Manipulation of Cell-Cell and Cell-Substratum Interactions in Mouse Mammary Tumor Epithelial Cells Using Broad Spectrum Antisera

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ABSTRACT Two antisera were raised in goats against material shed by two different mammary epithelial cell lines into serum-free tissue culture medium. These antisera, when added to the medium of intact, growing mouse mammary tumor cells in the absence of complement, cause distinct and dramatic alterations in cell morphology and adhesiveness. One antiserum (anti-SFM I) causes mouse mammary tumor epithelial cells to round and detach from the substratum. Treatment with the other antiserum (anti-SFM II) does not affect cell-substratum interactions, but causes the cells to convert from an epithelioid to a fibroblastic morphology. Statistical analysis of transmission electron micrographs of control and antibody-treated cells indicates that treatment with anti-SFM II is associated with a substantial reduction in the extent of intercellular junctions, particularly desmosomes. To identify the components with which the two antisera interact, nonionic detergent extracts of mouse mammary tumor cells were fractionated, and the ability of various fractions to block the morphological effects of either antiserum was determined. The whole Nonidet P40 (NP40) extract of the epithelial cells blocked the effects of both antisera. After the extract was subjected to ion exchange and lectin affinity chromatography, two separate fractions were obtained. One fraction blocks anti-SFM I induced rounding and detachment of cells from the substratum. The second fraction blocks the effects of both antisera. The isolation of the former fraction, which has highly restricted number of components, represents a significant first step toward identifying the surface membrane molecule(s) involved in cell-substratum adhesion in epithelial cells.

Knowledge of the molecular basis of cell-cell and cell-substratum interactions is fundamental to understanding the events that occur as cells migrate during embryogenesis or to understanding the molecular events that take place during metastasis. In the past, a great deal of work has been devoted to the study of molecules on either side of the surface membrane that may be involved in regulating cell-cell or cell-substratum interactions in a variety of cell types (reviewed in references 5, 17, 21, and 39). These studies have shown that fibronectin, along with certain types of collagen and proteoglycans, are the major components of an extracellular adhesive matrix to which fibroblasts adhere (5, 18, 27). In some cell types, noncollagenous glycoproteins other than, or in addition to, fibronectin may be important in adhesion or tissue organization. These include laminin (9, 35), a component of the basal lamina of several epithelial tissues; and CSP60, a glycoprotein that appears on the surface of vascular endothelial cells when they develop into a confluent, polygonal, nonoverlapping monolayer (36). In addition, a fibronectin-like protein termed chondronectin has been found to be involved in adhesion of differentiated chondrocytes to type II collagen (23).

Ultrastructural observation, immunofluorescence, and biochemical analyses of fibroblasts in culture have shown that cytoskeletal components, through their interactions with the cytoplasmic side of the surface membranes, also play a critical role in establishing and maintaining points of cell-substratum contact (1, 4, 11, 14, 15, 22, 25, 29, 30). Thus the organization of both the cytoskeleton and the extracellular matrix are important in the process of cell-substratum interaction in fibroblasts.

Epithelial cells, in addition to adhering to the substratum, have extensive cell-cell contacts. At the ultrastructural level,
epithelial cells are seen to adhere to one another by elaborate junctional complexes (10, 20, 28, 33). Each component of the junctional complex (tight junctions, intermediate junctions, desmosomes) has a distinctive ultrastructure, and each has a particular class of cytoskeletal filament associated with it (20, 33). Biochemical studies of junctional elements are not extensive. Gap junctions have been isolated and found to be enriched in a protein of 20,000–25,000 mol wt (13, 16). Partially purified desmosomes contain, in addition to the family of tonofilament proteins, glycoproteins ranging from 120,000–140,000 mol wt (7, 31). Nothing has been published to date on the identity of integral membrane components of tight or intermediate junctions.

Despite the progress in understanding cell-substratum interactions, particularly in fibroblasts, and in defining the ultrastructural elements involved in epithelial cell-cell adhesion, little is known about the integral membrane constituents involved in these interactions. In an attempt to identify membrane constituents involved in regulating cell-cell and cell-substratum interactions in epithelial cells, we have developed two broad-spectrum antisera that affect markedly these adhesive interactions. This report describes the effects of these antisera on mouse mammary tumor epithelial cells and presents a preliminary attempt to identify those cell surface molecules involved in regulating these complex cellular phenomena.

MATERIALS AND METHODS

Materials

d-[14C(U)]glucosamine hydrochloride (sp act. 285 mCi/mmol), l-[3H]amino acid mixture, Protosol, and the premixed scintillation solutions, Econofluor and Formula 963, were all purchased from New England Nuclear (Boston, Mass.).

[125I]serum was purchased from Amersham Corp. (Arlington Heights, Ill.), the detergent Nonidet P 40 (NP40) from Particle Data, Inc. (Elmhurst, Ill.), and Lens culinaris lectin (Lens) and wheat germ agglutinin (WGA) from Vector Laboratories (Burlingame, Calif.). Affi-Gel 102, Affi-Gel 10, and SM 2 Biobeads were purchased from Bio-Rad Laboratories (Richmond, Calif.). Dansyl chloride was purchased from Pierce Chemical Co. (Rockford, Ill.), trypsin and soybean trypsin inhibitor, from Worthington Biochemical Corp. (Freehold, N. J.). Other reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.). The mixture of seven different glycosidases, which had no detectable protease activity, was a gift from G. Ashwell at the National Institutes of Health (Bethesda, Md.).

Cells and Growth Condition

The target cell line used both to assess the effects of the two antisera discussed in this report and for purification of adhesion-related antigens was derived from a spontaneous BALB/c murine mammary tumor (This cell line was the gift of Dr. E. Lasfargues, Institute for Medical Research, Camden, N. J.). The cells were cloned in liquid medium and a clone displaying a well-defined polygonal arrangement of very flat cells was selected. These cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Flow Laboratories, Inc., Rockville, Md.), insulin (10 μg/ml), penicillin (50 μg/ml), and streptomycin (100 μg/ml). For experiments in which these cells were exposed to immune or pre-immune goat sera, the fetal bovine serum present in the culture medium was inactivated by heating at 50°C for 30 min. Cells were exposed to antiserum during the late log phase of growth and left in contact with antiserum or pre-immune goat serum for 16 h before fixation, unless otherwise indicated. To obtain sufficient material for fractionation, cells were grown in 490-cm² tissue culture roller bottles (Corning Glass Works, Corning, N. Y.) and harvested when confluent.

Preparation of Antisera

Two antisera were prepared for this study. The first was prepared against material shed into serum-free tissue culture medium by the same cells used as the target cell in these studies, i.e., the closed murine mammary tumor epithelial cells. This antiserum is referred to as anti-SFM I. The second antiserum, referred to as anti-SFM II, was prepared against material shed into serum-free tissue culture medium by the MCF-7 human mammary tumor cell line obtained from the Mason Research Institute (Rockville, Md.). This line was originally isolated and characterized by Soule et al. (32). In both cases, the serum-free medium antigen was prepared as follows. The cells were grown to near confluency, washed with phosphate-buffered saline (PBS) and then incubated for 4 h in tissue culture medium without serum. This medium was then discarded, the monolayers washed again with PBS, and fresh serum-free medium added. After 18 h, the medium was harvested, centrifuged (40,000 g, 1 h), dialyzed against 0.1× PBS, and lyophilized. The residue was dissolved in 1/10 vol of distilled water. For each injection, 1.5 ml of the reconstituted preparation containing ~1 mg protein was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into goats. Each goat received a series of three injections 2 wk apart. The goats were given booster inoculations routinely every 6–8 mo. Before use, antisera were heated to 56°C for 30 min to inactivate complement.

Preparation of Nonionic Detergent Extracts

Cells were labeled metabolically for 72–96 h with either d-[14C(U)]glucosamine hydrochloride or l-[3H]amino acid mixture before harvesting. Cells were harvested using l mM EDTA (disodium salt) in PBS (pH 7.4) containing 2 mM phenylmethylsulfonyl fluoride (PMSF) and washed three times with PBS. 40 roller bottles yielding a total of ~10⁶ cells were used for each preparation. Cells in 20 bottles were labeled with [14C]glucosamine (20 μCi/bottle) and those in the other 20 bottles with the l-[3H]amino acid mixture (50 μCi/bottle). After harvesting, the cell pellets were stored at −70°C until used. NP40 extracts of the cell pellet were prepared by thawing a pellet of 10⁶ cells in the presence of 40 ml of 0.5% NP40 and 2 mM PMSF in 0.05 M Tris acetate, pH 8.0. The material was kept on ice and frequently and vigorously pipetted over a 15-min period to facilitate extraction of detergent-soluble components. The extract was centrifuged for 50 min at 50,000 rpm (Beckman L5-75; Ti 75 rotor Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The 50,000 rpm supernatant was collected and SDS was added to a final concentration of 0.03%. The supernatant was either used immediately or rapidly frozen in acetone-dry ice and stored at −70°C until used. Such 50,000 rpm supernatants, containing ~75% of the [14C]glucosamine label and 45% of the l-[3H]amino acid label present in the original cell pellet, served as the starting material for fractionation studies.

Assay for Monitoring the Purification of Adhesion-related Molecules (Blocking Assay)

Mammary epithelial cells were plated in 96-well microtiter plates (Linbro, Hamden, Conn.) at a concentration of 5 × 10⁴ cells/well and were used 24 h later. Heat-inactivated goat anti-anti-SFM I or goat anti-SFM II serum was diluted in culture medium containing twice the usual amount each of amino acids, vitamins, glucose, fetal calf serum, penicillin, and streptomycin such that the final concentration of antiserum would induce the appropriate morphological response in 90–100% of the cells overnight. The optimum final concentrations of the two antisera were 1:200 for anti-SFM I and 1:32 for anti-SFM II. Fractions of NP40 extracts to be assayed were mixed 1:1 with heat-inactivated fetal calf serum, incubated with an equal volume of washed SM-2 Biobeads, and constantly agitated for 15 min. This treatment reduced the detergent concentration to a level that would not affect the morphology or viability of the cells. Various aliquots of the different fractions to be tested were added to 100 μl of the diluted antiserum, and the total volume was brought to 200 μl with PBS. The mixtures were added to cells and incubated overnight at 37°C. The next morning, the wells were examined for antiserum-induced morphological changes in the cells.

Fractionation of NP40 Cell Extracts on Affi-Gel 102

A water-jacketed column (1 × 30 cm) was cooled to 4°C. It contained ~18 ml of Affi-Gel 102 that was equilibrated in 0.05 M Tris acetate buffer containing 0.5% NP40 and 0.03% SDS (column buffer). Samples were applied, and the column was washed with column buffer until all unbound material was removed. Bound material was eluted sequentially with 0.05, 0.1, and 0.5 M NaCl in the column buffer. Fractions of 15 ml were collected. Radioactivity was monitored using an Intertechnique SL4000 liquid scintillation counter (IN/US, Fairfield, N. J.). Biological activity of each fraction was determined using the blocking...
fetal bovine serum. 16 h later, coverslips were rinsed briefly in Dulbecco's goat serum (1:18 dilution) in tissue culture medium containing heat-inactivated human serum. A 22-mm² coverslip. At late log phase, cell culture medium was replaced with fresh medium containing M199 and 10% fetal bovine serum. The sample was incubated for 3 min in a sample buffer containing 2% SDS and 5% 2-mercaptoethanol. The sample was incubated at 37°C for 45 min. Mercaptoethanol was added to a final concentration of 5% and the dansyl chloride-labeled samples were boiled for 3 min and analyzed by SDS PAGE.

**Polyacrylamide Gel Electrophoresis (PAGE)**

Samples were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (24). Separation was accomplished in a straight 8% polyacrylamide slab gel. Before application to the gel, samples were boiled for 3 min in a sample buffer containing 2% SDS and 5% 2-mercaptoethanol. Gels were sliced into 1-mm pieces, incubated in a 4-M Ecolonil:Protocol:water (3,500:260:25) mixture overnight at 37°C, and counted for 4 min in a liquid scintillation counter.

**Protein Determination**

Protein was measured by the method of Lowry et al. (26) using bovine serum albumin as a standard. When appropriate, detergents were added to the standard solutions in the same concentrations as contained in the samples. The white precipitate that formed in the presence of detergents was removed by centrifugation and did not appear to affect color formation significantly.

**Other Labeling Procedures**

Samples at various stages of the purification procedure were labeled with dansyl chloride in the following way: the samples were incubated in a buffer containing 0.1 M NaHCO₃, pH 8.0, and 1% SDS. 5 μl of 1% dansyl chloride solution in acetone was added per 200 μl of incubation mixture. The sample was incubated at 37°C for 45 min. Mercaptoethanol was added to a final concentration of 5% and the dansyl chloride-labeled samples were boiled for 3 min and analyzed by SDS PAGE.

**Electron Microscopy**

For scanning electron microscopy (SEM), cells were plated in 60-mm plastic petri dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) containing a 22-mm² coverslip. At late log phase, cell culture medium was replaced with anti-SFM I (1:125 dilution), anti-SFM II (1:18 dilution), or normal goat serum (1:18 dilution) in tissue culture medium containing heat-inactivated fetal bovine serum. 16 h later, coverslips were rinsed briefly in Dulbecco's modified PBS, and cells were fixed in 1.5% glutaraldehyde as described previously (6). After fixation and critical-point drying, these cells were examined in an SEM electron microscope.

**Transmission Electron Microscopy**

For transmission electron microscopy (TEM), cells were fixed in 1.5% glutaraldehyde as described previously (6). Cells were embedded in the plastic culture dishes in Araldite-Epon and sectioned either parallel or perpendicular to the substrate. Sections were stained with lead citrate and examined in a Zeiss EM-10 electron microscope.

**Freeze-Fracture Electron Microscopy**

Cells were fixed in situ with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 15 min. Fixed cells were scraped off the substrate and washed three times with 0.1 M phosphate buffer containing 5% sucrose. Cells were resuspended in 30% glycerol in the same buffer for 30 min, and centrifuged (1,000 g, 5 min). A drop of the concentrated cell suspension was frozen in Freon-22 in liquid nitrogen and fractured at -150°C in Balzers BAS-300 freeze-fracture machine (Balzers Corp., Hudson, N. H.). The specimen was shadowed using platinum carbon pellets, and the replicas processed by standard techniques.

**RESULTS**

**Morphology of Murine Mammary Tumor Epithelial Cells**

The cells used in these studies are epithelial by morphological criteria. They grow as islands of very flat, cobblestone-shaped cells which overlap one another at their edges (Fig. 1A). Their upper surfaces are covered with irregular microvilli. The TEM of cells sectioned perpendicular to the substrate (Fig. 1B) shows, in the region of cell-cell overlap, a slender sheetlike extension of one cell interacting with the upper surface of its neighbor. The extent of overlap increases with the density of culture. Intercellular junctions are present in the region of overlap (Fig. 1B and C). Further examples of such junctional specializations are seen in cells subjected to freeze-fracture (Fig. 2A) and in cells sectioned parallel to the substrate (Fig. 2B and C). Tight junctional elements, desmosomes, and gap junctions are clearly present in these cells. Furthermore, in sections cut parallel to the substrate in the zone which includes the overlap region (near the top of the monolayer) the surface membranes of neighboring mammary cells lie very close together and follow each others contours over extensive areas. Regions of surface membrane lying within the two sets of arrows (Fig. 2C) demarcate the close cell-cell contact present in these cells. Images similar to that in Fig. 2C are common and support the designation of these cells as epithelioid. Thus, Figs. 1 and 2 serve as a point of reference for examining the effects of anti-SFM I and anti-SFM II.

**Effects of Anti-SFM I and Anti-SFM II on Morphology of Murine Mammary Epithelial Cells**

When examined in the phase microscope, untreated mammary tumor epithelial cells appear polygonal in shape and tightly apposed to one another (Fig. 3A). Treatment with anti-SFM I causes the cells to round and detach from the substratum (Fig. 3B). After treatment with anti-SFM II, the cells remain attached to the substratum, but become fibroblastic in appearance (Fig. 3C). These effects on cell morphology are seen in greater detail if antibody-treated cells are examined by SEM (Fig. 4A and B; cf. Fig. 1A). In particular, it is apparent that treatment with anti-SFM II is associated with a dramatic reduction in the extent of cell-cell association. This reduction in cell-cell association was supported further at the TEM level.
by comparing the number of desmosomes found in serial thin sections, cut parallel to the substrate, of control and anti-SFM-II-treated monolayers. The number of desmosomes observed in these serial sections was reduced more than 10-fold by actual count in anti-SFM-II-treated cells compared with untreated cells. Conversion of cells from an epithelioid to a fibroblastic morphology by anti-SFM II is not an intermediate step in the process of cell rounding. This is demonstrated by the observations that at very low concentrations, anti-SFM I (rounding antiserum) does not induce a fibroblastic morphology and at high concentrations, anti-SFM II does not induce cell rounding. However, as will be shown later, anti-SFM I does contain...
FIGURE 2  TEM of control cells illustrating the variety of specific intercellular junctions displayed by this cell line. (A) TEM of a freeze-fracture replica of mouse mammary epithelial cells. Tight junctional elements (TJ) and a small gap junction (GJ) are present. x 75,000. (B) TEM of two cells cut parallel to the substrate near the top of the monolayer. The plasma membranes of the two cells lie close to one another over a considerable distance. Two desmosomes are clearly apparent (D). x 40,000. (C) TEM of portions of four cells in a monolayer cut parallel to the substrate near the top of the monolayer. The membranes of apposing cells follow the contours of each other closely over long distances. In particular, the membrane regions between the two sets of arrows are never more than 25 nm apart. x 50,000.
antibodies capable of disrupting cell-cell interactions. This activity can be detected only if the antibodies capable of disrupting cell-substratum interaction are blocked selectively.

Effects of Antiserum on Cell Growth and Viability

To determine if the effects of the antisera on cell morphology were the result of toxic factors, cells were grown in the presence of anti-SFM I or anti-SFM II (Fig. 5). Cells treated with anti-SFM I did not grow in the presence of the antiserum. This is not surprising because the cells were not adapted to growth in suspension. However, the cells remained viable throughout the 72-h exposure to anti-SFM I as judged by their ability to exclude trypan blue. Furthermore, after removal of the antibody, >95% of the cells were able to reattach to the substratum. The cells resumed growth after they had spread onto the substratum. The process of reattachment and spreading took 36-48 h (as compared with ~24 h for normally passaged cells). By that time, the cells had regained their epithelioid morphology. Cells induced to assume a spindle-shaped morphology by treatment with anti-SFM II continued to grow at the same rate as cells treated with an equal dilution of pre-immune serum. When the antiserum was removed, the cells reverted to the epithelial morphology within 24 h. These results indicate that the morphological effects induced by the antisera were completely reversible and that neither antiserum was toxic to cells even after 72 h of continuous exposure.

Blocking Assay for Detecting Surface Antigens Relevant to Adhesive Interactions in Mouse Mammary Tumor Epithelial Cells

In an attempt to determine the membrane components with which anti-SFM I and anti-SFM II interact to cause changes in cellular adhesion and morphology, cells were solubilized in NP40 and a blocking assay was developed to detect the presence of the appropriate antigens in the cell extracts. This assay was based on the premise that any material in an NP40 extract capable of blocking anti-SFM-I- or anti-SFM-II-induced disruption of cell-substratum or cell-cell interactions, must contain molecules relevant to the maintenance of such interactions in
these cells. The results of a typical blocking assay are shown in Fig. 6. In the absence of antiserum, the cells appeared polygonal and flat (Fig. 6A); in the presence of anti-SFM I, they appeared as rounded clumps (Fig. 6B); and in the presence of anti-SFM II (Fig. 6C), they appeared flat but spindle-shaped, indicating a substantial reduction in cell-cell interaction. The addition of an NP40 extract of mammary epithelial cells to either the anti-SFM I (Fig. 6E) or the anti-SFM II (Fig. 6F) serum inhibited antibody-induced morphological changes, indicating that the NP40 extract contained molecules relevant to the maintenance and/or control of both cell-cell and cell-substratum interactions. The NP40 containing buffer alone, which had been extracted with SM-2 beads, had no inhibitory effect on the activity of the antiserum. Furthermore, SM-2-extracted test fractions, when added to cells in the absence of antiserum, had no effect on cell morphology, verifying that the detergent content of the test samples had been lowered to nontoxic levels by treatment with SM-2 beads (Fig. 6D).

**Affi-Gel 102 Fractionation of NP40 Extracts from Mouse Mammary Tumor Cells**

As the first step in a scheme to separate the molecules in mouse mammary tumor cells with which the two antiseras react, an NP40 extract of these cells grown in the presence of D-[14C]glucosamine and H-amino acids was applied to Affi-Gel 102 and was eluted sequentially with 0.05, 0.1, and 0.5 M NaCl (Fig. 7). About 45% of the radioactive material associated with the D-[14C]glucosamine in the extract did not bind to the column. This material, designated “load-plus-wash” and material eluting with 0.05 M NaCl were both able to block the anti-SFM-I-induced rounding of the mammary epithelial cells. Neither of these fractions inhibited anti-SFM-II-induced disruption of cell-cell interactions. Material eluting from Affi-Gel 102 with 0.5 M NaCl blocked both anti-SFM-I-induced rounding and anti-SFM-II-induced disruption of cell-cell interactions. Material eluting with 0.1 M NaCl had no effect on the morphological changes induced by either anti-SFM I or anti-SFM II.

After dialysis against column buffer to remove NaCl, the load-plus-wash fractions and the 0.05-M NaCl fractions were pooled, applied to a smaller Affi-Gel 102 column, and eluted with 0.02, 0.04, 0.05, and 0.1 M NaCl. The material eluting between 0.02 and 0.04 M NaCl blocked only anti-SFM-I-induced rounding. None of the fractions inhibited the activity of anti-SFM II. This fraction is referred to subsequently as the “low-salt eluate”.

The active material eluting from the first Affi-Gel 102 column with 0.5 M NaCl, which blocked the effects of both anti-SFM I and anti-SFM II (Fig. 7, cross-hatched fraction) was dialyzed, reapplied to a smaller column of Affi-Gel, and eluted with 0.1, 0.2, 0.4, and 0.5 M NaCl. In this case, material eluting with 0.2 M NaCl was found to be the only active fraction. It was capable of blocking both anti-SFM-I-induced rounding and anti-SFM-II-induced disruption of epithelioid morphology. This fraction is referred to subsequently as the “high-salt eluate”.

**Lectin Affinity Chromatography of Affi-Gel Fractions Capable of Blocking Anti-SFM I and Anti-SFM II**

The active low-salt and high-salt Affi-Gel 102 eluates, re-
spectively, were fractionated further by lectin affinity chromatography. The low-salt eluate was passed over a column of Lens. Material containing 24% of the $^{14}$C-glucosamine-associated radioactivity present in the low-salt eluate bound to the lectin (Lens-positive material). This material was quantitatively eluted with 10% glucose and was capable of blocking anti-SFM-I-induced cell rounding and detachment. No blocking activity was detected in the Lens-negative material (Table I).

Because biological activity was not recovered from the high-salt eluate after fractionation on a Lens column, this material was passed over a WGA column (Table II). This lectin bound ~65% of the $^{3}$H-glucosamine and 5% of the $^{3}$H-amino acid-labeled material after three passes of the material over the column. The material that bound to the column (WGA-positive) did not block the effects of either antiserum, whereas the unbound material (WGA-negative), containing >80% of the

### Table I

| Starting material          | Total cpm ($^{3}$H x 10^{3}) | % Total applied to column | Blocking activity |
|---------------------------|-------------------------------|---------------------------|-------------------|
|                           | $^{3}$H | $^{14}$C | $^{3}$H | $^{14}$C | Anti-SFM I | Anti-SFM II |
| 0.04-M NaCl Affi-Gel 102 eluate (low-salt) | 5.8 | 16.2 | 100 | 100 | + | - |
| Unbound material (Lens-negative) | 5.6 | 11.5 | 97 | 71 | - | - |
| Bound material§ (Lens-positive) | 0.1 | 3.8 | 1.5 | 24 | + | - |

* Ability to block antibody-induced changes in cell morphological and adhesive properties.
† The bound material was eluted with 10% glucose.
§ The bound material was eluted with 10% glucose.

Fig. 8 shows the appearance in the blocking assay of mammary tumor epithelial cells treated with anti-SM I or anti-SFM II that had been blocked with the active fractions purified by the procedures described above. Fig. 8A shows cells rounded and detached by exposure to anti-SFM I. Fig. 8B shows cells treated with anti-SFM I that had been mixed with the low-salt Lens-positive material before its addition to the cells. Most of the cells remained attached to the substratum, indicating that the cell-substrate disrupting activity in the anti-SFM I serum had been inhibited by the low-salt Lens-positive material. However, the morphology of the cells was more fibroblastic than that of the untreated epithelioid control (see Fig. 6A). In contrast, cells exposed to anti-SFM I that had been blocked by the material in the high-salt WGA-negative fraction (Fig. 8C), retained the flat epithelioid appearance characteristic of control cells. Fig. 8D shows the fibroblastic shape of the epithelial cells exposed to anti-SFM II alone, whereas Fig. 8E demonstrates the ability of the high-salt WGA-negative fraction to block this anti-SFM-II-induced disruption of epithelioid morphology.

### Characterization of Fractions Active in the Blocking Assay

The extent of enrichment of molecules related to cell-cell or cell-substrate adhesion obtained by the fractionation scheme described above was determined by comparing the micrograms of protein in each active fraction required to block antibody-induced morphological changes, with that of the starting NP40 extract (Table III). The low-salt Lens-positive fraction was enriched about 40-fold in its ability to block the effects of anti-SFM I. Before the lectin affinity step, the high-salt eluate, which blocks both anti-SFM I and anti-SFM II, was enriched about 40-fold, respectively. However, subsequent passage of this material over a WGA affinity column actually reduced the specific blocking activity for anti-SFM II and provided no further enrichment for blocking activity against anti-SFM I.

The blocking activity of both samples was sensitive to trypsin treatment (Table IV). The blocking activity of the samples was not appreciably affected by treatment with mixed glycosidases under conditions that remove 60% of the $^{14}$C-glucosamine-labeled material. A 50% decrease in blocking activity of the high-salt eluate occurred as a result of boiling the sample for 5 min before testing. However, all the activity in the low-salt eluate was destroyed by this treatment. In both cases the blocking activity appeared to depend upon the integrity of a polypeptide.
Analysis of Active Fractions by SDS PAGE

The polypeptide composition of the final active fractions (low-salt Lens-positive and high-salt WGA-negative) was monitored by SDS PAGE. Fig. 9A shows the composition of the NP40 extract used as the starting material. After Lens culinaris affinity chromatography, the low-salt Lens-positive fraction contained a highly restricted group of glycoproteins migrating with an apparent molecular weight of 130,000–140,000 with a smaller peak at about 110,000 (Fig. 9B). The nonglycosylated proteins seen in the original NP40 extract (Fig. 9A) were removed from the preparation as a result of the lectin affinity chromatography step leaving only the ^3H-amino acid label that co-migrated with the ^14C-glucosamine radioactivity.

WGA affinity chromatography of the high-salt eluate resulted in the isolation of a single glycoprotein with a molecular weight of 130,000–140,000, which was the only glycoprotein present in the high-salt WGA-negative fraction (Table II). The nonglycosylated proteins seen in the original NP40 extract (Fig. 9A) were removed from the preparation as a result of the lectin affinity chromatography step leaving only the ^3H-amino acid label that co-migrated with the ^14C-glucosamine radioactivity.

**Table II**

| Starting material | Total cpm (X 10^6) | % Total applied to column | Blocking activity |
|-------------------|-------------------|--------------------------|------------------|
|                   | ^3H | ^14C | ^3H | ^14C | Anti-SFM I | Anti-SFM II |
| 0.2-M NaCl Affi-Gel 102 eluate (high-salt) | 4.4 | 5.3 | 100 | 100 | +* | - |
| Unbound fraction (WGA-negative) | 3.6 | 1.4 | 82 | 26 | + | + |
| Bound fraction‡ (WGA-positive) | 0.23 | 3.5 | 5.2 | 66 | −§ | − |

* Ability to block antibody-induced changes in cell morphological or adhesive properties.
† The bound material was eluted with 5% N-acetyl-D-glucosamine.
§ No blocking activity.

**Table III**

| Sample | mg To block anti-SFM | mg To block anti-SFM | Blocking activity | Enrichment in anti-SFM activity | Enrichment in anti-SFM II activity |
|--------|----------------------|----------------------|------------------|-------------------------------|----------------------------------|
| NP40 extract | 36 | 78 | 1 | 1 |
| Low-salt Affi-Gel 102 eluate | 4.5 | NA | 8 | NA |
| Lens-positive§ | 0.84 | NA | 43 | NA |
| High-salt Affi-Gel 102 eluate | 8.1 | 8.2 | 4 | 10 |
| WGA-negative¶ | 7.5 | 14.7 | 5 | 5 |

The amount of protein in each sample was determined by the method of Lowry (26). The minimum number of micrograms of protein required to block completely antibody-induced changes in cellular morphology or adhesive properties was determined by the blocking assay as described in the text.

* Ratio of µg protein in NP40 extract to block anti-SFM I or anti-SFM II to µg protein in sample to block anti-SFM I or anti-SFM II. 
† NA = not applicable.
§ Lens-positive refers to material in the 0.04-M NaCl Affi-Gel 102 eluate that binds to and is eluted from Lens culinaris by glucose.
¶ WGA-negative refers to material in the 0.2-M NaCl Affi-Gel 102 eluate that does not bind to wheat germ agglutinin.

**Table IV**

| Sample | Treatment | Blocking activity after treatment |
|--------|-----------|----------------------------------|
| Lens-positive | Heat* | -† |
| | Trypsin§ | NA § |
| | Mixed glycosidase¶ | +** | NA |
| WGA-negative | Heat* | ±|| |
| | Trypsin§ | - | ± |
| | Mixed glycosidase¶ | + | ± |

| Sample | Treatment | Blocking activity after treatment |
|--------|-----------|----------------------------------|
| Lens-positive | Heat* | -† |
| | Trypsin§ | NA § |
| | Mixed glycosidase¶ | +** | NA |
| WGA-negative | Heat* | ±|| |
| | Trypsin§ | - | ± |
| | Mixed glycosidase¶ | + | ± |

* 100°C for 5 min.
† Complete loss of blocking activity after treatment.
§ NA = not applicable.
|| 10 µg, 37°C for 2 h; trypsin activity stopped by the addition of soybean trypsin inhibitors. Control samples incubated at 37°C for 2 h followed by the addition of soybean trypsin inhibitors showed no loss of blocking activity.
¶ A mixture of glycosidases from Diphobococcus.
** Blocking activity equal to that of control sample.
|| 50% decrease in blocking activity when compared to control sample after treatment.

**Figure 8** Results of a blocking assay to determine the ability of low-salt Lens-positive and high-salt WGA-negative fractions of an NP40 extract to block anti-SFM I- and anti-SFM-II-induced effects on morphology and adhesiveness of mouse mammary tumor epithelial cells. (A) Cells treated with anti-SFM I. (B) Cells treated with anti-SFM I mixed with the low-salt Lens-positive fraction (Table I). (C) Cells treated with anti-SFM I mixed with the WGA-negative fraction (Table II). (D) Cells treated with anti-SFM II. (E) Cells treated with anti-SFM II mixed with a WGA-negative fraction (Table II).
sulted in a fraction that blocked both anti-SFM I and anti-SFM II. This material did not bind to WGA and contained little $^{14}$C-glucosamine-labeled material. The electrophoretic pattern of the metabolic label showed that this fraction was

![Figure 9](image_url)  
**FIGURE 9** Electrophoretic analysis of the fractionated NP40 extracts of cells and fractions after lectin affinity chromatography that are capable of blocking anti-SFM I or anti-SFM II. Mammary tumor epithelial cells labeled with $^3$H-amino (---) and $^3$H-amino (---) were extracted with NP40 and fractionated on Affi-Gel 102 as described in Materials and Methods. Molecular weight markers used in SDS PAGE were actin (43,000), bovine serum albumin (68,000), $\alpha$-actinin (95,000), and myosin (200,000). (a) NP40 extract of mammary tumor epithelial cells. This material blocks the effects of both antisera. (b) Lens-positive material from the low-salt Affi-Gel 102 eluate (Table I). This material blocks anti-SFM I-induced rounding of mammary tumor epithelial cells. (c) WGA-negative material from the high-salt Affi-Gel 102 eluate (Table II). This material blocks the effects of both antisera.

![Figure 10](image_url)  
**FIGURE 10** Comparison of components present in the low-salt Lens-positive fraction as detected by Coomassie Blue, dansyl chloride, and $^{125}$I. Aliquots of the low-salt Affi-Gel 102 eluate before and after Lens affinity chromatography were labeled either with dansyl chloride or with $^{125}$I before electrophoresis on 8% polyacrylamide gels containing SDS as described in Materials and Methods. The composite of the electrophoretic analysis shows (A and B), the components as visualized by Coomassie Blue staining, in the low-salt eluate and the Lens-positive material from this eluate containing blocking activity for anti-SFM I; (C and D) the negative image of the dansyl chloride-labeled components in the low-salt eluate and the Lens-positive material from this eluate; (E and F) an autoradiogram of the $^{125}$I-labeled components present in the low-salt eluate and the Lens-positive material from this eluate.

still quite complex and appeared to be enriched in polypeptides with molecular weights of 97,000, 93,000, 60,000, and 50,000 (Fig. 9 C). Only the 97,000-dalton component contained appreciable $^{14}$C-glucosamine-labeled material.

To detect any additional components that might have been present in the low-salt Lens-positive fraction, but that contained too little radioactivity to be detected, this fraction was labeled with either dansyl chloride or $^{125}$I. The Coomassie Blue staining patterns of the material present in the low-salt eluate before and after lectin affinity chromatography are shown in Fig. 10 A and B, respectively. The 130,000-140,000-dalton band is the principal component present; a faint band can be seen at 110,000 (Fig. 10 B). The Coomassie Blue staining pattern therefore agrees with the metabolic labeling pattern as shown in Fig. 9 B. Fig. 10 C and D reveal the components in these same samples that are labeled with dansyl chloride. The dansyl chloride labeling pattern corresponds precisely to the Coomassie Blue staining pattern. Fig. 10 E shows the low-salt eluate labeled with $^{125}$I, whereas Fig. 10 F shows the iodinated components present in the Lens-positive material from the low-salt eluate. Again, components migrating with apparent molecular weights of 130,000-140,000 and 110,000 are the major labeled constituents.

**DISCUSSION**

Antisera have been used in several systems as tools to probe the interactions of cells with themselves and with various substrates (2, 3, 6, 8, 12, 19, 34, 38). Such antisera provide nontoxic agents that can be used to alter cellular behavior reversibly. Because of antibody specificity, they are potentially useful in identifying the membrane constituents involved in certain aspects of cell behavior. For example, using such probes, Edelman and his colleagues (3, 34) have identified
material with apparent molecular weight 140,000 which is involved in embryonic neural retina cell aggregation in vitro. Immunologically distinct material with a molecular weight of 68,000 has been implicated in embryonic liver cell aggregation (2). Hsieh and Sueoka (19) have prepared antiserum against a rat neural tumor cell line. This antiserum causes cells to round and detach from culture plates and also interferes with cell spreading. They have correlated, by adsorption experiments, the biological activity of this antiserum with the presence of proteins of 120,000 and 80,000-90,000 daltons.

In the work described here we have used antiseras to probe the cell surface to gain some understanding of the membrane constituents that influence cell-substratum or cell-cell interactions in epithelial cells. Because there are no clues as to which constituent(s) may be of paramount importance in these phenomena, we have followed the approach used previously by us to probe for molecules involved in cell-substratum adhesion in hamster fibroblasts (6, 38) of preparing broad-spectrum antiseras that can alter cellular behavior in a specific and nontoxic manner. Two such antiseras, anti-SFM I and anti-SFM II, were raised in goats against components shed into serum-free tissue culture medium by two different tumor epithelial cell lines. These data document the effects of the two antiseras upon the morphology of mouse mammary epithelial cells in culture and describe an approach for the preliminary identification of those membrane constituents that may play some role in regulating cell-cell and cell-substratum adhesion.

It was important at the outset to establish that the mouse mammary tumor cells used as the target cells in this study were, in fact, epithelioid. Figs. 1 and 2 illustrate the polygonal array of these cells in confluent monolayers and the existence of intercellular junctional elements commonly associated with epithelial cells (10, 28, 33). Our assessment was based on morphological criteria and we did not attempt to study the physiological properties of these cells.

Figs. 3 and 4 indicate that the two antiseras have dramatically different effects on the morphology and adhesiveness of mammary tumor epithelial cells. Anti-SFM I clearly affects cell-substratum interactions, and under the appropriate circumstances, it is possible to show that it is also capable of disrupting cell-cell interactions (Fig. 8 B). It therefore appears to be a more complex antiserum than anti-SFM II in terms of its effect on cell behavior. Anti-SFM II causes a dramatic reduction in the extent of cell-cell association. This is apparent from the appearance of control and anti-SFM II-treated cells by phase microscopy (Fig. 3 C) and SEM (Fig. 4 B). In addition, monolayers of anti-SFM-II-treated cells showed more than a 10-fold reduction in the number of desmosomes when compared to control monolayers. The antiserum probably also has effects on junctional elements other than desmosomes. In serial sections of monolayers cut parallel to the substratum, a zone was observed near the top of the control monolayer in which the fractional surface area of plasma membrane of cells in close contact with that of their neighbors (see Fig. 2 C) was ~0.6 (37). No such zone enriched in close cell-cell contact was observed in serial sections of anti-SFM-II-treated cells. These data suggest that the control monolayers have an extensive (but not necessarily continuous) belt of tight and/or intermediate junctions which is affected by anti-SFM II treatment. Comparison by TEM of control and anti-SFM-II-treated cell monolayers sectioned perpendicular to the substrate showed no indication that anti-SFM II treatment affected cell-substratum interactions (data not shown). Thus, anti-SFM II treatment is associated with a selective disruption of cell-cell interactions, which permits the cells to assume a fibroblastic shape. Because the antiseras are complex, it is not possible to determine precisely how each produces its characteristic effect on cells. For example, it will not be possible, until more specific antiseras are obtained, to determine whether anti-SFM II acts directly upon regions of the cell surface involved in junctional specializations, or whether it interacts with other surface antigens that affect the ability of these cells to maintain their intercellular adhesive interactions.

The selectivity in the morphological alterations produced in the same target cell, suggests that the two antiseras are interacting with distinct populations of molecules on the cell surface. To document this further, and to begin to focus on surface molecules likely to be important in adhesive behavior, we developed a blocking assay to identify the presence of adhesion-related antigens in nonionic detergent extracts of cells. This assay was used to follow the fate of adhesion-related molecules while a scheme for their isolation was developed. In this manner, two fractions of interest were identified. One of these fractions, the low-salt Lens-positive material (Table I; Fig. 8 B), could block only anti-SFM-I-induced cell rounding and detachment from the substratum. SDS PAGE analysis of the material in this fraction revealed a major population of glycoproteins of 130,000-140,000 daltons detectable by all the labeling procedures used and a less readily labeled constituent at 110,000 daltons (Figs. 9 and 10). That these molecules are capable of direct interaction with anti-SFM I can be demonstrated by the fact that they bind to an immunoaffinity column containing immobilized anti-SFM I immunoglobulin and can be eluted in a form that is still capable of blocking anti-SFM-I-induced cell rounding and detachment from the substratum. Analysis by SDS PAGE shows that the 130,000- to 140,000-dalton band is still the major component in the eluted material (data not shown).

Evidence that the antigens in the low-salt Lens-positive eluate are involved in regulating cell-substratum interaction and not cell-cell interaction is gained from examining the behavior of cells that have been exposed to anti-SFM I mixed with these antigens (Fig. 8). The ability of anti-SFM I to induce the rounding and detachment of cells from the substratum is blocked, but the cells assume a fibroblastic appearance similar to that of cells treated only with anti-SFM II (see Fig. 8 B and D). This suggests that both anti-SFM I serum contains antibodies that are able to interfere with cell-cell interactions as well as antibodies capable of disrupting cell-substratum adhesion, and that the restricted number of components found in the low-salt Lens-positive fraction contains molecules involved in blocking only those antibodies in the anti-SFM I serum that react with surface molecules related to cell-substratum adhesion. Thus, we have restricted to one fraction a population of glycoproteins involved in regulating cell-substratum interactions in epithelial cells. The observation that this fraction does not interfere with anti-SFM II-induced disruption of cell-cell interactions also supports this hypothesis.

The picture with respect to the antigens in the second active fraction which blocks the effects of both antiseras is much less clear. This fraction consists of a large number of either nonglycosylated or poorly glycosylated proteins (Fig. 9 C), none of which seems to resemble material in the low-salt Lens-positive eluate which blocks anti-SFM I (Fig. 9 B). Several attempts 3 Damsky, C. H. Unpublished observations.
have been made to fractionate this material further in order to separate the blocking activities against the two antisera. However, the two activities remain associated after differential precipitation with acetone or acetic acid, and after immunoadsorption with either immobilized anti-SFM II or anti-SFM I.

The fact that both high-salt WGA-negative and the low-salt Lens-positive material are able to block anti-SFM I may seem somewhat puzzling. The very different behavior exhibited by the two fractions during the Affi-Gel 102 and lectin fractionation steps and their different polypeptide compositions on SDS PAGE makes it unlikely that the two fractions have an antigen in common that blocks anti-SFM I. Although we have not ruled out that possibility, our results could also be explained by assuming that more than one protein or glycoprotein is involved in regulating a phenomenon as complex as cell-substratum adhesion, and that anti-SFM I can induce cell rounding and detachment only if antibodies bind to all the relevant molecules. The blocking of antibody binding to any one potentially active molecule would then be sufficient to block the entire antibody-induced morphological alteration. Progress in deciding among these and other possible explanations will require development of additional purification procedures and more specific antibodies.

In conclusion, isolation of the low-salt Lens-positive fraction represents a significant first step toward identifying the molecule(s) relevant to cell-substratum adhesion in epithelial cells in that it permits us to focus on a highly restricted population of membrane glycoproteins, at least one of which is clearly involved in cell-substratum adhesion. Experiments are in progress to produce monospecific antibodies to the individual components present in this fraction. These specific probes should permit us to localize the distribution of the antigen(s) involved in regulating cell-substratum adhesion both in vivo and in vitro and should enable us to affinity purify sufficient amounts of the relevant antigen(s) for structural studies.

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