typically ~300 μg/well. Nonspecific (background) ^{125}I-protein A binding (200–300 dpm/well), determined from control epithelia not exposed to MAb, had been subtracted from all data presented here.

RESULTS

Production and Characterization of MAb's

Of the 960 microtiter wells seeded in the hybridization protocol (see Materials and Methods), 427 were found to contain living hybridomas. Supernates from 82 of these screened positive against the apical membranes of confluent A6 epithelia. A subgroup of 65 positive hybridoma supernates were also tested for anti-A6 lipid activity using the solid-phase radioimmunoassay described in Materials and Methods. Of

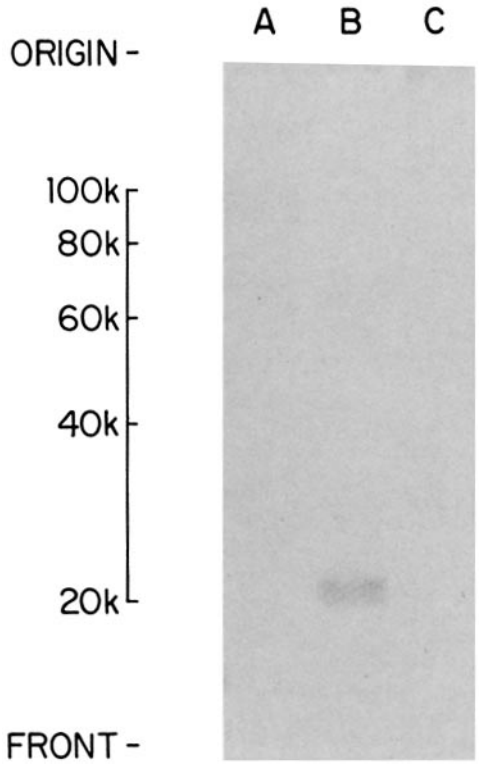

FIGURE 1 Identification of the protein antigen associated with the anti-A6 plasma membrane monoclonal antibody 17D7. The figure shows an autoradiograph of a 12.5% SDS polyacrylamide gel on which MAb immunoprecipitates from a detergent extract of [^{35}S] methionine-labeled A6 cells were run (see text for details). The immunoprecipitates from 18C3, 17D7, and control ascites were run in lanes *A*, *B*, and *C*, respectively.

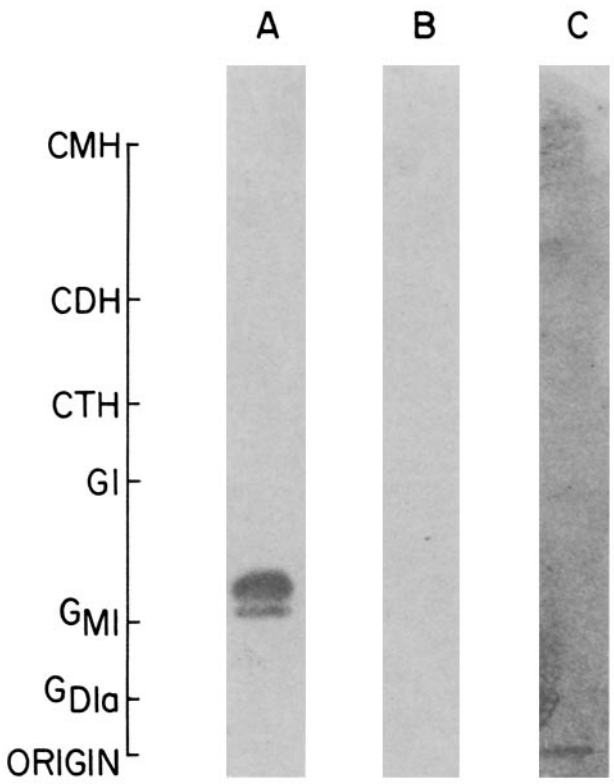

FIGURE 2 Identification of the glycolipid antigen associated with the anti-A6 apical membrane monoclonal antibody 18C3. The figure shows an autoradiograph of a thin layer chromatograph of A6 lipids reacted first with MAb then with ^{125}I-labeled rabbit anti-mouse F(ab)$_2$ fragments (see text for details). Lanes *A*, *B*, and *C* were reacted with 18C3, 17D7, and control ascites, respectively. The positions of some simultaneously chromatographed standard gangliosides and neutral glycolipids are shown on the left. The abbreviations are: *CMH*, Galβ1-1Ceramide; *CDH*,Galβ1-4Glcβ1-1Ceramide; *CTH*, Galα1-4Galβ1-4Glcβ1-1Ceramide; *GI*, globoside; G_{M1}, Galβ1-3GalNAcβ1-4[NeuNAc2-3]Galβ1-4Glcβ1-1Ceramide; G_{D1a}, NeuNAcα2-3Galβ1-3GalNAcβ1-4[NeuNAcα2-3]Galβ1-4Glcβ1-1Ceramide.

these, 52% screened positive. Since the probability that a given well contained more than one cell line secreting anti-A6 apical membrane antibody was <1%, this result indicates that ~50% of the antibodies detected were directed against A6 apical membrane lipids.

Two MAb's, which we refer to as 17D7 and 18C3, were selected for detailed study. The hybridoma cell lines secreting these antibodies have been cloned to monoclonality by limiting dilution and the corresponding antibodies produced in quantity as ascites fluid for these studies. The concentration of MAb in ascites fluid was typically 1–2 mg/ml. Experiments dealing with the identification, localization, and behavior of the 17D7- and 18C3-antigens are reported below.

Identification of 17D7- and 18C3-Antigens

The detergent extract of A6 cells grown in [^{35}S]methionine was immunoprecipitated with 18C3, 17D7, and control ascites fluid (Bethesda Research Laboratories No. 9401), and the precipitates were electrophoresed on SDS polyacrylamide gels. Fig. 1 shows an autoradiograph of such a gel. A single 23-kD band associated with MAb 17D7 (Fig. 1, lane *B*) is clearly seen, whereas no significant immunoprecipitation was produced by either 18C3 or control ascites (Fig. 1, lanes *A* and *C*, respectively). Taken together with the fact that 17D7 screens positive against intact A6 cells, this gel provides convincing evidence that this antibody is directed against a single 23-kD membrane peptide. The counts immunoprecipitated by MAb 17D7 represented ~0.1% of the total [^{35}S]methionine counts associated with the detergent-solubilized cell extract.

When 17D7 and 18C3 were tested for activity against an A6 lipid extract using the solid-phase radioimmunoassay described in Materials and Methods, 17D7 screened negative whereas 18C3 screened positive (data not shown). A more detailed test of interactions of these antibodies with A6 lipids is shown in Fig. 2. This figure is an autoradiograph of a thin layer chromatogram of A6 lipid extract reacted with MAb then with ^{125}I-labeled rabbit anti-mouse F(ab)$_2$ fragments. Neither 17D7 (Fig. 2, lane *B*) nor control ascites (lane *C*) show any reaction with the chromatogram, whereas 18C3 specifically labels two closely spaced bands. This doublet is presumably due to the reaction of 18C3 with two closely related lipid species with small differences in the length of their ceramide portions. It is worth pointing out that even a highly hydrophobic protein, which was soluble in chloroform/methanol and thus present in the lipid extract, would be expected to remain at the origin of this thin layer chromatograph. Thus the mobility of the 18C3 antigen illustrated in Fig. 2 provides strong evidence that this compound is a lipid.

Presence of Antigens in the Apical and Basolateral Membranes

It is interesting to ask whether the antigens recognized by

TABLE I. *Presence of 17D7- and 18C3-Antigens in the Apical and Basolateral Membranes of A6 Epithelia**

	Group A Attached monolayers (2 h)	Group B Attached monolayers (1 h) + suspended fragments (1 h)	*n*
18C3	17.3 ± 1.9	17.6 ± 0.6	3
	39.7 ± 4.5	40.9 ± 3.7	4
	48.8 ± 7.9	38.5 ± 6.0	5
17D7	18.6 ± 3.5	34.5 ± 3.0	3
	17.9 ± 2.3	76.7 ± 6.5	3
	18.4 ± 2.7	59.0 ± 11.3	4

* Intact attached A6 monolayers were incubated for 1 h at 4°C with MAb. Epithelia in group B were then suspended in the MAb solution by gentle scraping and left in their wells side by side with intact (unscraped) monolayers (group A). Incubation in the same MAb solution was continued for a second hour, then cells from group B were transferred to 1.5-ml centrifuge tubes for washing and determination of ^{125}I-protein A binding (1 h at 4°C). Cells from group A were washed in the cluster-24 plate, then suspended in 500 μl A6 Ringer's, transferred to 1.5-ml centrifuge tubes, and thereafter treated identically to group B. All incubations and washes were carried out at 4°C to prevent endocytosis and recycing of membrane components during the experiment. Results are expressed as ^{125}I disintegrations per microgram cell protein ± SD. The number of wells in each group is indicated by *n*. The results from three independent experiments are shown.

17D7 and 18C3 are expressed only in the apical membrane of A6 epithelia or whether they are distributed over both the apical and basolateral membranes. Table I shows the results of an experiment designed to answer this question. Intact A6 epithelia in cluster-24 wells were incubated with MAb for 1 h at 4°C to label apical sites, then cells in group B were scraped into suspension to expose the basolateral membrane to the MAb, and cells in group A were left as attached epithelia. Incubation with MAb was then continued for a second hour, after which antibody binding to both groups of cells was assayed with ^{125}I-protein A. Results from three independent experiments for each MAb are illustrated in Table I. These data show that there is no significant difference in the binding of MAb 18C3 between groups A and B, and thus that, within the limits of accuracy of our experiment (~10%), the 18C3-antigen is localized exclusively to the apical membrane of the A6 cells. On the other hand, the binding of MAb 17D7 to suspended fragments (group B) is two to three times as large as to attached monolayers (group A). Thus the 17D7-antigen is obviously present on both sides of the A6 epithelium. Owing to the size of the epithelial fragments used in these experiments (2–4 mm^2), possible artifacts due to damaged cells at the edges of the fragments should be small since these will necessarily represent a small fraction of the total number of cells.

The localization of the 17D7- and 18C3-antigens illustrated in Table I was also confirmed by comparing MAb binding to intact attached A6 epithelia with binding to attached A6 monolayers in which the tight junctions between cells were opened by calcium chelation (incubation with 1 mM EGTA in magnesium and calcium free A6 Ringer's solution for 30 min at 4°C). In these experiments (data not shown) the binding of MAb 17D7 to chelated monolayers was typically 50% greater than to intact monolayers. No difference in binding was observed for MAb 18C3.

Evidence for Internalization of MAb-Antigen Complexes

Fig. 3 shows the results of an experiment in which each of

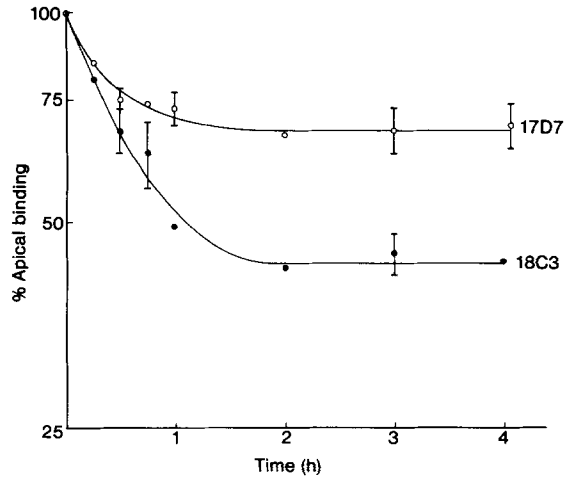

FIGURE 3 Disappearance of 17D7- and 18C3-MAb-antigen complexes from the apical surface of intact attached A6 epithelia was monitored for periods of decreasing length as follows. Cluster-24 plates containing attached A6 epithelia incubating in CL-2/OV were placed on an orbital shaker in a tissue culture incubator equilibrated with room air at 28°C. At various times duplicate wells were washed and MAb in CL-2/OV was added. After a 15-min incubation with MAb the wells were washed and reincubated with CL-2/OV for the times indicated on the figure. In this way all epithelia remained on the shaker for the same total time (4.25 h), but the period of incubation in CL-2/OV after MAb binding was varied. At the end of these incubations the entire plate was washed in cold A6 Ringer's and assayed for ^{125}I-protein A binding at 4°C. All results have been normalized to the binding observed at time zero (9,960 and 8,330 dpm/well for 18C3 and 17D7, respectively).

the MAb's 17D7 and 18C3, were bound to intact attached A6 epithelia, and the epithelia were then rinsed and left in CL-2/OV at 28°C for various periods of time before the amount of antibody remaining on the apical surface was assayed using ^{125}I-protein A. In each case the antibody initially disappears rapidly from the apical cell surface. After 1–2 h, however, the residual apical binding of both MAb's approaches a constant value corresponding to ~70% of initial binding for 17D7 and 40% for 18C3. This initial rapid disappearance of apical MAb was not observed with fixed cells or with cells maintained at 4°C (data not shown). Also, when epithelia treated as in Fig. 3 were solubilized (in buffer SB) after 0 or 3 h of incubation in CL-2/OV and assayed for fluid-phase MAb concentration (see Materials and Methods), no significant change in total cell antibody content with time could be detected for either 17D7 or 18C3 (data not shown). Taken together with the fact that residual apical MAb binding remains constant for several hours (compare Fig. 3) these data provide strong evidence that the initial disappearance of both antibodies from the apical surface is not due to MAb dissociation but rather to internalization (endocytosis) of MAb-antigen complexes by the cells.

Recycling of MAb-Antigen Complexes

Table II shows the results of an experiment in which we have assayed for the possible recycling of internalized MAb-antigen complexes to the apical surface of A6 epithelia. In this experiment intact A6 monolayers in cluster-24 wells were incubated with MAb for 1 h at 28°C then left for an additional 2 h at 28°C to allow the (putative) internalized MAb-antigen complexes enough time to distribute among intracellular

TABLE II. *Recycling of MAb-Antigen Complexes to the Apical Surface of A6 Epithelia**

	Control (before protein A)	Test (after protein A at $t = 0$)	
		$t = 0$	$t = 1$ h
18C3	3,484 ± 150	365 ± 14	1,536 ± 71
17D7	3,854 ± 287	155 ± 13	136 ± 25

* Intact attached A6 epithelia were incubated with MAb for 1 h at 28°C then washed and left shaking (2 cycles/s) for 2 h in CL-2/OV at 28°C. After this, cells were washed and incubated for 10 min at 4°C in either CL-2/OV (Control) or CL-2/OV containing 2 μg/ml unlabeled protein A (Test). The control wells and half of the test wells were then immediately assayed for ^{125}I-protein A binding (10 min at 28°C), and the remaining test wells were incubated for 1 h at 28°C before ^{125}I-protein A binding was measured. The experiment was carried out in triplicate. Results are expressed as disintegrations per well ± SD.

pools (compare Fig. 3). At the end of this period "control" wells were assayed for apical MAb binding while test cells were incubated with unlabeled protein A to saturate apically exposed MAb. Half the test wells were then assayed for ^{125}I-protein A binding to check the effectiveness of this maneuver and the rest were reincubated in CL-2/OV for 1 h at 28°C before ^{125}I-protein A binding was measured. Table II illustrates that for epithelia treated with 18C3 there is a dramatic increase in apical ^{125}I-protein A binding after this reincubation period. No increase is observed, however, for cells treated with 17D7. These results are consistent with the hypothesis that the 18C3-antigen complex is internalized by the A6 cells and subsequently recycled back to the apical cell surface. No recycling of 17D7-antigen complexes is apparent from this experiment.

In the experiment summarized in Table III we have assayed for the recycling of apical MAb-antigen complexes to the basolateral surface of the A6 cells. Epithelia were divided into eight groups for each MAb. Intact monolayers were incubated with MAb (20 min at 4°C) then either tested immediately as described below or incubated for 2 h in CL-2/OV at 28°C (to allow internalization of MAb-antigen complexes) before treatment; half of the wells in each of these groups were then incubated with unlabeled protein A to saturate apically exposed MAb; finally, each of the above four groups (zero time ± unlabeled protein A, 2 h ± unlabeled protein A) was halved according to whether ^{125}I-protein A binding was carried out on attached monolayers (apical binding only) or on suspended monolayer fragments (apical plus basolateral binding). Further details are given in the legend to Table III. This experiment allowed us to determine the amount of MAb-antigen complex present on both the apical and basolateral surfaces of the cells immediately after the application of the MAb and after two subsequent hours of internalization.

Referring first to the data for 18C3 in Table III we see that ~75% of the MAb present on the apical surface of the cells at time zero is no longer accessible from the apical side after 2 h of incubation in CL-2/OV at 28°C (35.0 ± 2.0 dpm/μg at time zero vs. 7.9 ± 0.6 dpm/μg at 2 h). As expected, there is no significant difference in ^{125}I-protein A binding between attached and suspended epithelia at time zero (35.0 ± 2.0 dpm/μg for attached cells vs. 28.6 ± 3.2 dpm/μg for suspended cells). However, when the same comparison is made for cells that were incubated at 28°C for 2 h after MAb treatment, significantly more binding is observed for suspended cells than for attached cells (16.3 ± 2.0 dpm/μg for suspended cells vs. 7.9 ± 0.6 dpm/μg for attached cells). These results indicate

TABLE III. *Appearance of Antigen-MAb Complexes on Basolateral Surface of A6 Epithelia**

	Time for internalization	Cells attached (A) or suspended (S) for determination of ^{125}I-protein A binding	Apical sites saturated with unlabeled protein A	
			−	+
	h			
18C3	0	A	35.0 ± 2.0	1.0 ± 0.4
	2	A	7.9 ± 0.6	1.0 ± 0.2
	0	S	28.6 ± 3.2	1.2 ± 0.2
	2	S	16.3 ± 2.0	9.2 ± 1.3
17D7	0	A	25.6 ± 0.4	3.8 ± 0.6
	2	A	15.8 ± 0.6	3.4 ± 0.4
	0	S	23.0 ± 5.2	2.4 ± 0.4
	2	S	12.2 ± 1.6	3.0 ± 0.6

* Intact attached A6 epithelia were incubated with MAb for 20 min at 4°C then washed and either treated immediately as described below (time = 0 h) or left shaking for 2 h in CL-2/OV at 28°C (time = 2 h) before treatment. Half of the epithelia in each of these groups were then incubated for 10 min at 4°C in CL-2/OV containing 2 μg/ml unlabeled protein A (+) and the rest were incubated in CL-2/OV alone (−). After the monolayers were rinsed in cold A6 Ringer's, ^{125}I-protein A was added to all wells, and half of the wells in each of the above four groups were scraped into suspension. ^{125}I-Protein A binding was allowed to proceed for 1 h at 4°C with attached epithelia (*A*) and suspended epithelial fragments (*S*) incubating side by side on an orbital shaker. Suspended fragments were subsequently transferred to microcentrifuge tubes for washing, whereas intact epithelia were washed in the cluster-24 wells then scraped into A6 Ringer's and also transferred to microcentrifuge tubes. ^{125}I-Radioactivity and protein were determined identically for all cells. Results are expressed as ^{125}I disintegrations per minute per microgram protein ± SD. The experiment was carried out in triplicate.

that 18C3 MAb-antigen complexes have appeared on the basolateral surface. When apical sites are saturated with unlabeled protein A before basolateral ^{125}I-protein A binding in suspension is measured, the presence of these basolateral complexes is seen directly (9.2 ± 1.3 dpm/μg).

In contrast to the results discussed above for 18C3, no recycling of apical 17D7-MAb antigen complexes to the basolateral surface of A6 epithelia is detectable in the experiment illustrated in Table III.

DISCUSSION

As already stressed in the introduction, little is known about the degree of polarization of epithelial membrane constituents or about its genesis and maintenance. Although the morphological and functional polarity of epithelial cells is now well documented, and it is well established that certain proteins are localized to either the apical or basolateral membrane, little detailed information about the actual protein composition of these membranes is available. It is not known, for example, whether there is significant overlap between the protein constituents of these two membranes or whether most proteins are uniquely localized to one or the other cell surface. Information concerning epithelial lipid composition and polarity is even more limited.

Louvard (4) has studied the recycling of an apical membrane enzyme, aminopeptidase (α-aminoacyl-peptide hydrolase) in the kidney epithelial cell line MDCK (Madin-Darby

canine kidney). Using indirect immunofluorescent techniques with polyclonal antibodies to aminopeptidase he was able to demonstrate that aminopeptidase is localized to the apical membrane of MDCK cells and that the antibody–aminopeptidase complex is endocytosed and subsequently reappears on the apical cell surface, primarily in the region of cell-to-cell contact. Louvard observed that the total apical membrane content of aminopeptidase was endocytosed within 30 min.

Van Meer and Simons (22) have harvested influenza virus and vesicular stomatitis virus from infected MDCK monolayers. These viruses are known to bud exclusively from the apical and basolateral membrane of MDCK cells, respectively (23). Van Meer and Simons found that the phospholipid compositions of the two viruses were markedly different, presumably reflecting differences in apical and basolateral lipid composition. This conclusion was substantiated by the further observation that the phospholipid compositions of the two viruses were very similar when harvested from unpolarized MDCK cells obtained by disrupting the epithelium with EDTA and trypsin.

Dragsten et al. (5) have studied the diffusion of fluorescently labeled lipid probes in the apical and basolateral membranes of the A6, LLC-PK1, and MDCK epithelial cell lines. They found evidence that lipid probes that partitioned only into the outer leaflet of the membrane bilayer did not pass through the tight junction whereas those probes that could flip-flop to the inner leaflet did pass through. Thus their results imply that the tight junction may form a barrier to passage of lipids only in the outer membrane leaflet.

In this report we have employed two monoclonal antibodies, 17D7 and 18C3, as probes of epithelial polarity in the cultured toad kidney cell line A6. These antibodies were generated using a plasma membrane preparation from the A6 cells and screened against the apical membrane of intact A6 epithelia. Accordingly, both of these antibodies were expected to be directed against externally oriented membrane components on the apical surface of the A6 cells. As we demonstrate above (Table I), the 17D7 antigen is in fact expressed on both the apical and basolateral surface of these cells whereas the 18C3-antigen is localized to the apical membrane only. We have identified the 17D7-antigen as a 23-kD peptide by immunoprecipitation from [^{35}S]methionine-labeled cells followed by SDS PAGE and autoradiography (Fig. 1). No significant ^{35}S-labeled proteins were immunoprecipitated by 18C3. However, this MAb did screen positive to a lipid extract from the A6 cells and subsequently was found to react specifically with a doublet on a thin layer chromatograph of this lipid extract (Fig. 2). Owing to its solubility in organic solvents and its mobility on the thin layer chromatograph, it is highly unlikely that this antigen is a protein. However, we cannot exclude the possibility that the 18C3 antigenic site (presumably located on the glycosylated portion of the 18C3 lipid antigen) may be expressed on a glycoprotein as well. This protein may not be detected in Fig. 1 because it represents a small fraction of the total membrane protein or because it does not incorporate [^{35}S]methionine.

The results illustrated in Fig. 3 and Tables II and III document the handling of the 18C3 antibody-antigen complex by the A6 cells. These experiments characterize the transition from an initial situation where the complexes are localized to the apical membrane to a final steady state where they are redistributed among various cell compartments. The data presented here are consistent with the following scenario. The 18C3 antibody-antigen complexes are rapidly internalized (Fig. 3) into an intracellular compartment from which they are recycled to both the apical (Table II) and the basolateral membrane (Table III), reaching steady-state in 1–2 h. Since there is no loss of total cell antibody content during the internalization and recycling process (see Results) lysosomes are presumably not involved. The possibility that apical 18C3-antigen complexes reach the basolateral surface by flip-flopping past the tight junction (5) is unlikely since free 18C3-antigen obviously does not behave in this manner and since the increased size and mass resulting from antibody binding should impede rather than facilitate this process. Also, the data presented in Table III indicate that the total amount of antibody associated with the cell surface at 2 h is ~50% of that present at time zero, further substantiating the existence of an intracellular pool of antibody–antigen complexes.

The behavior of the 17D7 antibody–antigen complex is quite different from that observed for 18C3. Although this complex is apparently internalized, since it disappears from the cell surface but it still detected in solubilized cell extracts (see Results), no evidence for recycling is observed. Taken together, the results shown in Fig. 3 and Tables II and III indicate that there are two populations of 17D7-antigen complexes associated with the A6 apical membrane. One population (30–40% of the total) is rapidly internalized (complete in ~1 h) but not recycled by the cells, while the other is either anchored on the cell surface or internalized at a much slower rate.

As already stressed, information concerning the character and maintenance of epithelial lipid polarity is very limited. The results presented here provide several novel insights. The first is the identification of a lipid which is localized to one surface of an epithelium. Owing to its antigenicity, this lipid is presumably a glycolipid and thus localized to the outer leaflet of the apical membrane (24). Previous reports have demonstrated differences in lipid properties (25, 26) and in relative lipid composition (22, 25) of epithelial apical and basolateral membranes; however, to our knowledge this is the first demonstration of a localized lipid species. Although the differences in lipid composition referred to above could be accounted for by relatively nonspecific mechanisms such as asymmetric apical to basal and basal to apical passage of membrane lipids past the tight junction, the apical localization of a single lipid species indicates the existence of a specific sorting and insertion process, at least for this lipid. We also demonstrate that this sorting mechanism can be perturbed by MAb binding so that the 18C3 antibody–antigen complex is recycled to both the apical and the basolateral membrane. Although the underlying cause of this interference remains to be determined, we anticipate that it will provide a powerful probe of the lipid sorting machinery.

We thank Drs. John Kung, Lawrence Samelson, and Keiko Ozato for advice on monoclonal antibody technology, Dr. Jean Pierre Kinet for advice and assistance with SDS PAGE, Drs. John Magnani and Victor Ginsberg for advice and assistance with lipid chromatography and autoradiography, Mr. Chester Williams and Ms. Janet N. George for their technical skills, and Ms. Anne Hansen-Johnston for secretarial assistance.

This work was supported in part by a generous grant (to R. J. Turner) from the Kidney Foundation of Canada.

Received for publication 2 May 1985, and in revised form 19 August 1985.

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