THE MOLECULAR NATURE OF THE CELL SURFACE-CYTOSKELETON INTERACTION IN MICROVILLI OF 13762 RAT MAMMARY ADENOCARCINOMA CELLS

By

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DEDICATION

To my parents, Bong-Hyun and Jung-Sook,

for their love.

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NOMENCLATURE

ASGP	-	Ascites sialoglycoprotein
ATP	-	Adenosine triphosphate
BSA	-	Bovine serum albumin
^{o}C	-	Degrees centigrade
CAG	-	Cytoskeletal associated glycoprotein
cm	-	Centimeter
Con A	-	Concanavalin A
DNase	-	Deoxyribonuclease
DOC	-	Deoxycholate
DPM	-	Disintegrations per minute
EDTA	-	Ethylenediamine tetraacetate
EGTA	-	Ethyleneglycol-bis-(β-amino-ethylether)N,N'-tetraacetate
F-Actin	-	Filamentous actin
g	-	Gravity
G-Actin	-	Globular actin
GEM	-	5 mM glycine, 1 mM EDTA, 5 mM β-mercaptoethanol, pH 9.5
>	-	More than
hr	-	Hour(s)
IEF	-	Isoelectrofocusing
K	-	Thousand
<	-	Less than
M	-	Molar
α-MM	-	Alpha methylmannoside

mM - Millimolar

Min - Minute(s)

Mr - Molecular weight

MAT - Mammary ascites tumor

mCi - Millicurie

mg - Milligram

ml - Milliliter

OA - Ovalbumin

PAGE - Polyacrylamide gel electrophoresis

PBS - Phosphate buffered saline (120 mM NaCl, 5 mM KCl, 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.4)

PIPES - Piperazine-N,N'-bis[2-ethane-sulfonic acid]

PMSF - Phenylmethyl sulfonyl fluoride

PNA - Peanut agglutinin

rpm - Revolution per minute

S buffer - 5 mM Tris, 0.15 M NaCl, 2 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM dithioerythreitol, 0.5% Triton X-100, 0.1 mM PMSF, pH 7.6 buffer

SDS - Sodium dodecyl sulfate

sec - Second(s)

TEM - Transmission electron microscopy

TES - N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid

v - Volume

w - Weight

µCi - Microcurie

µg - Microgram

µl - Microliter

µm - Micrometer(s)

CHAPTER I

INTRODUCTION

The organization of the mammalian cell surface components plays a significant role in a variety of physiological properties, which directly relate to neoplastic transformation, such as cell growth, communication, development, differentiation, division, movement, and escape from immune destruction (91, 92, 97). The control of organization of the cell surface molecules is widely believed to depend at least in part on a transmembrane interaction between these molecules and a submembrane cytoskeleton composed of actin and its associated proteins. Direct and indirect evidences for the connections of the submembrane cytoskeleton to the membrane come from several types of studies. 1) Cell surface proteins of the mature human erythrocyte are relatively immobile (2, 105, 118). Some of these proteins are linked to a submembrane cytoskeleton composed of spectrin, actin and associated proteins (3, 11, 12, 50). If this cytoskeleton is disrupted, the mobility of the cell surface components is increased (32, 33, 90). 2) Ligand-induced directed movements of cell surface components (capping) are altered by agents that modify cytoskeletal elements (91). 3) Actin and its associated proteins are collected immediately beneath the plasma membrane region containing the capped ligands during the capping (9, 27, 94, 116). 4) Some cell surface components are concentrated in regions of the plasma membrane containing organized

arrays of microfilaments (28). 5) Cell surface specializations are often associated with organized submembrane cytoskeletal elements (8, 29, 38, 59, 62, 76). However, these elements are not always microfilaments (8, 59). Thus it seems likely that there are multiple mechanisms for cell surface-cytoskeleton interactions.

Evidence for a linkage of plasma membrane components to cytoskeletal elements is largely circumstantial except in the case of the erythrocyte. The major problem is to obtain cell surface fractions which retain recognizable membrane-cytoskeleton linkages. Most methods for preparing animal cell plasma membranes suffer from difficulties in recognizing and removing intracellular organelles (23). Intracellular vesicles and granules may contain actin and/or actin binding sites (100). Thus, the isolation of a plasma membrane fraction containing actin does not guarantee that one has a cell surface-cytoskeleton interaction site. To circumvent this problem, most studies of membrane-cytoskeleton interactions have used specialized cells or cell surface regions. The primary systems studied have been the erythrocyte membrane, intestinal brush border microvilli, and fibroblast adhesion sites. Unfortunately, these all suffer from two problems; they are highly specialized membrane regions and they are regions of low membrane component mobility. Thus they may not be appropriate models for dynamic regions of the cell surface.

The erythrocyte membrane is the only case in which the interactions between the cytoskeleton and the membrane can be described in molecular detail (11, 12). The cytoskeleton is composed of spectrin tetramers (48, 74) and possibly higher (84) polymers associated with actin oligomers (24) and band 4.1 (11, 12). The spectrin is linked

to the membrane via ankyrin (4), which also is associated with the integral membrane protein involved in anion movements, band 3 (2, 3, 11, 12, 50). Interestingly, only 10-20% of the membrane band 3 molecules appear bound to ankyrin. There may be other loci of interaction of the cytoskeleton with membrane components. For example, PAS-2 remains associated with cytoskeletal residues after nonionic detergent extraction (85).

The erythrocyte membrane has been an excellent system for developing concepts and technologies for studying membrane-cytoskeleton interactions. How applicable the molecular details are to more complex cells, particularly those with dynamic membranes, remains to be seen. Much attention has been paid recently to the discovery of spectrin and spectrin-related (fodrin) proteins (43, 49, 71, 87, 101) and ankyrin-like proteins (5, 26) in many other cell types. Discovery of these proteins in membrane preparations has led to the speculation that they may perform the same function in other cells that they do in the erythrocyte. However, most plasma membrane preparations do not have nearly as much spectrin as the erythrocyte membrane, and the spectrin/actin ratio is far different. Thus, if spectrin, ankyrin and actin are forming the linkage between the membrane and cytoskeleton in these systems, they must be organized differently than in the erythrocyte.

The intestinal brush border is another system which has been used extensively for investigations of the submembrane cytoskeleton (13, 76, 82, 120). Brush border microvilli contain actin microfilaments associated into bundles by fimbrin (68 K) and villin (95 K) (13, 15, 77, 120). Interestingly, the villin bundling activity is present only at low calcium concentrations. Above micromolar calcium villin severs

microfilaments (44, 45). Microvillar microfilaments appear to be attached in two ways. At the tips of the microvilli there is a dense plaque into which the microfilaments appear to insert (82, 83). Lateral connections of the bundles to the plasma membrane are observed by electron microscopy (76, 83). Correlative electron microscopy and sodium dodecyl sulfate polyacrylamide gel electrophoresis studies suggest that the linker is a 110 K polypeptide (76, 78), which is a calmodulin-binding protein (46, 57). The microfilament bundles extend into the terminal web region of the cell, where they interact with myosin, α-actinin, tropomyosin and spectrin-like proteins (39). The organization of this region of the cell is much more complex than the brush border microvilli (56).

Fibroblast adhesion sites, which are the termini of stress fibers (7), are clearly sites where microfilaments interact with membranes. By immunofluorescence and immunoelectron microscopy, vinculin (130 K) (40) and α-actinin (100 K) (68) have been shown to be associated with these sites. Vinculin has been shown to be closer to the membrane than α-actinin (39, 41), and has been proposed as the membrane-linking protein. However, it also appears to have actin bundling activity (63), and may simply act to organize actin filaments near the membrane. Vinculin has been reported to bind to the ends of actin filaments (73), and a larger form (150 K) of vinculin with more hydrophobic properties has been reported (113).

Proteins which bind to actin must be important to the interaction of microfilaments with membranes. A variety of actin-binding proteins have been isolated and characterized (25, 67, 121). These can be divided into four functional classes (Table I): crosslinking proteins,

TABLE I

CLASSIFICATION OF ACTIN-BINDING PROTEINS*

Function	Mr X 10^{-3}	Ca^{2+}-sensitivity
Crosslinking Proteins		
Bundling Proteins		
Fascin	58	-
Fimbrin	68	-
Vinculin	130	+
Network Forming Proteins		
Filamin	250-270	-
α-actinin	100	+
Capping and Fragmenting Proteins		
Gelsolin	90	+
Villin[a]	95	+
Fragmin	42	+
G-actin Binding Proteins		
Profilin	14-16	-

*Data taken from Reference 121.

[a]Also network forming protein.

capping proteins, fragmenting proteins and monomer binding proteins. Two primary types of crosslinking proteins have been found. Bundling proteins, such as fimbrin (14, 42) or fascin (58 K) (17), are usually monomers, while network-forming proteins, such as filamin (250 K subunit) (51, 119) or α-actinin (117), are usually dimers of large subunits. Capping, fragmenting and monomer binding proteins all may be involved in the regulation of microfilament length in different systems by shifting monomer-polymer, monomer-oligomer or oligomer-polymer equilibria (25). Any protein which binds to actin filaments could potentially be involved in binding them to membranes, e.g. a capping protein, which binds the filament end, could link the filament to a membrane (73). Lin and coworkers have isolated large protein complexes from erythrocyte and other membranes that promote actin polymerization. These might be membrane sites for regulating actin polymerization (72, 73). However, they might also be fragments of membrane-associated F-actin eluted from the membrane with associated proteins.

Polymerized actin arrays have been studied ultrastructurally in a number of systems, including elegant structural analyses of microfilament bundles in stereocilia (29) and brush border microvilli (76). Fewer studies are available on the biochemistry of such systems, the most thorough being on the brush border microvilli, as described earlier. Characterizations of actin-associated proteins from invertebrate eggs (17) and acrosomes (119) have been reported. Changes in polymerized actin and associated proteins have been described to occur during platelet activation (36, 47, 86, 98, 102). A number of studies of cell cytoskeletons have described proteins remaining after nonionic

detergent extraction (16, 34, 54, 95, 106). Such results are difficult to interpret without further investigations of particular protein-protein interactions or fractionations (88, 114). One promising approach has been to isolate selectively tropomyosin-containing stress fibers using anti-tropomyosin (79).

Membrane-associated cytoskeletons or lamina (37, 69) have been prepared by detergent extractions of various types of membrane preparations for identification and partial characterization of the proteins (80, 81). In another approach Koch and Smith (66) have demonstrated association of H-2 histocompatibility antigens with microfilaments of filopodia by myosin affinity precipitation. HLA-A2 antigen, purified by affinity isolation, contains variable amounts of actin unless depolymerization of the cytoskeleton is carried out before antigen solubilization (99). Fluorescence studies indicate HLA-A2 antigen interacts with cytoskeletal components, but the molecular nature of the interacting component is unknown. Association of cell surface glycoproteins with cytoskeletal preparations after treatment of cells with Con A has been demonstrated (96, 111). It was postulated that Con A induces attachment of the membrane glycoproteins to the cytoskeleton, but no information was given about the site or mechanism of attachment. One problem with studying detergent-insoluble cytoskeletons is that proteins other than cytoskeletal proteins may be insoluble in the detergents. Also actin is often a nonspecific contaminant of immunoprecipitations. Thus it is necessary to use alternative methods to demonstrate that any particular component is really associated with the cytoskeleton.

A major problem in studying the more generally applicable systems

is the difficulty of obtaining discrete, highly purified cell surface or plasma membrane fractions with the attached cytoskeletal elements. Carraway's laboratory has circumvented many of the problems of investigating membrane-cytoskeleton interactions and the submembrane cytoskeleton by using two sublines of the 13762 ascites mammary adenocarcinoma tumor cells (Figure 1) which represent the extremes of cell surface behavior (Table II). The cell surfaces of the MAT-C1 cells are covered with the highly branched microvilli and are xenotransplantable. The MAT-C1 cell surface Concanavalin A receptors are essentially immobile. In contrast the MAT-B1 cells have the unbranched microvilli and are not xenotransplantable. The MAT-B1 cell surface Concanavalin A receptors are quite mobile (20, 60). Huggins et al. (60) have observed that the microvilli on MAT-C1 cells are more stable to such treatments as hypotonic swelling or cytochalasin perturbations than are the microvilli on MAT-B1 cells or other ascites or cultured cells. Microvilli, a defined cell surface fraction could be isolated without cell disruption and contamination from intracellular elements (18, 21). Like brush border, these microvilli have an internal core of microfilaments. However, this core is not highly structured as in the brush border microvilli (18). Moreover, the major cytoskeleton proteins from the microvilli are those expected to be found near dynamic regions of the plasma membrane and are not the same as found in brush border microvilli, except for actin (21). Lactoperoxidase labeling shows that the major accessible cell surface proteins are not different in the microvilli from the intact cells (18). When microvilli membranes are solubilized in an actin-stabilizing buffer, a substantial fraction of the actin is found by sucrose density

Figure 1. 13762 Cells.

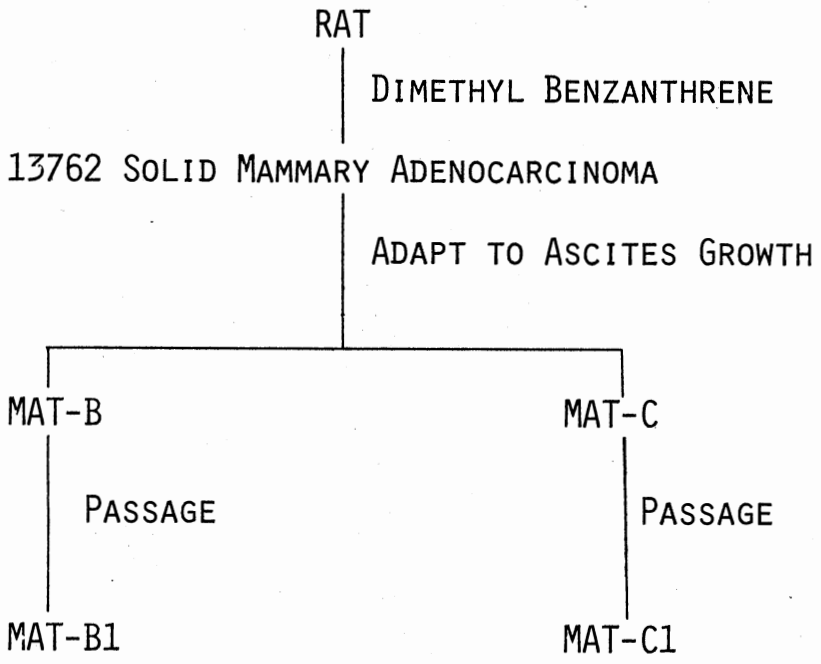

RAT
DIMETHYL BENZANTHRENE
13762 SOLID MAMMARY ADENOCARCINOMA
ADAPT TO ASCITES GROWTH
MAT-B
MAT-C
PASSAGE
PASSAGE
MAT-B1
MAT-C1

TABLE II

PROPERTIES OF 13762 ASCITES TUMOR CELLS

	MAT-B1	MAT-C1
Xenotransplantability	-	+
Con A Receptor Redistribution	+	-
Branched Microvilli	-	+
Stability to Cytochalasin	-	+
Stability to Hypotonic	-	+
58 K Polypeptide	-	+

gradient centrifugation or gel filtration to be intermediate (oligomeric) in size between monomeric and filamentous actin (18).

The objective of this study was to investigate the membrane-cytoskeleton interactions. In the present investigation, we have isolated the stable actin-containing transmembrane complexes, a putative membrane-cytoskeleton interaction site, from both of these sublines and demonstrated that the complexes from the two sublines differ by a single protein component. In addition we have used Con A crosslinking to demonstrate the actin-glycoprotein interaction by CsCl gradient centrifugation, taking advantage of the high density of the ASGP-1/ASGP-2 complex to shift the transmembrane complex on the gradient after linkage of the two complexes by Con A. We have provided evidence that the sialoglycoprotein complex (ASGP-1/ASGP-2) becomes linked to the cytoskeleton by Con A crosslinking of ASGP-2, which is a major Con A binding protein, of the sialoglycoprotein complex to CAG of the transmembrane complex. CAG-cytoskeleton interaction has also been demonstrated by myosin precipitation, DNase-induced changes in CAG sedimentation (19), and rate-zonal sucrose density gradient centrifugation.

CHAPTER II

MATERIALS AND METHODS

Materials

1-^{14}C-D-glucosamine (40 mCi/mmol), 1-^{14}C-L-leucine (56 mCi/mmol), and 4,5-^{3}H-L-leucine (39 Ci/mmol) were obtained from ICN. Chemicals for electrophoresis were from Bio-Rad. All enzymes and chemicals for buffers, chromatography, and extractions were products of Sigma. Instagel was from Packard. Delume was from Isolab. CsCl (99.95% purity, optical grade) was a product of Varlacoid.

Methods

Cells

MAT-B1 and MAT-C1, 13762 rat mammary gland ascites tumor sublines, were maintained by weekly intraperitoneal injection of approximately 1.0 X 10^6 (MAT-B1) and 1.4 X 10^6 (MAT-C1) cells in 0.1 ml of 0.9% NaCl into 60-90-day-old female Fisher 344 rats. On the seventh day after injection the rats were sacrificed and ascites cells were recovered from the peritoneal cavity by aspiration and washed two or three times by suspending in cold PBS (phosphate-buffered saline, 120 mM NaCl, 2 mM NaH_2PO_4, 20 mM Na_2HPO_4, pH 7.4) containing 2 mM $MgCl_2$ and 0.1% glucose and by centrifuging at 120 X g for 3 min. Metabolic labeling with ^{14}C-glucosamine (10 μCi), ^{14}C-leucine (10 μCi), or ^{3}H-leucine

(100 μCi) was accomplished by injection of the compound in 0.3 ml of 0.9% saline into the peritoneal cavity of a tumor-bearing rat approximately 16 hr prior to killing and recovery of the cells.

Isolation of Microvilli

The microvilli from both MAT-B1 and MAT-C1 sublines were isolated by the procedure outlined in Figure 2. Cells were incubated at 37°C in PBS-Mg^{2+}-glucose containing 20% calf serum for 30 minutes. After shearing of microvilli from cells by 12-14 passages through a 14-gauge syringe needle, the suspensions were centrifuged at 750 X g for 5 min to remove cell bodies. The supernates were then centrifuged at 40,000 X g for 30 min to pellet the microvilli. The microvilli were suspended in PBS-$MgCl_2$ and washed once.

Preparation of Microvillar Membranes

To prepare the microvillar membranes, the washed microvilli were incubated in GEM buffer (5 mM glycine-1 mM EDTA-5 mM β-mercaptoethanol, pH 9.5) at room temperature for 30 minutes, followed by homogenization in a Dounce homogenizer fitted with a tight pestle (30-35 strokes). The homogenate was centrifuged at 10,000 X g for 15 min at 4°C to sediment the residual microvilli and large fragments. The supernate was then centrifuged at 150,000 X g for 1 hr to give membrane fractions, which were washed once. The outline is described in Figure 3.

Extraction of Microvilli and Microvillar Membranes

Detergent extractions of microvilli and microvillar membranes were

Figure 2. Microvilli Isolation.

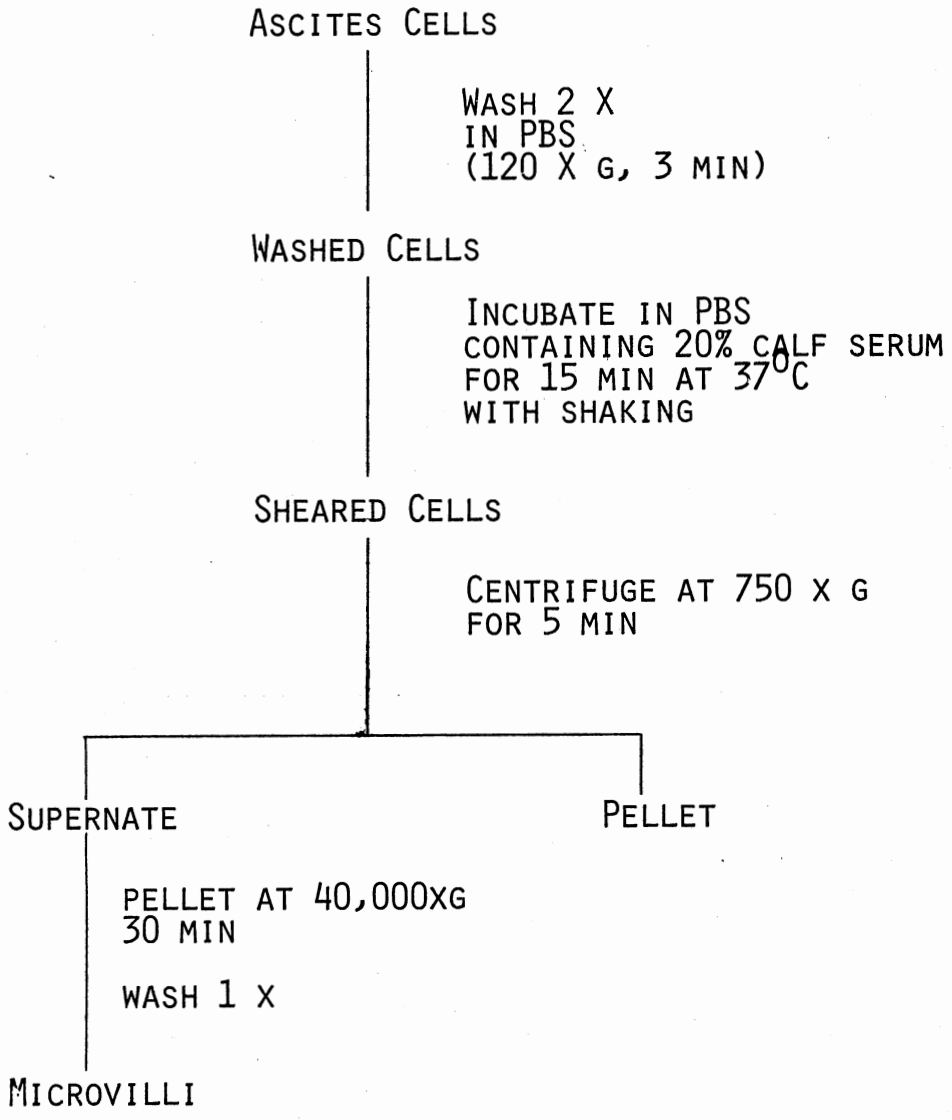
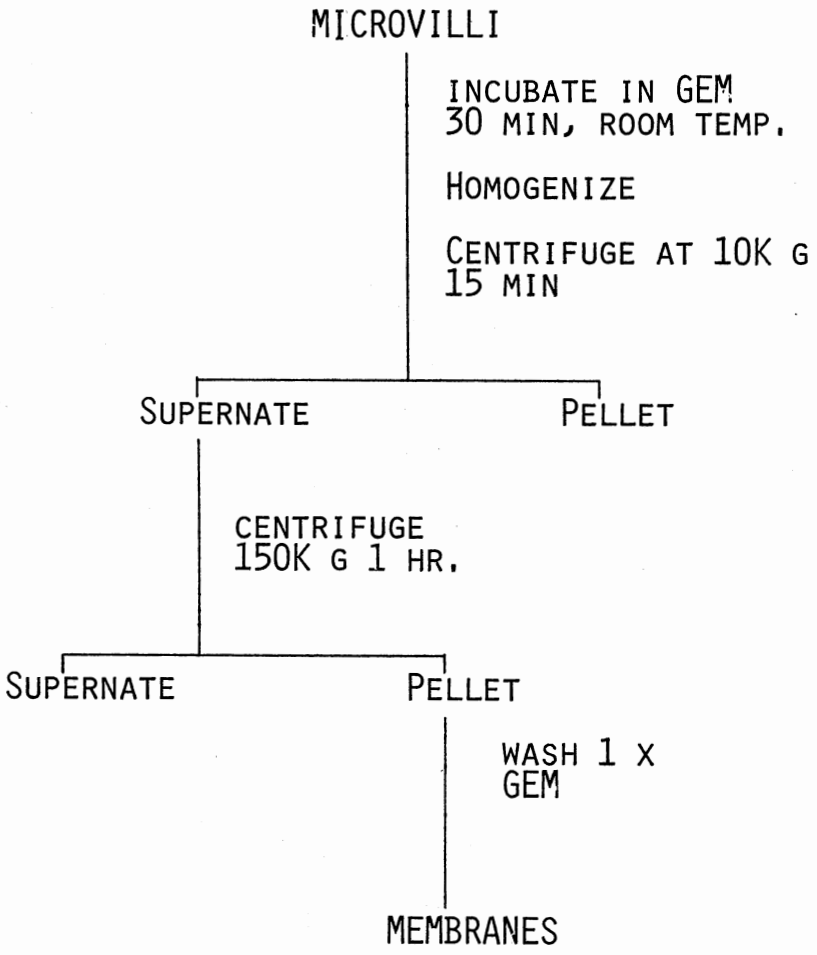

ASCITES CELLS
WASH 2 X
IN PBS
(120 X G, 3 MIN)
WASHED CELLS
INCUBATE IN PBS
CONTAINING 20% CALF SERUM
FOR 15 MIN AT 37°C
WITH SHAKING
SHEARED CELLS
CENTRIFUGE AT 750 X G
FOR 5 MIN
SUPERNATE
PELLET
PELLET AT 40,000XG
30 MIN
WASH 1 X
MICROVILLI

Figure 3. The Preparation of Microvillar Membrane.


MICROVILLI
INCUBATE IN GEM
30 MIN, ROOM TEMP.
HOMOGENIZE
CENTRIFUGE AT 10K G
15 MIN
SUPERNATE
PELLET
CENTRIFUGE
150K G 1 HR.
SUPERNATE
PELLET
WASH 1 X
GEM
MEMBRANES

performed in an appropriate buffer containing Triton X-100 or deoxycholate (DOC) for 15 min at room temperature and at 37°C for 10 min. The exact conditions used are described in the figure legends.

Transmission Electron Microscopy

This was performed as described by Hinkley (55). The pelleted samples were gently resuspended in a small volume of appropriate buffer and transferred to Formvar-carbon coated 200 mesh grids. The samples on grids were rinsed in rapid succession with three drops each 1 mg of cytochrome c per ml in the distilled water, distilled water, and 5% uranyl acetate prepared from a saturated solution just before use. The last drop of uranyl acetate was allowed to stand on the grid for 30 sec before carefully blotting out with filter paper. The grids were air-dried and then examined in a Phillips 300 electron microscope operating at 60 KV.

Chemical Treatments

The microvilli and the microvillar membrane fractions were incubated at 37°C in both the absence and presence of Concanavalin A (Con A) in PBS, pH 7.4 for 15 min in a water bath. The addition of α-methylmannoside (α-MM) as a competitive inhibitor of Con A served as a control for specific Con A binding. The unbound reagents were removed by centrifugation at 40,000 X g for 15 min. The amounts of α-MM and Con A used are shown in each figure legend.

Lactoperoxidase-catalyzed iodination of the microvilli was performed according to the method of Hubbard and Cohn (58). For neuraminidase treatments, microvilli were incubated at 37°C for 30 min

with 0.5 unit of neuraminidase in 2 ml of Dulbecco's PBS.

Gel Filtration

Gel filtration was performed on a 0.75 cm X 75 cm Sepharose CL-2B column preequilibrated with S buffer. Approximately 1 ml of S buffer-solubilized membranes were applied on the column and chromatographed, and 1.2 ml fractions were collected. 100 µl aliquots of each fraction were measured for radioactivity in Instagel, and the remaining fractions were dialyzed against 2 X distilled water containing 1 mM EGTA and 0.1 mM phenylmethylsulfonylfluoride to minimize proteolysis and lyophilized. Then they were solubilized in electrophoresis buffer and subjected to SDS PAGE and IEF/SDS PAGE.

Density Gradient Centrifugation

CsCl. Preformed discontinuous CsCl gradients in Triton-PBS or in S buffer were prepared by gently underlaying 2.0 ml each of buffer containing 1.60, 2.00, 2.40, 2.80, 3.20 M CsCl in the centrifuge tube (5/8 X 3 inches). Approximately 1.2 ml of Triton-PBS-solubilized microvillar extracts and S buffer extracts from microvillar membranes were gently layered onto each gradient. Centrifugations were performed at 50,000 rpm for 5 hr and 18 hr with a T-875 rotor (Beckman).

Sucrose. S buffer extracts (approximately 1.0 ml) of microvillar membranes and the supernates from the 10,000 X g centrifugation of Triton-PBS-solubilized microvilli were applied on the top of gradients of 7-25% sucrose, and were centrifuged at 80,000 X g and 4°C for 18 hr using a SW 40 rotor. Fractions were extruded from the top of the

tube by pumping with 60% sucrose solution.

Analytical Methods

For sodium dodecyl sulfate polyacrylamide gel electrophoresis, the sample was prepared by solubilizing the pelleted material in the electrophoresis sample buffer (2% w/v SDS, 5% v/v β-mercaptoethanol, 1 mM EDTA and 5% v/v glycerol). For 2-dimensional isoelectrofocusing/SDS PAGE, sample was solubilized and boiled for 3 min in one volume of buffer A (pH 6.8) containing 2% w/v SDS, 5% v/v β-mercaptoethanol and 60 mM Tris. The solubilized sample was cooled down to room temperature and mixed with two volumes of buffer B containing 9.5 M urea, 5% v/v β-mercaptoethanol, 0.8% v/v NP-40, 0.4% v/v 3-10 ampholines, 0.8% v/v 4-6 ampholines, and 0.8% v/v 5-7 ampholines. SDS PAGE was performed on the appropriate % slab gels using the procedure outlined by King and Laemmli (65). IEF/SDS PAGE was performed by the method of Rubin and Leonardi (104).

For radioactivity determination, aliquots of column fractions were mixed with 3 ml of Instagel and counted in LKB 1212 or Beckman LS 2800 liquid scintillation counter. Bands of slab gels were excised. The polyacrylamide gel slices were incubated with Soluene-350 to solubilize the proteins at 50°C for 5 hr, and mixed with Instagel. 100 µl of Delume was added to each mixture to prevent chemiluminescence, and the mixtures were placed overnight in the dark prior to counting.

For myosin affinity analysis, the method employed was based on that of Koch and Smith (66). Myosin filaments were prepared by diluting rabbit skeletal muscle myosin (in 0.6 M KCl and 50% glycerol) into 10 mM TES buffer, pH 7.5, to a final concentration of 1 mg per ml. Filaments,

which formed within 15 min at 4°C, were centrifuged at 1,000 X g and the pelleted myosin filaments were washed in TES buffer. The washed pellet was resuspended at a concentration of 1 mg per ml in 10 mM TES containing 0.2% Triton X-100. For binding experiments, 500 µg myosin filaments were incubated with 1 ml of Triton-PBS-solubilized microvilli at room temperature for 20 min and then centrifuged at 1,000 X g for 5 min. The pellets were washed three times with PBS. The addition of 0.4 M ATP served as a control to release actin and its associated protein(s) from the myosin filaments. All samples were later analyzed by SDS PAGE.

The methods of Bradford (10) and Lowry et al. (75) were routinely used for the determination of protein concentrations.

CHAPTER III

RESULTS

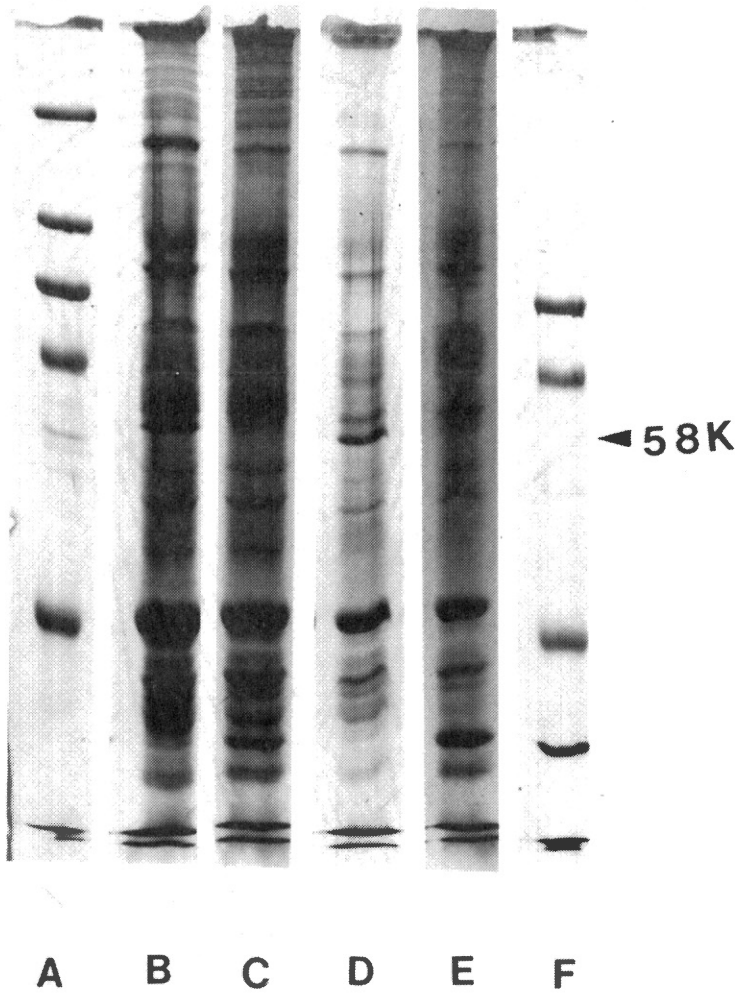
Characterization of Microvilli and Microvillar Membranes

Microvilli were removed from both MAT-B1 and MAT-C1 cells by a gentle procedure, isolated by differential centrifugation, and then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Actin was found to be a major polypeptide constituent. Alpha-actinin, as identified by using ^{125}I-labeled anti-alpha-actinin (21), was not as abundant as actin, but was also a major component. The qualitative comparisons of MAT-B1 and MAT-C1 microvilli by SDS PAGE showed that they differed primarily by one polypeptide with an apparent molecular weight of 58,000-daltons (58 K), which was found in MAT-C1 microvilli but not in MAT-B1 microvilli (Figure 4).

Microvillar membranes were prepared under hypotonic (GEM) buffer) and alkaline (pH 9.5) conditions which depolymerize filamentous actin of the microfilaments of the microvillus core structure. SDS PAGE (Figure 4) showed not only the presence of the 58 K component in MAT-C1 microvillar membranes, but also a great enhancement of the amount of the 58K polypeptide relative to actin in the membrane fractions compared to the microvilli. By transmission electron microscopy of thin sections, no microfilaments could be observed in the microvillar membranes (22). The major Coomassie blue-staining

Figure 4. SDS PAGE Gel of Microvilli and Microvillar Membranes.

A) Standard: myosin (200 K), β-galactosidase (116 K) phosphorylase a (92.5 K), bovine serum albumin (68 K), ovalbumin (42 K); B) MAT-C1 microvilli; C) MAT-B1 microvilli; D) MAT-C1 microvillar membranes; E) MAT-B1 microvillar membranes; F) standard: phosphorylase a, BSA, OA, carbonic anhydrase (30 K).


58K
A
B
C
D
E
F

polypeptide components of MAT-C1 microvillar membranes were found to be actin and 58 K polypeptide. In contrast the major elements of MAT-C1 microvillar membranes were found to be actin and 58 K polypeptide. In contrast the major elements of MAT-B1 microvillar membranes were actin and a Mr 30,000-dalton polypeptide. Compared to microvilli, alpha-actinin was greatly diminished in the membrane fractions (>90%).

If MAT-B1 and MAT-C1 cells were labeled with ^{125}I and lactoperoxidase, essentially no labeling of actin or 58 K was observed in the intact microvilli or in the membrane fractions. However, both components were heavily labeled in the presence of deoxycholate (18). These experiments indicate that the microvilli and microvillar membrane vesicles are sealed, and that actin and 58 K are located on the cytoplasmic side of the membrane permeability barrier. The fact that the 58 K polypeptide remains in the membrane fractions after depolymerization of the actin filaments suggests that it is not associated directly with the actin filaments but could still be associated with another form of actin.

Preparation and Characterization of Cytoskeleton Fractions

The following strategy is based on the capacity of non-ionic detergents to dissolve readily most membrane proteins, while preserving the cytoskeleton as an insoluble residue. To investigate the cytoskeleton-associated components of the microvilli, the MAT-C1 microvilli were extracted with 0.2% Triton X-100 in PBS, pH 7.4, at room temperature for 15 min and centrifuged at 100,000 X g for 1 hr to obtain the

insoluble cytoskeletal residues. Previous studies showed that these extraction conditions removed essentially all of the lipid, more than 90% of membrane associated glycoproteins and more than 85% of the proteins, as detected by ^{14}C-glucosamine and ^{3}H-leucine labeling (18).

By transmission electron microscopy microfilaments and some amorphous materials are observed (Figure 5). Most of the microfilaments exist as loose networks but not compact bundles. SDS PAGE pattern of the cytoskeleton reveals three major components and quite a few minor components (Figure 6) as compared to many components in the whole microvilli. Two of these components had been previously identified as actin and the 58,000-dalton polypeptide which is associated with both cytoskeleton and membrane fractions. The third component is a broad band with a molecular weight of approximately 75,000-80,000-daltons and stains poorly with Coomassie blue. The third component was further characterized by two dimensional isoelectrofocusing/sodium dodecyl sulfate polyacrylamide gel electrophoresis. A multiplet of spots descending with decreasing size and increasing pH is observed on a silver-stained gel of IEF/SDS PAGE (Figure 7). The multiplet of the spots suggests that this component could be a glycoprotein containing sialic acids on the terminal residues of the oligosaccharide side chains. The sialic acid residues are thought to be responsible for charge heterogeneity. Since it is in the cytoskeletal residues obtained from the microvilli, this third component has been termed a cytoskeleton-associated glycoprotein (CAG).

To confirm that the CAG is linked to sialic acid residues, two types of experiments were conducted. First, Triton residues were prepared from microvilli of MAT-C1 cells that were metabolically

Figure 5. TEM of MAT-Cl Microvillar Cytoskeleton.

Magnification: A) 33,000 X; B) 88,000 X. Bars = 0.5 μm.

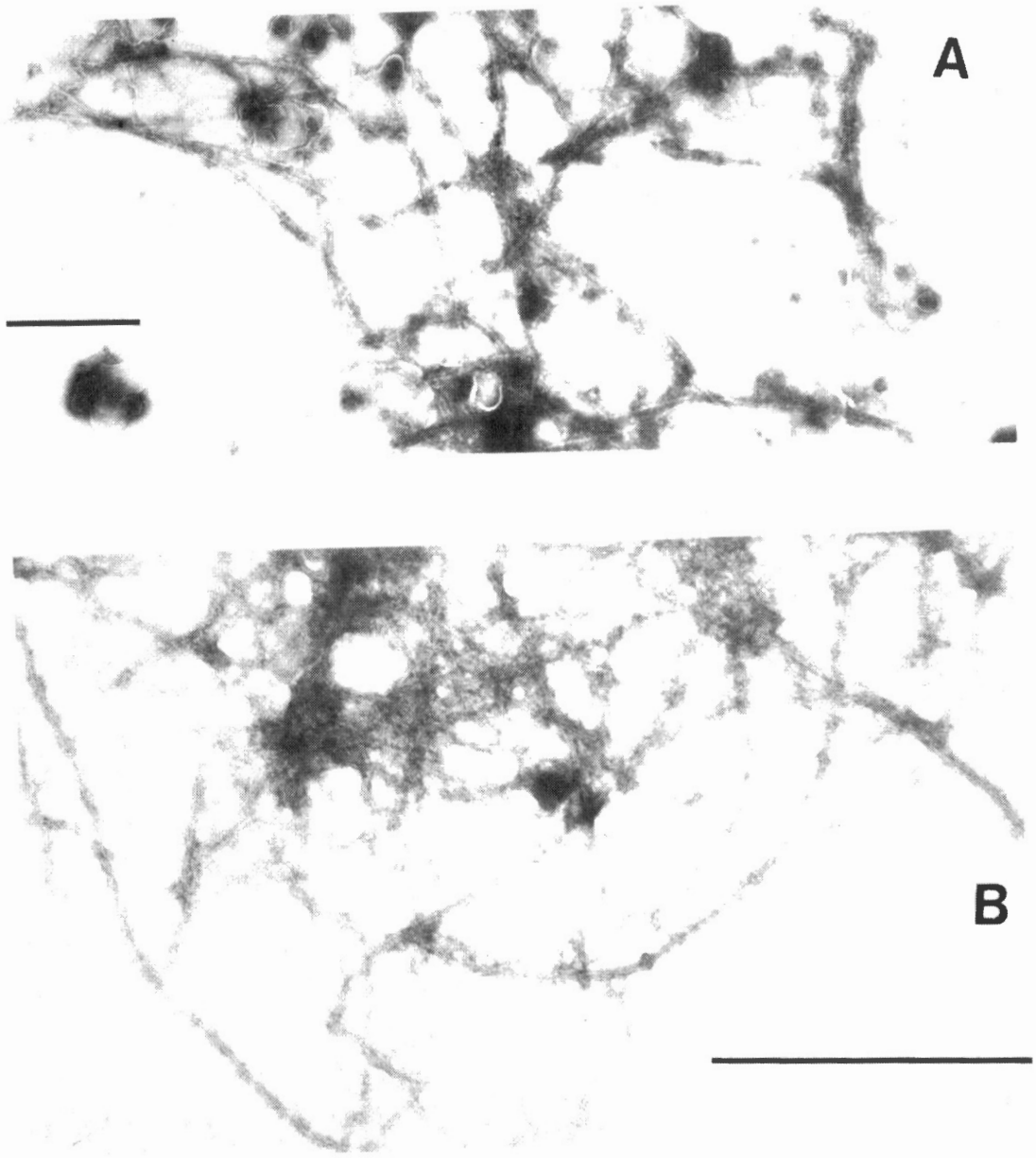

A
B

Figure 6. SDS PAGE Gel of Microvilli and 100,000 g Pellet.

A) MAT-Cl microvilli; B) 100,000 g pellet of microvilli treated with Triton-PBS.

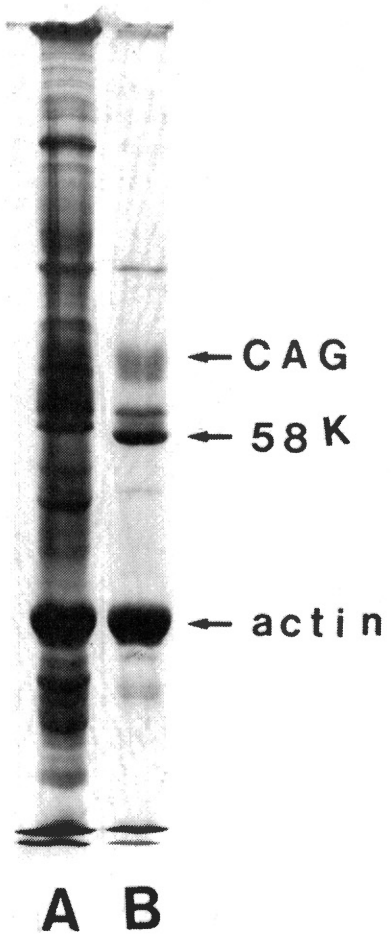
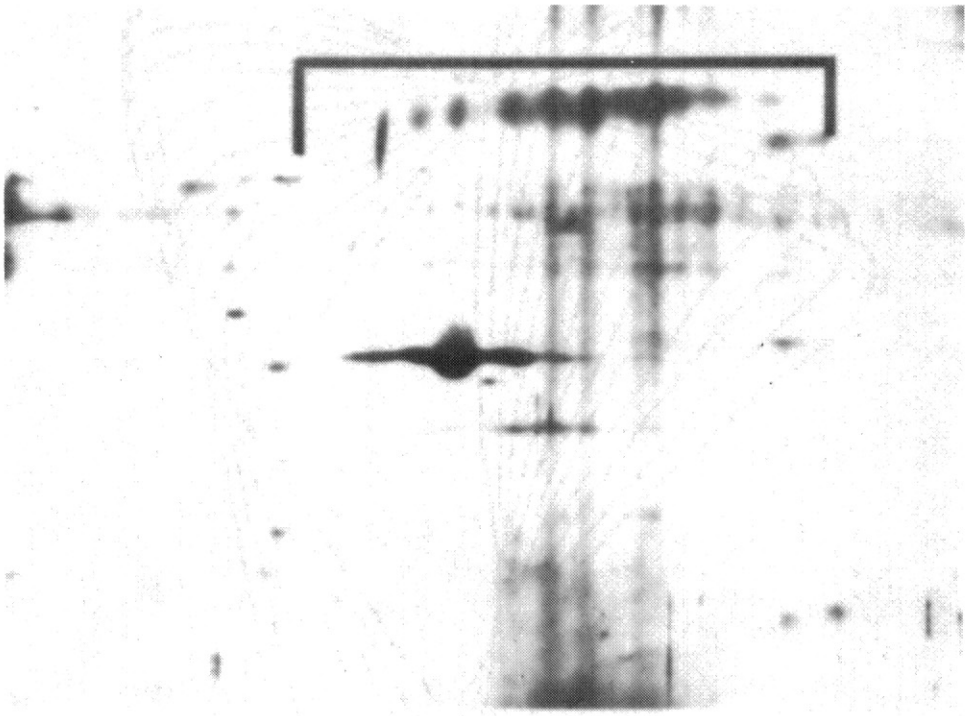

CAG
58K
actin
A B

Figure 7. IEF/SDS PAGE Stained with Silver of 100,000 g Residue of Microvilli Extracted with Triton-PBS.

CAG is shown in bracket.



dual-labeled with ^{14}C-glucosamine and ^{3}H-leucine. Bands of the 58 K polypeptide and CAG were cut out from the SDS PAGE gel and counted for radioactivity. As shown in Table III, only CAG has a significant amount of glucosamine label. This result supports in part the proposal that the CAG is sialoglycoprotein because sialic acid is driven from the glucosamine.

Secondly, microvilli were treated with 0.5 unit of neuraminidase in 2 ml of Dulbecco's PBS at 37°C for 30 min, which specifically cleaves off sialic acid. The cytoskeletal residues were obtained from the neuraminidase-treated microvilli, and then analyzed on IEF/SDS PAGE. Multiple spots of CAG still remained, but a significant shift of the spots to higher pH values was observed (Figure 8). This shift indicates that the acidic character of CAG is decreased due to the loss of sialic acids from CAG. Both results, obtained from the glucosamine labeling and the neuraminidase treatment, strongly suggest that CAG is a sialoglycoprotein. The failure to reduce the pattern to a single dot by the neuraminidase treatment may imply that either desialation is incomplete or that factors other than sialic acid contribute to the charge heterogeneity of CAG.

Because the intact microvilli are impermeable to enzymes (18), the neuraminidase effect on microvilli indicates that CAG is at the external surface of microvilli, as expected for a sialoglycoprotein. To provide further evidence for the presence of CAG at the cell surface, MAT-C1 microvilli were iodinated by Na-^{125}I and lactoperoxidase under conditions in which actin and the 58 K polypeptide were not labeled, and the cytoskeletal residues from the iodinated microvilli were analyzed by SDS PAGE and autoradiography. The autoradiogram shows

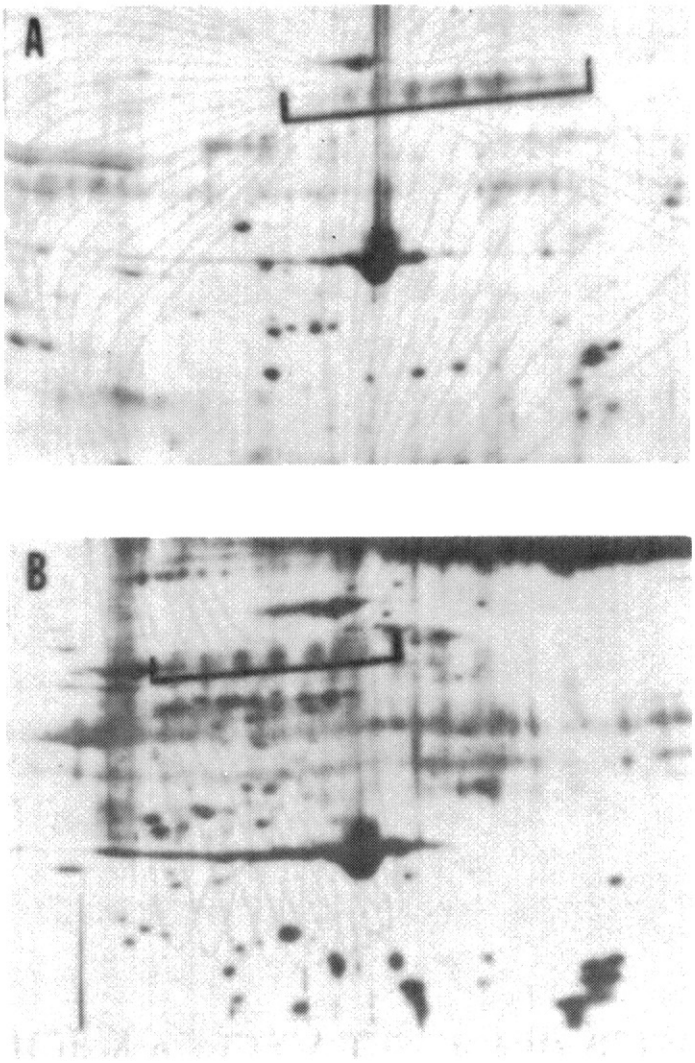
TABLE III

^{14}C-GLUCOSAMINE AND ^{3}H-LEUCINE LABELING OF CAG AND 58 K POLYPEPTIDE IN MICROVILLAR TRITON RESIDUES

	^{14}C-glucosamine (dpm)	^{3}H-leucine (dpm)	$\frac{^{14}C}{^{3}H}$
CAG	56	241	0.23
58 K	2	282	0.01

Figure 8. Effect of Neuraminidase Treatment of Microvilli on CAG (Shown in Brackets).

A) Untreated microvillar Triton residue; B) Neuraminidase-treated microvilli.


A
B

that CAG are iodinated, but that actin and the 58 K polypeptide are not labeled (Figure 9). However, when the microvilli were treated with deoxycholate, which made them permeable to the enzyme, followed by the iodination of the deoxycholate-treated microvilli, all three components - actin, 58 K polypeptide, and CAG - were heavily iodinated (Figure 9). These iodination results strongly support the proposal that CAG is located on the cell surface. Along with the result that CAG remains in the insoluble cytoskeletal residues after extraction with non-ionic detergent, the observations presented above suggest that CAG is a membrane-spanning transmembrane glycoprotein, which could interact directly or indirectly via other protein member(s), with submembrane cytoskeleton. However, the mode of association of cell surface component (CAG) with cytoskeleton remains to be studied.

Isolation and Characterization of Actin-Containing Transmembrane Complexes

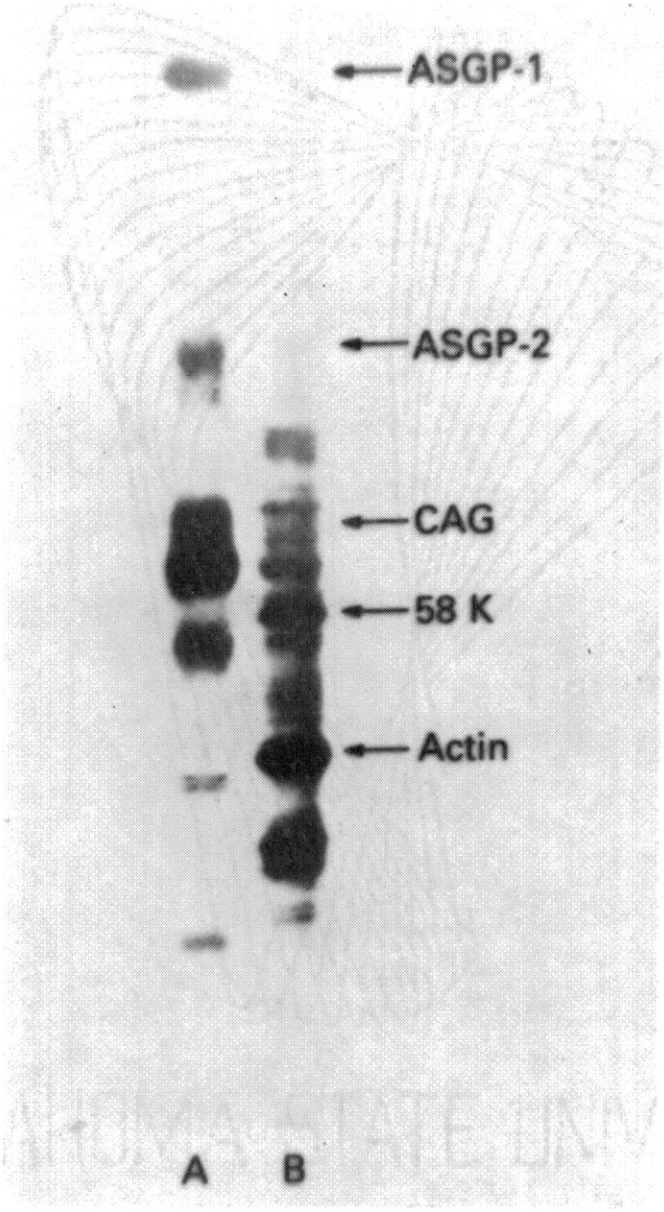
To study the molecular nature of the interaction of CAG with the non-ionic detergent insoluble cytoskeleton, membrane fractions were isolated from both MAT-C1 and MAT-B1 microvilli by a procedure that breaks down actin filaments and examined by non-ionic detergent extractions and fractionations.

Extractions of Microvilli and Microvillar Membranes

For extractions of the microvilli and microvillar membranes, two kinds of Triton X-100-containing buffers were used and compared.

Figure 9. Autoradiograms of Lactoperoxidase Iodination of Intact and DOC-Treated Microvilli.

A) Intact, sealed microvilli; B) deoxycholate-treated microvilli. (Note lack of labeling of 58 K polypeptide and actin in A compared to B. CAG is labeled in both.)

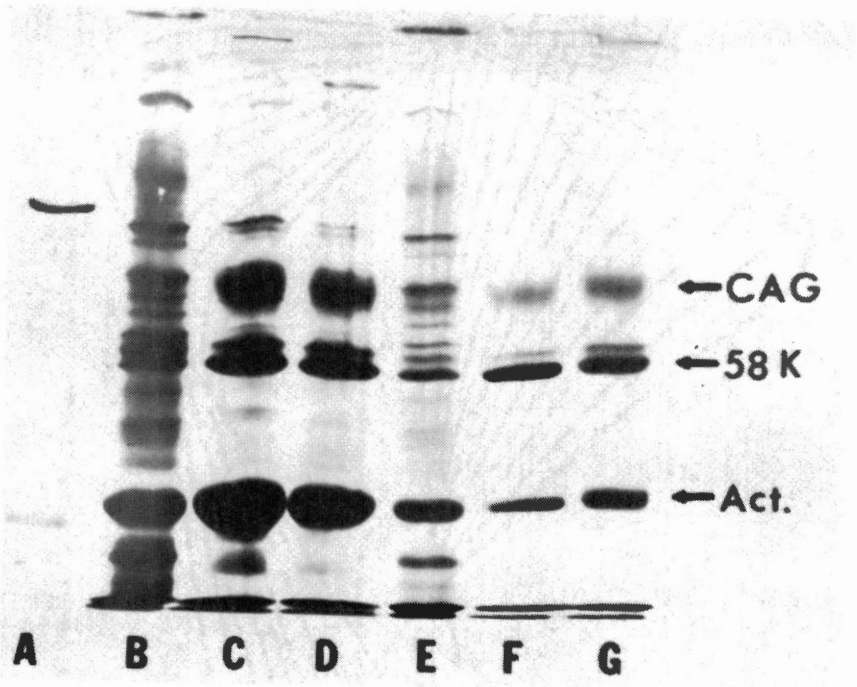

ASGP-1
ASGP-2
CAG
58 K
Actin
A
B

S buffer (5 mM Tris, 0.15 M NaCl, 2 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM dithioerythreitol, 0.5% Triton X-100, 0.1 mM PMSF, pH 7.6), is designed to stabilize the actin microfilaments and to retard monomeric (G-) and filamentous (F-) actin interconversions (115), and Triton X-100/PBS, pH 7.4, is a more commonly used extractant for preparing cytoskeletal residues. The qualitative and quantitative comparisons between microvilli and microvillar membranes were of particular significance, because microfilaments have been observed in microvilli but not in microvillar membranes, even though actin was observed as a major component of the microfilament-depleted membranes. Both the microvilli and the microvillar membranes were extracted under the identical conditions except the concentrations of protein.

Microvilli (not more than 1 mg protein per ml and not lower than 200 µg protein per ml) and membranes (<200 µg protein per ml but >100 µg protein per ml) were extracted with each of the extraction buffers at room temperature for 15 min. The extracts were chilled on ice, immediately centrifuged at 100,000 X g for 1 hr to pellet cytoskeleton residues, and then subjected to SDS PAGE analysis. As previously reported, for MAT-C1 microvillar residues three major components are present - actin, 58 K polypeptide, and CAG (Figure 10). For MAT-C1 microvillar membranes the same three major components are also observed in residues after extraction with S buffer or Triton X-100/PBS (Figure 10). In S buffer other minor higher molecular weight components have been largely extracted. One exception is a polypeptide migrating slightly more slowly than the 58 K polypeptide. It has a molecular weight of about 60,000-dalton, coisolates with the 58 K polypeptide under a variety of conditions, comigrates with the 58 K

Figure 10. S Buffer and Triton-PBS Extract Pellets of MAT-C1 Microvilli and Microvillar Membranes.

High speed pellets of microvilli (B-D) and microvillar membranes (E-G). A) Standard containing β-galactosidase and OA; B) whole microvilli; C) S buffer pellet; D) Triton-PBS pellet; E) whole membranes; F) S buffer pellet; G) Triton-PBS pellet.


CAG
58 K
Act.
A
B
C
D
E
F
G

on isoelectrofocusing in urea/NP-40 (indicating the same pI as the 58 K), but is present in only 10-20% the amount of the 58 K. Thus it appears to be an alternative form of 58 K, possibly with an extended polypeptide chain having no net charge, or very tightly bound component not released by the strong denaturant urea.

Both MAT-B1 microvilli and microvillar membrane cytoskeletal residues obtained after extraction with S buffer or Triton/PBS are composed of three major proteins - actin, CAG, and 30,000-daltons polypeptide (Figure 11). But the MAT-B1 residues has small polypeptide of Mr 30 K-daltons instead of 58 K polypeptide that was found in the MAT-C1 residues. The results above raise questions about the nature of the 30 K polypeptide from the MAT-B1 cells and its relationship to the 58 K polypeptide of MAT-C1 subline. To see if there is any relationship between the two polypeptides, one dimensional peptide mapping was conducted. The peptide mapping studies showed that the 30 K and the 58 K had no common major peptides (data not shown). This result suggests that the 30 K polypeptide is not a fragment of the 58 K polypeptide.

The amount of three major components in Triton insoluble residues from both MAT-C1 microvilli and microvillar membranes as well as that in whole microvilli and microvillar membranes were determined by counting for radioactivity of the excised gel bands of metabolically ^{3}H-leucine-labeled samples (Table IV). It was found that for whole microvilli actin was eighteen times more prominent than the 58 K polypeptide. However, the S buffer residue of the microvilli incorporated fifteen times as much actin as the 58 K and the Triton/PBS residue of the microvilli had seven times the quantity of the 58 K.

Figure 11. 100,000 g Pellet of MAT-B1 Microvilli and Microvillar Membranes.

Microvilli and membranes were extracted with S buffer and Triton-PBS, centrifuged at 100 K g. A) Standards containing β-galactosidase, BSA, and OA; B) whole microvilli; C) S buffer pellet of microvilli, D) Triton-PBS pellet of microvilli; E) whole membranes; F) S buffer pellet of membranes; G) Triton-PBS pellet of membranes.

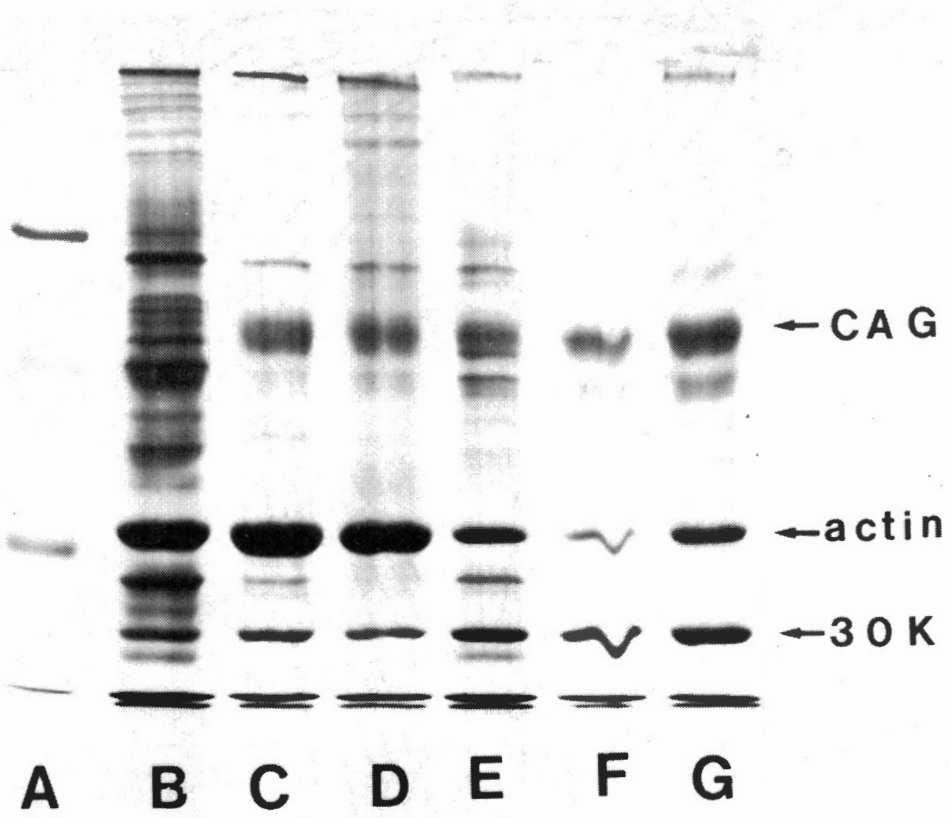

CAG
actin
30K
A
B
C
D
E
F
G

TABLE IV

RATIOS OF ACTIN TO CAG AND ACTIN TO THE 58 K POLYPEPTIDE IN MICROVILLI, MICROVILLAR MEMBRANES, AND TRITON X-100-INSOLUBLE RESIDUES

Preparation	Actin/CAG	Actin/58 K
Microvilli		
Whole microvilli	ND	18
S buffer residue	7.0	15
Triton X-100/PBS residue	3.5	7
Microvillar membranes		
Whole membranes	ND	5
S buffer residue	1.3	1.5
Triton X-100/PBS residue	1.0	1.0

ND, not determined because other bands were present in the CAG region of the gel in unextracted microvilli and membranes.

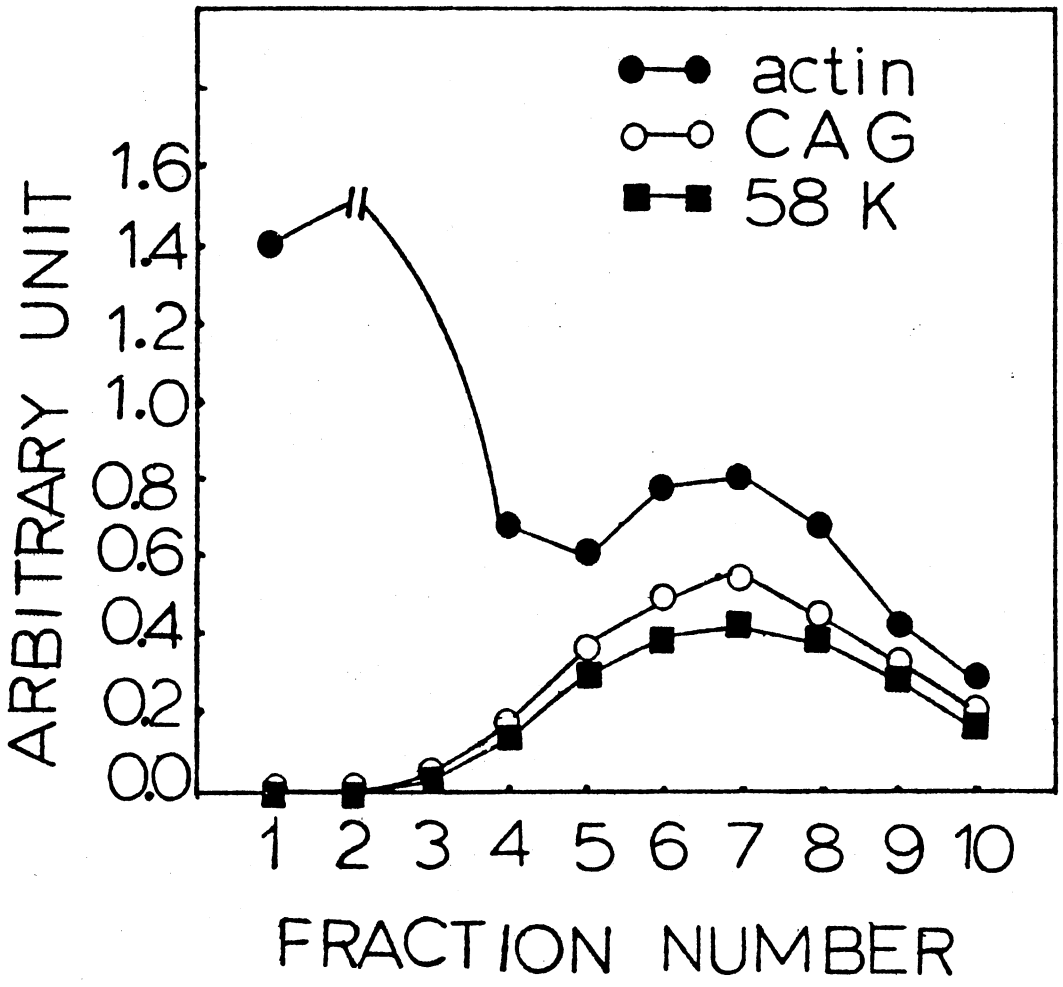
The microvillar S buffer residue has an actin/CAG ratio of 7.0 whereas the Triton/PBS residue shows half as much actin/CAG. Furthermore, the ratios mentioned above were also analyzed in microvillar membranes. The actin/58 K ratio in whole microvillar membranes is five. For the S buffer and Triton/PBS residues, the actin/58 K ratio and actin/CAG ratio approached one. As already mentioned, the amount of actin relative to the other two components is much higher in microvilli residues than in microvillar membrane residues. Also the results show that only 10-20% of the membrane actin remains in the S buffer-insoluble residues. The fact that the ratios among the three components approach equimolar in the membrane residues suggests the presence of a defined complex that might be demonstrated and purified by further fractionations.

Fractionations of S Buffer-Solubilized Microvillar Membranes

To fractionate the detergent-insoluble cytoskeletal residues containing the putative complex of three components further, S buffer-solubilized microvillar membranes from MAT-C1 cells metabolically labeled with ^{3}H-leucine were subjected to rate-zonal sucrose density gradient centrifugation on a linear 7-25% gradient prepared in S buffer. Centrifugation was performed at 80,000 X g and 4^{o}C for 18 hr, followed by fractionations, and the fractions were analyzed on SDS PAGE. The results (Figure 12) show almost complete separation of the 58,000-daltons polypeptide and CAG from the smaller and soluble components, including much of actin and the MAT ascites cell major glycoproteins ASGP-1 and ASGP-2 (110). The observation demonstrates the comigration

Figure 12. Cosedimentation Profile of CAG, 58 K, and Actin of Sucrose Density Gradient.

S buffer extracts of microvillar membranes were applied on the top of 7-25% sucrose gradient and centrifuged at 80,000 g for 18 hr using a SW 40 rotor. CAG (○); 58 K (■); actin (●). Quantification was obtained from densitometric traces of SDS PAGE gel of gradient fractions.


actin
CAG
58 K
ARBITRARY UNIT
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10
FRACTION NUMBER

of a portion of actin, the 58 K polypeptide, and CAG, implying the specific association of these three components in a complex. The remainder of membrane-associated actin in the S buffer extract was in the lighter sucrose fractions with other membrane glycoproteins and polypeptides, as expected from the previous observations on the size of this released actin (22).

To rule out fortuitous cosedimentation of homoaggregates of the three components, ^{3}H-leucine-labeled MAT-C1 microvillar membranes were solubilized in S buffer and chromatographed on Sepharose CL-2B column (0.75 cm X 75 cm). Fractions were collected, dialyzed, lyophilized and then subjected to SDS PAGE. Figure 13 shows that only the three components - a portion of actin, 58 K polypeptide, and CAG - elute directly after the void volume of the column (fractions 10-13). Thus the three components appear to behave as a complex on both sucrose density gradient centrifugation and gel filtration. Samples from fractions 11 and 12 were pooled, dialyzed, lyophilized and analyzed by IEF/SDS PAGE (Figure 14). The gel contains only the three major components (A:CAG, B:58 K, C:actin).

Because the MAT-B1 subline did not have the 58 K polypeptide in its microvilli, it was of interest to determine whether a complex of actin and CAG could be demonstrated in microvillar membranes obtained from MAT-B1 cells. Therefore, S buffer extracts of MAT-B1 microvillar membranes were applied over the Sepharose CL-2B column chromatography and eluted. The elution profile (Figure 15) is very similar to that observed in the analogous experiment for MAT-C1 microvillar membranes (Figure 16). Fractions are dialyzed, lyophilized, and analyzed by SDS PAGE. And the peak containing fractions 11 and 12 was examined by

Figure 13. SDS PAGE of Sepharose 2B Column Fractions of MAT-C1 Microvillar Membranes.

MAT-C1 microvillar membranes were extracted with S buffer and chromatographed. 1.2 ml fractions were collected. The far left lane contains standards of β-galactosidase, BSA, OA.

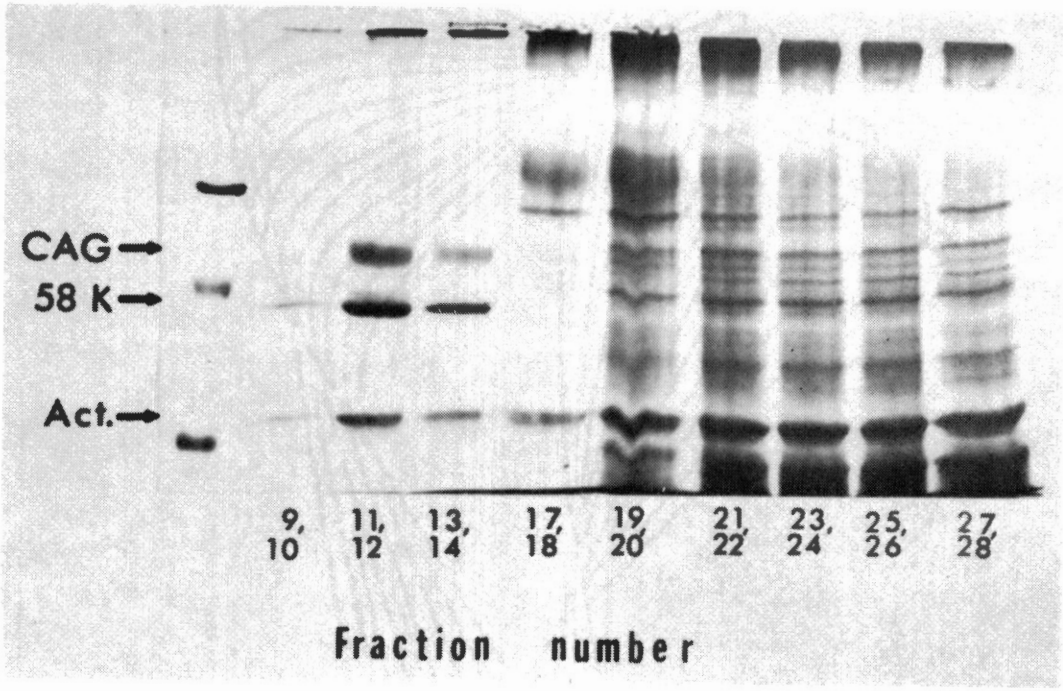

CAG
58 K
Act.
9, 10
11, 12
13, 14
17, 18
19, 20
21, 22
23, 24
25, 26
27, 28
Fraction number

Figure 14. IEF/SDS PAGE of MAT-C1 Transmembrane Complex.

Samples from fractions 11 and 12 shown in Figure 13 were pooled, dialyzed, lyophilized, and analyzed by IEF/SDS PAGE. A:CAG, B:58 K, C:actin.

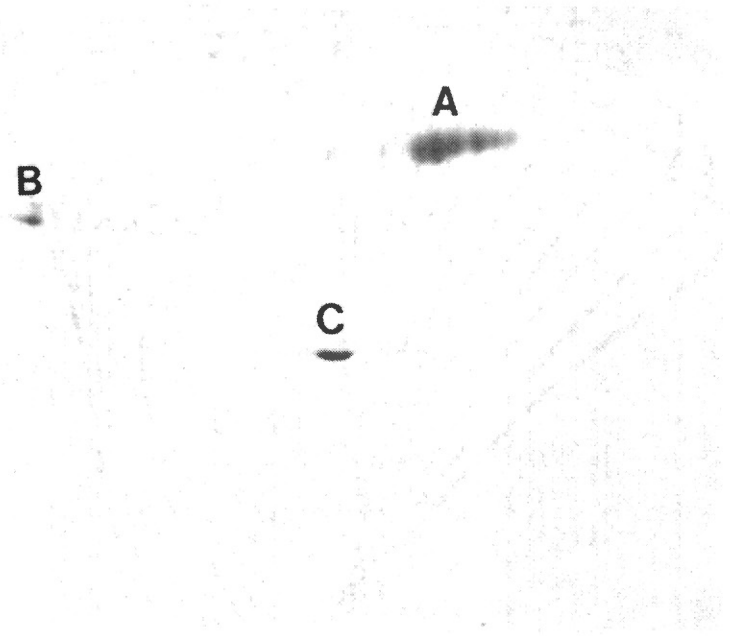

A
B
C

Figure 15. Sepharose 2B Chromatography Elution Profile of MAT-B1 Microvillar Membranes.

MAT-B1 microvillar membranes were extracted with S buffer and chromatographed on Sepharose CL-2B. 1.2 ml fractions were collected.

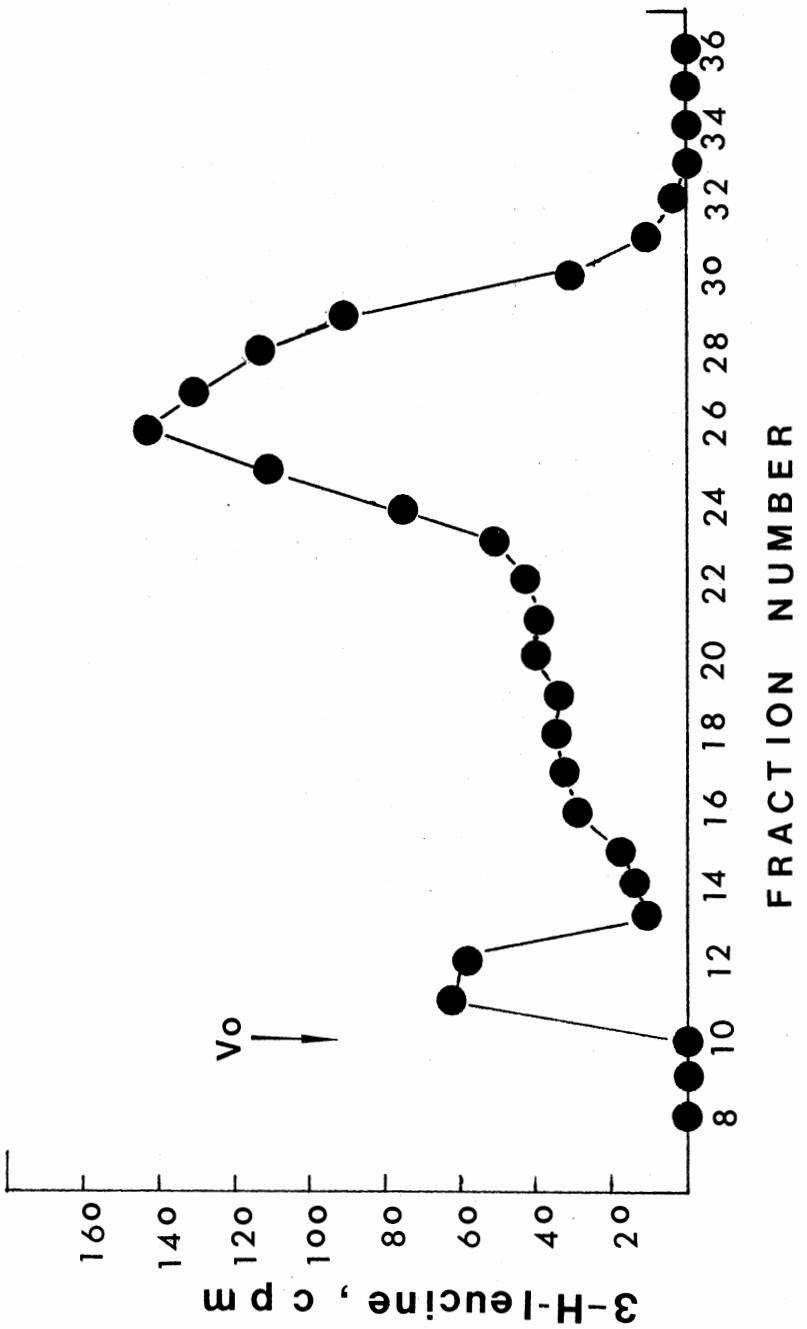
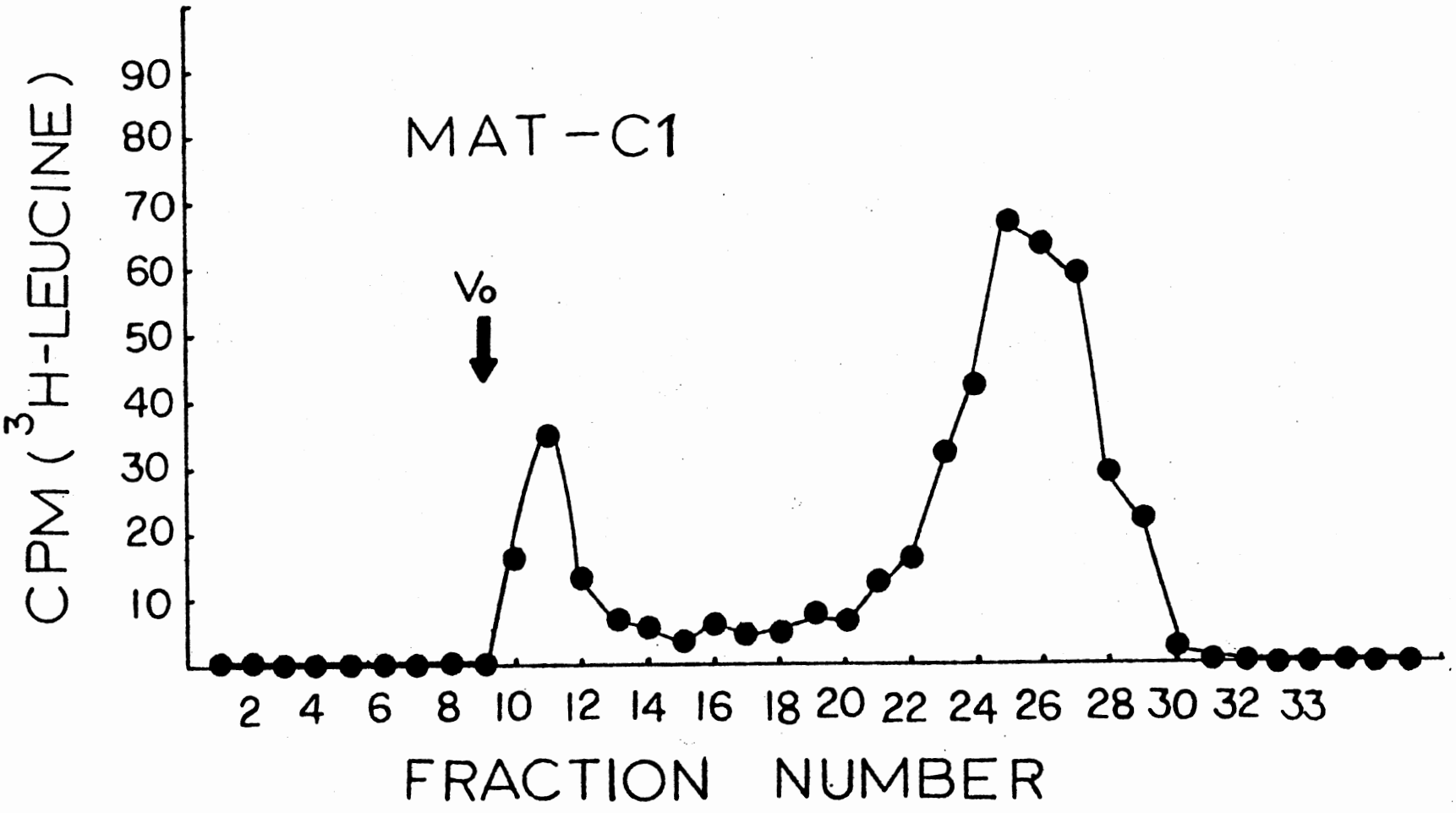

3-H-leucine, cpm
160
140
120
100
80
60
40
20
Vo
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
FRACTION NUMBER

Figure 16. Sepharose 2B Elution Profile of S Buffer-Extracted MAT-C1 Microvillar Membranes.

100 µl aliquots of each fraction were measured for radioactivity in Instagel.


MAT-C1
CPM (^{3}H-LEUCINE)
90
80
70
60
50
40
30
20
10
Vo
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 33
FRACTION NUMBER

IEF/SDS PAGE and was shown to contain only actin and CAG as significant components (Figure 17, A:CAG, B:actin).

To investigate the stability of the three component complex from MAT-C1 microvillar membranes, the Triton residues were extracted in various concentrations of guanidine-hydrochloride in 30 mM Na-PO_4, pH 7.6. 1-2 M guanidine-HC1 released the 58 K polypeptide (>75%), but released little actin or CAG (Figure 18 and Table V). The association of actin and CAG is stable at high pH, low ionic strength, and high ionic strength and appears to be disrupted only by strong denaturants, such as urea (data not shown), sodium dodecyl sulfate, and guanidine hydrochloride. These observations indicate a stable and specific association among the components of the complexes. All the studies described here provide strong evidence for a direct linkage between a cell surface glycoprotein (CAG) and actin. Thus the complex has been termed as an actin-containing transmembrane or simply transmembrane complex.

The Association of the Transmembrane Complex to the Microfilament

The fact that the complex contains actin as one of its components raises a question if the complex is directly linked to the microfilament (filamentous actin) via the complex actin. To investigate the possible association between the transmembrane complex and the microfilament, two types of studies were performed. First, ^{14}C-leucine-labeled MAT-C1 microvilli were extracted in PBS containing 0.2% Triton X-100, and examined by differential centrifugation. The extracts were centrifuged at 10,000 X g and 4^{o}C for 15 min, and the supernates were

Figure 17. IEF/SDS PAGE of MAT-B1 Isolated Transmembrane Complex.

The peak (fractions 11 and 12) shown in Figure 15 was pooled and analyzed by IEF/SDS PAGE. A) CAG; B) actin.

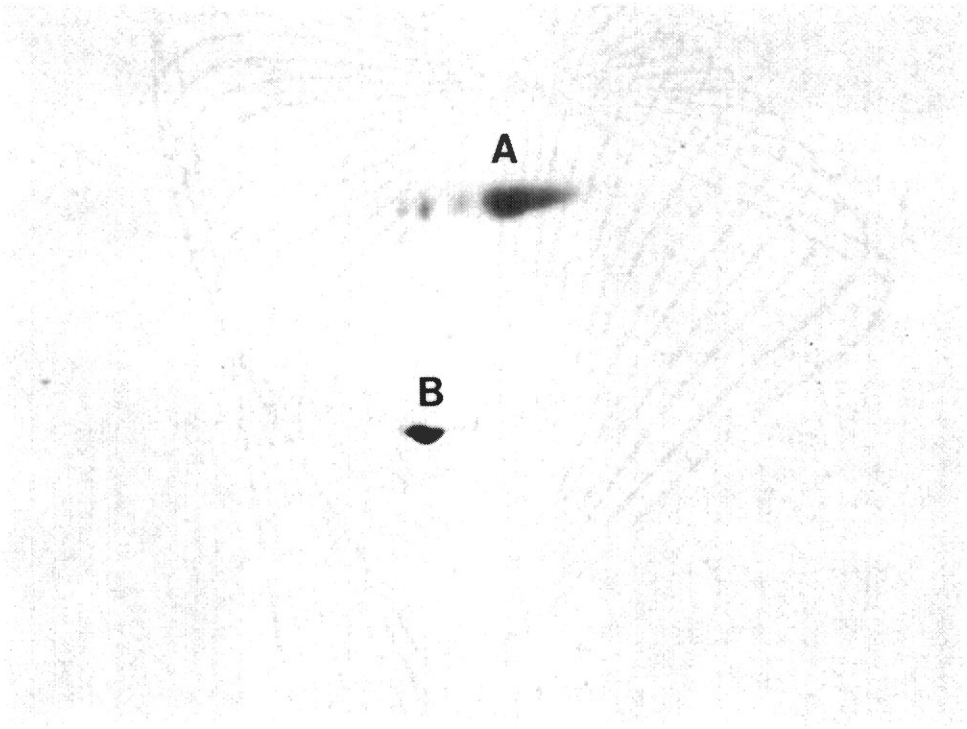

A
B

Figure 18. Extraction of MAT-C1 Transmembrane Complex with Guanidine-Hydrochloride.

The Triton residues were extracted in various concentrations of guanidine-hydrochloride in 30 mM Na-PO_4 (pH 7.6) at room temperature for 30 min, centrifuged at 100 K g for 1 hr in SW 50.1 rotor. Pellet and supernate fractions were analyzed by SDS PAGE (7.5% gel). A) Standards: β-galactosidase, BSA, and OA; 1S) 1 M guanidine supernate; 2S) 2 M supernate; 4S) 4 M supernate; 6S) 6 M supernate; 1P) 1 M guanidine pellet; 2P) 2 M pellet; 4P) 4 M pellet; 6P) 6 M pellet. Top arrowhead shows position of CAG, middle arrowhead indicates 58 K, and low arrowhead designates actin.

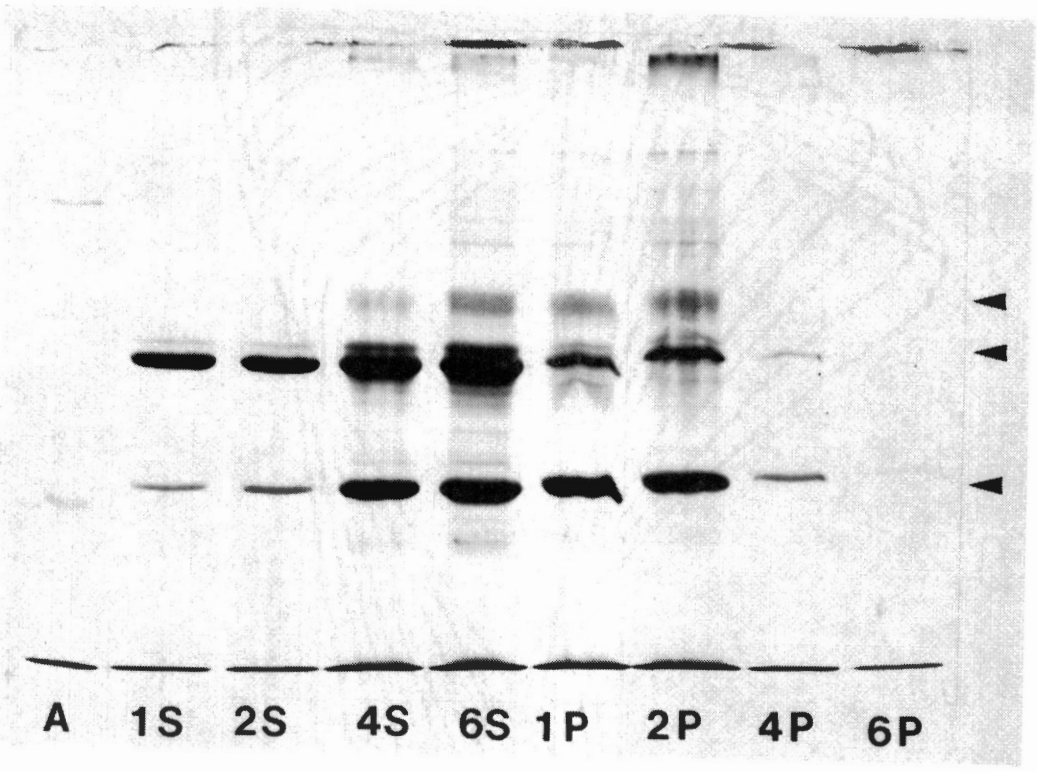

A
1S
2S
4S
6S
1P
2P
4P
6P

TABLE V

EXTRACTION OF MAT-Cl TRANSMEMBRANE COMPLEX
WITH GUANIDINE-HYDROCHLORIDE

Guanidine-HCl (Molar)	% Extracted		
	Actin	58K	CAG
1	<10	75	<5
2	10	75	<5
4	90	95	90
6	>95	>99	>95

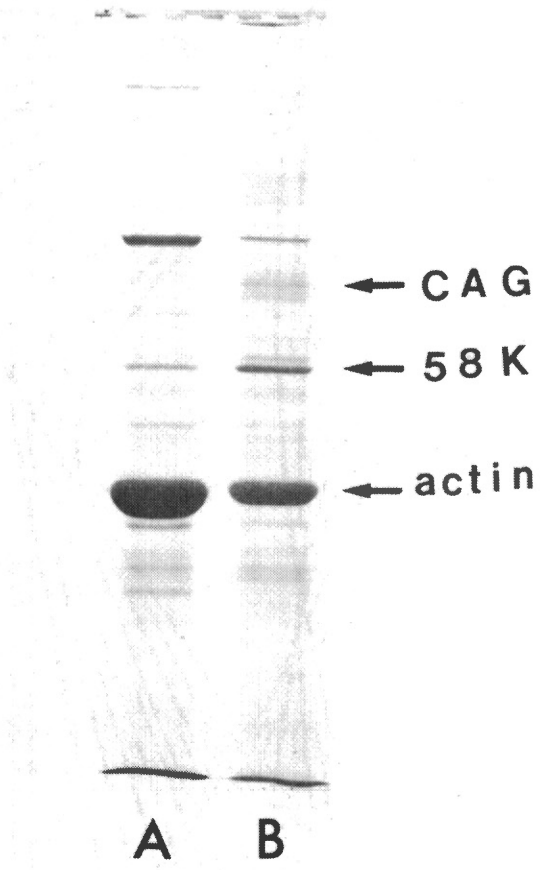
recentrifuged at 100,000 X g for 1 hr. Both low speed pellet (10,000 X g) and high speed pellet (100,000 X g) were analyzed by SDS PAGE. Interestingly the low speed pellets contain the same component that was found in the residues obtained by 100,000 X g centrifugation of the whole microvilli Triton extracts (Figure 19). The high speed pellets of low speed supernates also show actin, 58 K polypeptide, and CAG (Figure 19). It is found that only 30-40% of the 58 K polypeptide is sedimented at 10,000 X g centrifugation and >60% of the 58 K polypeptide is in the high speed pellets.

The fact that the 58 K polypeptide is present in both the low speed pellet and the high speed pellet, appears to indicate that the transmembrane complex is associated with the microfilament, but it may imply that the complex has simply co-sedimented with filamentous actin. Therefore, based solely on this result, it is difficult to distinguish its specific anchorage to microfilaments from fortuitous co-precipitation with the filament.

Therefore, a myosin affinity analysis was conducted. This technique is uses of the strong specific affinity of myosin filaments for actin. Thus if a sample contains filamentous actin in association with some other protein, it should be possible to precipitate the latter with myosin filaments through the actin. For this study, MAT-C1 microvilli were extracted in Triton-PBS at room temperature for 15 min, and the extracts were mixed with the myosin filaments. The addition of 0.4 M ATP served as a control for the nonspecific binding of the 58 K polypeptide to the myosin filaments. The mixtures were incubated at room temperature for 20 min, centrifuged at 1,000 X g for 5 min to pellet the myosin filament and its associated polypeptide(s), and

Figure 19. SDS PAGE of Fractions of Triton-PBS-Extracted Microvilli.

The extracts were centrifuged at 10 K g for 15 min to give pellet (lane A) and supernates. The supernates were further centrifuged at 100 K g for 1 hr to give pellets (lane B). Samples were equivalently loaded.


CAG
58K
actin
A
B

washed three times to remove the loosely and unspecifically bound proteins. In the absence of ATP, approximately 60% of actin, >50% of the 58 K and a few other minor polypeptides were bound to myosin filaments, whereas in the presence of ATP about 30% of actin and <5% of the 58 K were precipitated with the myosin filaments. The results of a similar experiment performed on 10,000 X g supernates of Triton-PBS-extracted microvilli showed that >30% of actin and about 70% of the 58 K were precipitated with the myosin filaments in the absence of ATP, but only about 10% actin and none of the 58 K were bound to the myosin filament in the presence of ATP (Figure 20 and Table VI). CAG was hardly visible because several minor polypeptide bands, which probably originate from myosin as contaminants and/or degraded products, migrate in the same region as CAG. These observations indicate that a substantial fraction of the 58 K polypeptide is precipitated by the myosin filaments. Since the 58 K is associated with the transmembrane complex, the results support the proposal that the transmembrane complex is associated with the microfilaments composed of filamentous actin and its associated proteins.

Additional evidence for the association of the transmembrane complex with the cytoskeleton was obtained by DNase treatments of the microvilli in Triton-containing PBS (19). It is known that DNase depolymerizes actin microfilaments (107). For MAT-C1 microvilli, only about 20% of the actin in DNase-treated samples was sedimented at 100,000 X g centrifugation for 1 hr, compared to approximately 60-70% in the untreated control. Concomitant with this release of actin, a shift of CAG was observed from the 10,000 X g pellet to 100,000 X g pellet in DNase-treated samples but not in DNase-non-

Figure 20. Myosin Affinity Analysis.

Triton-PBS-extracted MAT-C1 microvilli (A and B) and 10 K g supernates of Triton extracts (C and D) were mixed with the myosin filament both in the absence and presence of ATP. The mixtures were centrifuged at 1,000 g to pellet myosin and its associated protein(s). The pellets were analyzed by SDS PAGE. A) Extracts; B) extracts and ATP; C) supernates; D) supernates and ATP. All pellets were loaded equivalently.

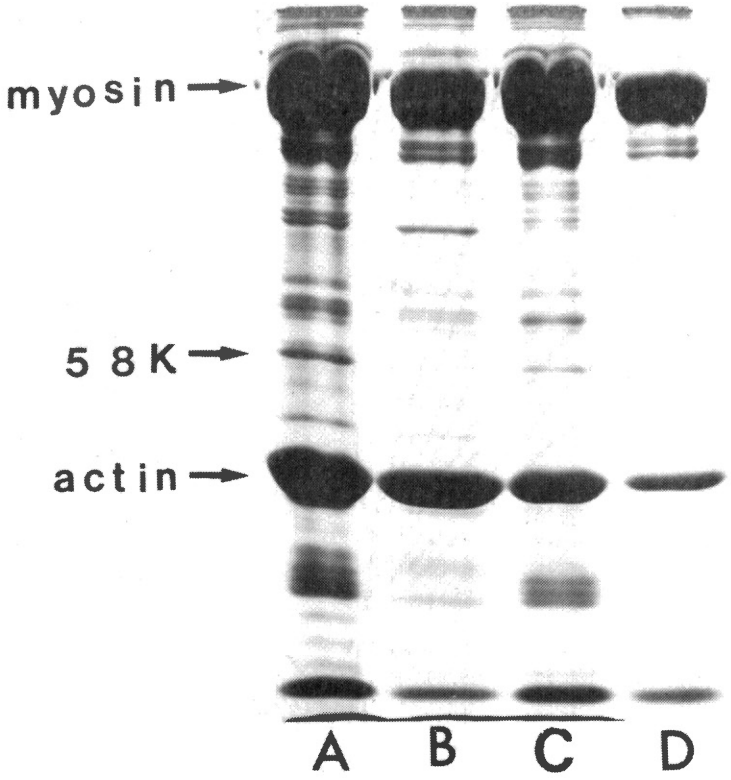

myosin
58K
actin
A
B
C
D

TABLE VI

SEDIMENTABILITY OF ACTIN AND THE 58 K WITH THE MYOSIN FILAMENTS

	% Sedimentability	
	Actin	58 K
Microvillar Extracts	63	55
Microvillar Extracts/ATP	30	<5
10,000 g Supernates	35	70
10,000 g Supernates/ATP	10	<5

treated samples. In combination with all of these results, other strong evidence from sucrose density gradient centrifugation studies to be mentioned later propose that the actin-containing transmembrane complex is associated with the microfilaments.

Con A-Induced Anchorage of ASGP-1/ASGP-2 Complex to Submembrane Cytoskeleton

More than 90% of labeled glucosamine incorporated into glycoproteins in MAT-B1 and MAT-C1 cells is found in two cell components, ASGP-1, a peanut agglutinin (PNA)-binding sialomucin, and ASGP-2, a Concanavalin A-binding protein (109). These are present as a complex at the cell surface (53, 110), and are termed the sialoglycoprotein complex (ASGP-1/ASGP-2). Capping studies with fluorescent Con A and PNA have shown that the glycoprotein complex is immobile in one subline (MAT-C1) and mobile in the other subline (MAT-B1) (20, 60).

These observations suggest a Con A-mediated linkage of the sialoglycoprotein complex to the cytoskeleton, because the organization and reorganization of cell surface components is widely believed to be controlled by a submembrane actin-containing cytoskeleton (91). Two questions concerning the control of the organization of the major cell surface components of these ascites cells are raised. 1) Does the linkage of the sialoglycoprotein complex (ASGP-1/ASGP-2) occur by direct linkage to the cytoskeleton, or does it occur indirectly by a Con A bridge between two Con A-binding proteins, ASGP-2 and CAG? 2) Can the difference in Con A receptor mobility between the MAT-B1 and MAT-C1 sublines be explained by the differences in anchorage of the Con A receptors, e.g., ASGP-2?

Microvilli from ^{14}C-glucosamine-labeled MAT-B1 and MAT-C1 cells were incubated with Con A in PBS, pH 7.4, for 30 min at 37°C and washed to remove unbound material. The Con A-treated microvilli were extracted with PBS containing Triton X-100 and centrifuged at 100,000 X g and 4°C for 1 hr to pellet the cytoskeleton, and the pelleted residues were analyzed by SDS PAGE. SDS PAGE (Figure 21) shows that ASGP-1 and ASGP-2 are largely retained. To investigate whether there is any significant difference in the proportions of ASGP-2 associated with cytoskeletal residues of MAT-B1 and MAT-C1 microvilli in the presence of Con A, ASGP-2 was quantified by gel band excision and counting for radioactivity. MAT-B1 and MAT-C1 residues shows 77 and 84%, respectively (Table VII). Thus the proportion of ASGP-2 retained to the cytoskeleton does not differ substantially between the two sublines, even though they differ qualitatively in their receptor redistribution.

As already mentioned, there are two likely ways in which the ASGP-1/ASGP-2 complex could be attached to a submembrane cytoskeleton. 1) The ASGP-1/ASGP-2 complex could become linked to the cytoskeleton as a result of the interaction of ASGP-2 with Con A. 2) Alternatively, the ASGP-1/ASGP-2 complex could become linked to a second cell surface molecule (CAG), a component of the transmembrane complex, which is already attached to the submembrane cytoskeleton.

To determine if Con A is linking the ASGP-1/ASGP-2 complex to the transmembrane complex, Con A-treated or untreated microvillar membranes were extracted with S buffer and sedimented by 100,000 X g centrifugation. In the Con A-treated samples the residues from membranes of both sublines have ASGP-1 and ASGP-2 (Figure 22), in addition to the

Figure 21. The Cytoskeletal Residues of Con A-Treated and Untreated MAT-B1 and MAT-C1 Microvilli.

Con A-treated and untreated ^{14}C-glucosamine-labeled MAT-B1 and MAT-C1 microvilli were extracted with Triton-PBS. The extracts were centrifuged at 100,000 g for 1 hr to pellet cytoskeletons. The cytoskeletons were electrophoresed on 7.5% polyacrylamide gel.
A) Con A-untreated MAT-B1; B) Con A-treated MAT-B1;
C) Con A-untreated MAT-C1; D) Con A-treated MAT-C1.

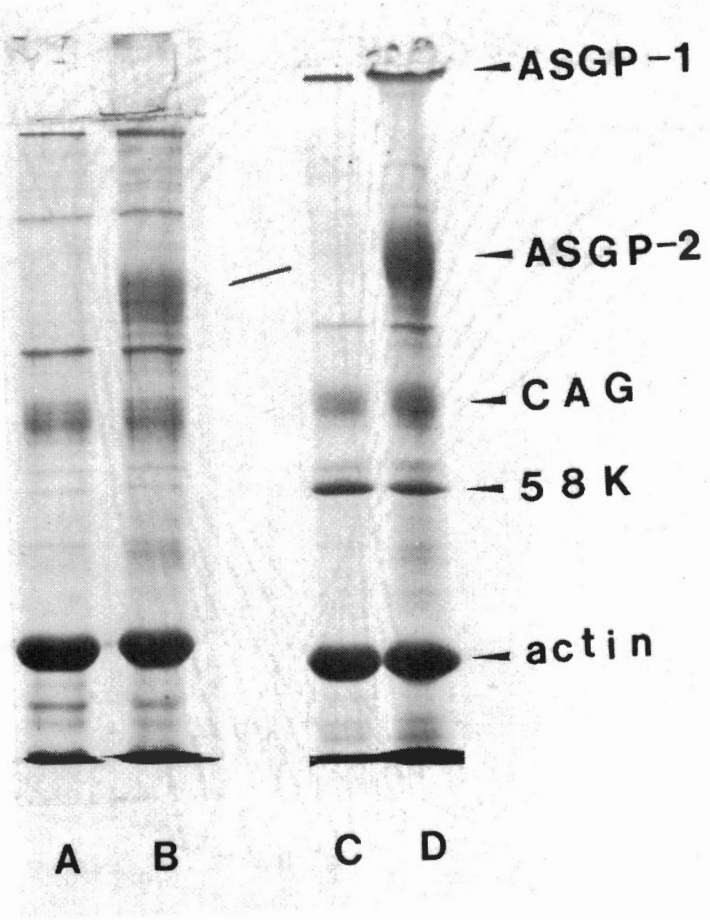

ASGP-1
ASGP-2
CAG
58K
actin
A
B
C
D

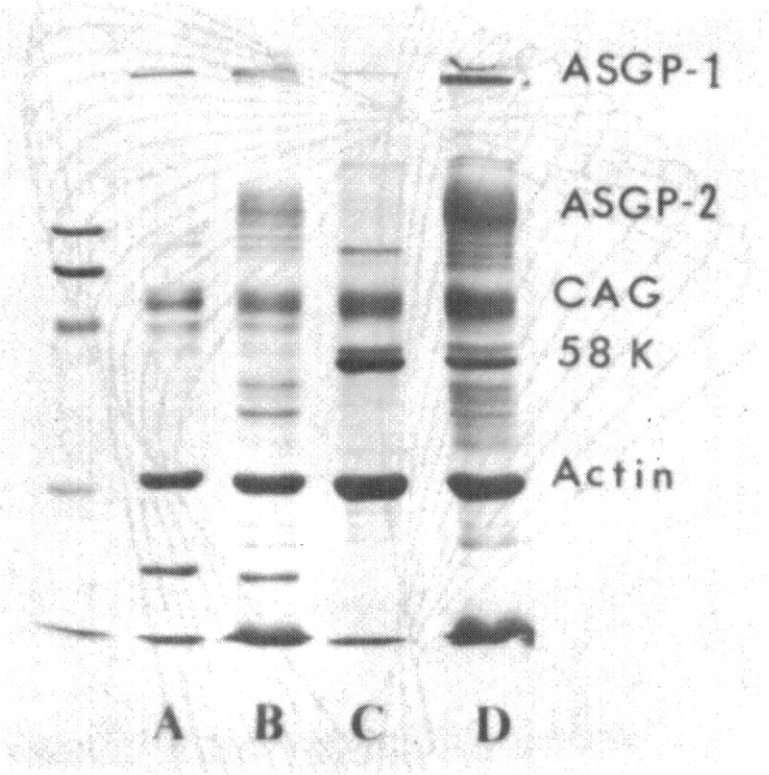
TABLE VII

CON A EFFECT ON THE AMOUNT OF ASGP-2 REMAINING IN CYTOSKELETON

Microvilli	dpm (^{14}C-glucosamine)		% Remaining
	Microvillar Extract	100 K g Pellet	
MAT-B1-Con A	453	349	77
MAT-C1-Con A	845	715	84

Figure 22. Comparisons of S Buffer Residues of Con A-Treated and Untreated MAT-B1 and MAT-C1 Microvillar Membranes.

A) Untreated MAT-B1; B) treated MAT-B1; C) untreated MAT-C1; D) treated MAT-C1. The unlettered lane on the left contains molecular weight standards: myosin, β-galactosidase, phosphorylase a, BSA, and OA.


ASGP-1
ASGP-2
CAG
58 K
Actin
A
B
C
D

actin, the 58 K polypeptide, and CAG (MAT-C1, Figure 22), and actin, and CAG (MAT-B1, Figure 22) found in the Con A-untreated controls. This association of the ASGP-1/ASGP-2 complex with the transmembrane complex does not result from a stabilization of the submembrane cytoskeleton, retaining more actin to which the ASGP-1/ASGP-2 complex could bind, because the ratios of actin to CAG and actin to 58 K are not increased in the Con A-treated samples compared to the untreated controls (Table VIII). The most reasonable explanation for these results is that the ASGP-1/ASGP-2 complex is linked by Con A bridge to CAG of the transmembrane complex. This explanation is supported by the fact that Con A binding to CAG can be demonstrated by overlays of fluorescent Con A on SDS PAGE gels of the S buffer residues of microvillar membranes (R. Helm, unpublished observation).

To demonstrate conclusively that there is an association by Con A between the transmembrane complex and the ASGP-1/ASGP-2 complex and that two complexes are not simply co-sedimenting, Con A-treated or untreated microvilli from ^{3}H-leucine-labeled MAT-C1 cells were treated after Triton X-100 treatment by CsCl density gradient centrifugation. The extracted microvilli were centrifuged for only 5 hr on a preformed gradient to retard dissociation of the 58 K polypeptide from the transmembrane complexes, but depolymerization of actin by the high salt conditions still occurred. This centrifugation time is sufficient for the ASGP-1/ASGP-2 complex to approach equilibrium, as indicated by its presence at a density near 1.4 (109). If the sialoglycoprotein and the transmembrane complexes are linked by Con A, the components of transmembrane complex should be shifted to a higher density in the presence of Con A, reflecting the greater carbohydrate content

TABLE VIII

RATIOS OF DPM'S OF LEUCINE OF MAJOR COMPONENTS OF S BUFFER RESIDUES FROM CON A-TREATED AND UNTREATED MICROVILLAR MEMBRANES

Treatment	Cell	58 K/Actin	CAG/Actin	ASGP-2/Actin
None	MAT-B1	0	0.6	0
Con A	MAT-B1	0	0.6	1.2
None	MAT-C1	0.6	0.5	0
Con A	MAT-C1	0.55	0.6	1.8

contributed by ASGP-1 (109-110). That this occurs is demonstrated in Figures 23 and 24. The sialoglycoprotein complex is found at densities 1.3-1.4 (fractions 1-4) in the Con A-treated (Figures 23 and 24) sample and control (Con A-treated in the presence of 0.5 M α-methyl-mannoside (Figure 24). CAG and actin are observed to be coincident with ASGP-2 only in the presence of Con A, as shown in Figures 23 and 24. Since the only means of shifting the actin and CAG to a higher density is through interaction with a highly glycosylated component, ASGP-1, the results provide strong evidence for a Con A-mediated association between the transmembrane complex and the sialoglycoprotein complex.

The 58 K polypeptide is slightly displaced from CAG on the gradient, indicating that it is slowly being dissociated from the transmembrane complex on the CsCl gradient. If centrifugations are performed for 18 hr, the 58 K polypeptide in both cases of the Con A-treated and Con A plus α-MM-treated samples is completely displaced to lower densities, but actin and CAG still remain coincident with ASGP-2 only in the Con A-treated samples (Figures 25 and 27) and not in controls (Figures 26 and 27). These results confirm previous observations on the stability of the actin-CAG interaction. These observations rule out the direct linkage of the sialoglycoprotein complex to the cytoskeleton, supporting the alternative suggestion that the association of ASGP-1/ASGP-2 complex to the cytoskeleton occurs by Con A crosslinking of ASGP-2 to CAG which is presumed to be linked to the cytoskeleton via its actin component.

Because CsCl gradient depolymerizes actin filaments, the CsCl density gradients do not indicate whether the two complexes are

Figure 23. SDS PAGE of Fractionations from CsCl Gradient of Triton-PBS Extracts of Con A-Treated Microvilli.

MAT-Cl microvilli were treated with 100 µg Con A per ml as described in the experimental procedures and extracted with Triton-PBS. The extract was centrifuged for 5 hr, and fractions were collected for SDS PAGE. The top of the gradient is on the right.

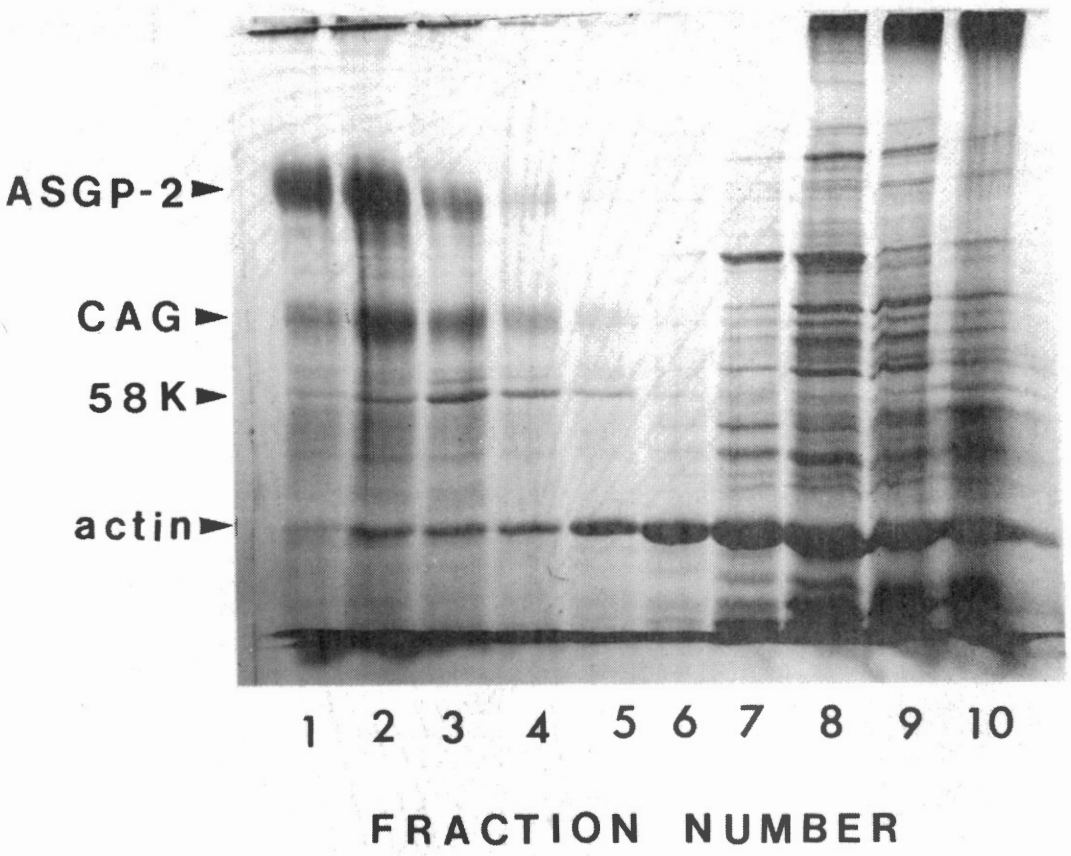

ASGP-2
CAG
58K
actin
1 2 3 4 5 6 7 8 9 10
FRACTION NUMBER

Figure 24. CsCl Gradient Profiles.

The controls were treated with Con A and 0.5 M α-methyl-mannoside. The samples and gels were prepared as in Figure 23, and the appropriate bands were excised and counted. The gradient was approximately linear from fraction 1 (density 1.4) to fraction 5 (density 1.3).

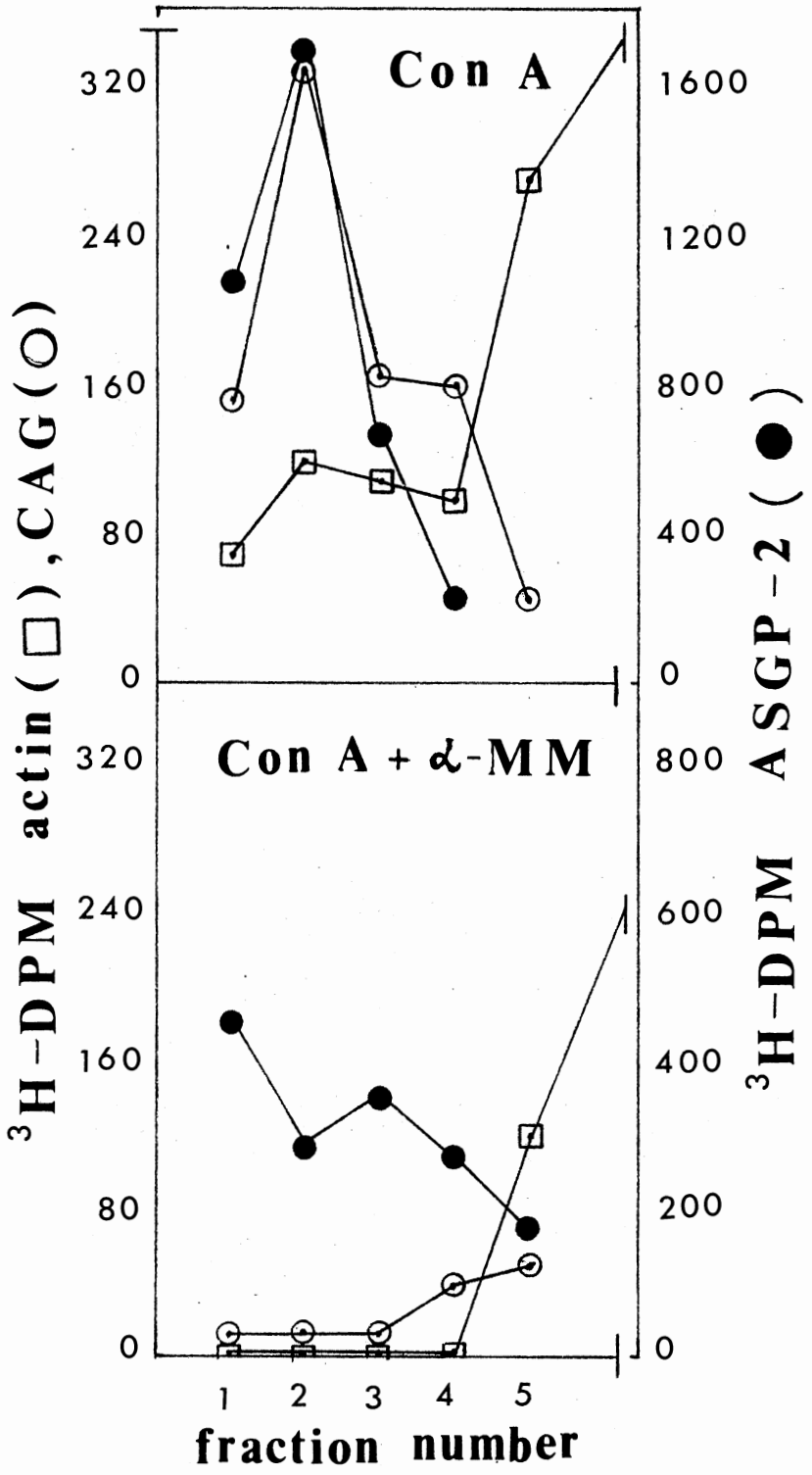
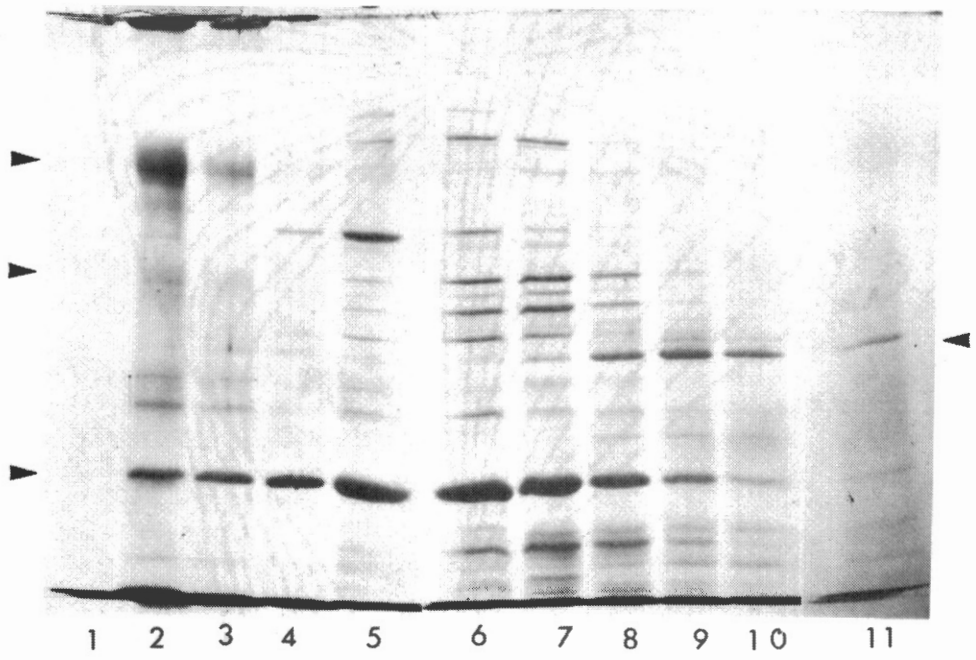

Con A
Con A + α-MM
320
240
160
80
0
1600
1200
800
400
600
200
1 2 3 4 5
fraction number
^{3}H-DPM actin (□), CAG (○)
^{3}H-DPM ASGP-2 (●)

Figure 25. SDS PAGE of Fractions from CsCl Gradient of S Buffer Extracts of Con A-Treated Microvillar Membranes.

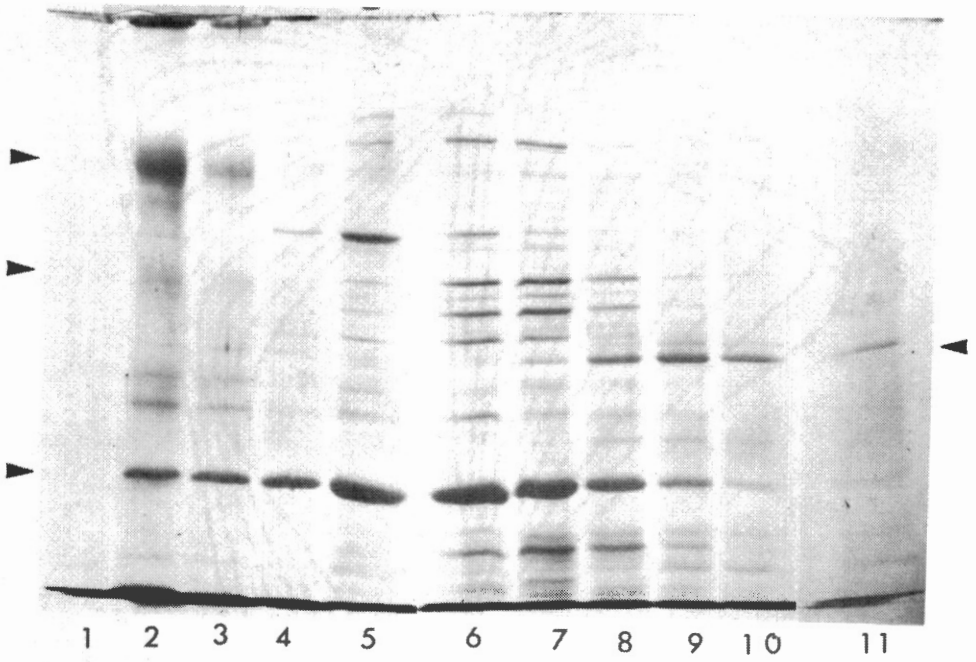
The extracts were centrifuged at 50 K rpm for 18 hr using T-875 rotor. Top of the gradient is on the right. The left top arrowhead indicates ASGP-2, left middle arrowhead shows the position of CAG, and left low arrowhead designates actin. Right arrowhead indicates 58 K.



FRACTION NUMBER

Figure 26. SDS PAGE of Fractions from CsCl Gradient of S Buffer Extracts of Con A Plus α-MM-treated Microvillar Membranes.

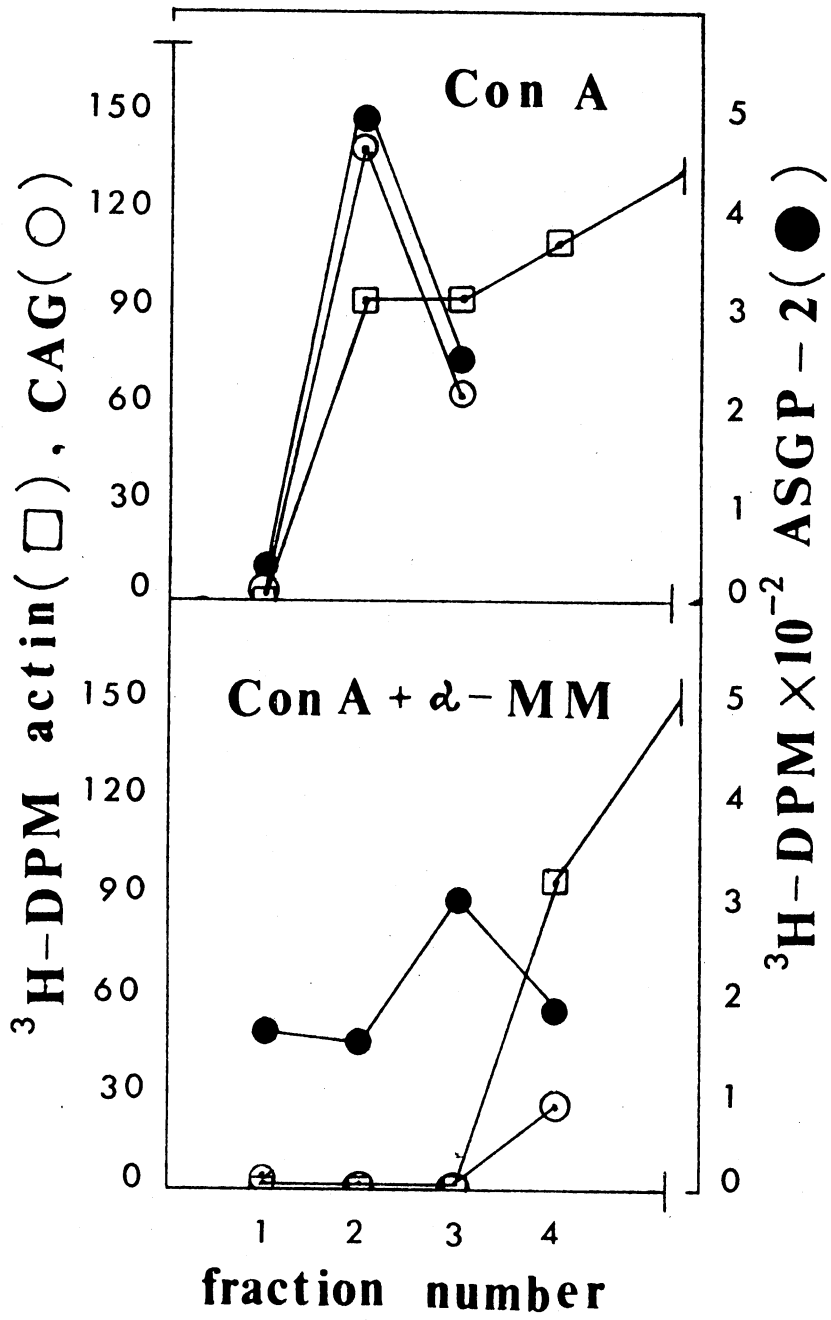
Membranes were obtained from MAT-C1 ^{3}H-Leucine-labeled microvilli treated with Con A plus 0.5 M α-methylmannoside, and extracted with S buffer. The extracts were centrifuged at 50 K rpm for 18 hr using T-875 rotor. The top of the gradient is on the right.



FRACTION NUMBER

Figure 27. CsCl Gradient Profiles for S Buffer Extracts of Microvillar Membranes Treated with Con A or Con A Plus α-MM.

The appropriate bands of SDS PAGE gels of Figures 25 and 26 were excised and counted for radioactivity.


Con A
Con A + α-MM
150
120
90
60
30
0
5
4
3
2
1
0
1
2
3
4
fraction number
^{3}H-DPM actin(□), CAG(○)
^{3}H-DPM ×10^{-2} ASGP-2(●)

associated with additional microvillar cytoskeletal elements. To examine this question, Con A-treated and untreated microvilli from ^{3}H-leucine-labeled MAT-C1 cells were extracted with PBS containing 0.2% Triton X-100 and the extracts were fractionated by differential centrifugation. Centrifugation at 10,000 X g for 15 min gave a microfilament core containing actin and α-actinin as the major components (Figure 28). Recentrifugation of the supernate at 100,000 X g for an hour gave a pellet containing actin, the 58 K polypeptide, and CAG as major components. Con A and ASGP-2 were associated with both pellet fractions of the Con A-treated microvilli (Figure 28). Somewhat surprisingly, the amount of actin in the low speed pellet was decreased by Con A treatment (Table IX). This shift in actin from the low speed pellet to the high speed residue may suggest that Con A treatments might disrupt the filament crosslinking to produce the uncrosslinked actin filaments. Con A treatment did not depolymerize the microfilaments, since the amount of soluble actin in the Triton high speed supernates remained unchanged.

Since the high speed pellet contains both transmembrane complex and polymerized actin, the 10,000 X g supernates were fractionated by rate-zonal sucrose density gradient centrifugation (7-25% sucrose gradient in PBS containing 0.2% Triton X-100, 80,000 X g, 15 hr, 4^{o}C) to determine whether an association between the polymerized actin and the transmembrane complex could be demonstrated. Since these gradients separate on the basis of size rather than density, co-sedimentation of CAG and actin, in excess of the amount of actin in the transmembrane complex, would indicate an association of transmembrane complex with the polymerized actin. Since sialoglycoprotein complex is smaller

Figure 28. SDS PAGE of Fractions of Triton-Extracted Microvilli Treated with Con A or Con A Plus α-Methylmannoside.

The extracts of MAT-Cl microvilli were centrifuged at 10,000 g for 15 min to give pellets (lanes A and B) and supernates. The supernates were recentrifuged at 100,000 g for 1 hr to give pellets (lanes C and D) and supernates (lanes E and F). A, C, and E: treated with Con A. B, D, and F: treated with Con A plus 0.5 M α-MM. The samples were equivalently loaded so that protein and glycoprotein differences are readily apparent.

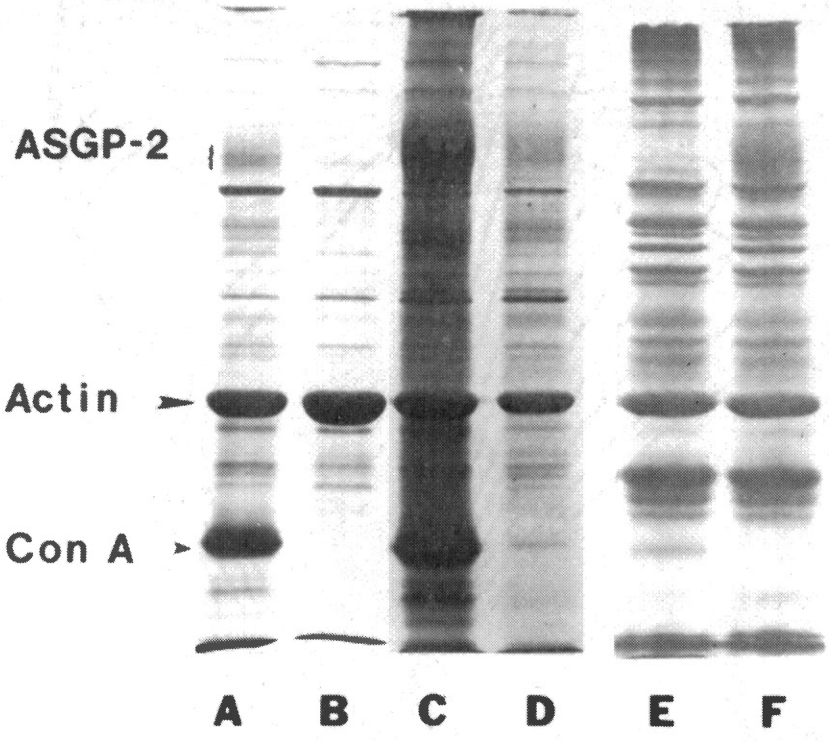

ASGP-2
Actin
Con A
A
B
C
D
E
F

TABLE IX

CON A EFFECT ON ACTIN DISTRIBUTION IN TRITON EXTRACTS BY DIFFERENTIAL CENTRIFUGATION

	10 K g Pellet	100 K g Pellet	100 K g Supernate
	Actin, % of Total		
Control (no Con A)	51	15	34
Con A	30	33	37
Con A plus α-MM	49	16	35

than the transmembrane complex, Con A-mediated association of the two complexes should result in a dramatic shift of ASGP-2 further into the gradient.

Gradient fractions obtained after 80,000 X g centrifugation were analyzed by SDS PAGE, as shown in Figures 29 and 30. The bands for actin, CAG and ASGP-2 were excised for counting radioactivity. The profiles of these components are shown in Figure 31. In the control the soluble proteins, including the soluble actin and ASGP-2, are found in the top half of the gradient (fractions 1-6). The remainder of the actin is found concentrated around the fractions 8-9, and its profile is nearly coincident with that of CAG. In the presence of Con A, the migration of the soluble proteins, including soluble actin, is unchanged except for ASGP-2. The Con A crosslinking has caused ASGP-2 to be shifted into the gradient to a broad peak with a maximum at fraction 9. Moreover, the actin and CAG have also been slightly shifted such that they are nearly coincident with the ASGP-2. The simplest explanation for this result is that the crosslinking of ASGP-2 to CAG causes the shift of all three species (actin, CAG, and ASGP-2) on the gradient. Since the ratio of actin to CAG in the peaks from the sucrose gradients in both of Con A-treated samples and Con A plus α-MM-treated controls is much higher than that found for the transmembrane complexes, the actin must be in polymerized form, not just as part of the transmembrane complexes. These results provide strong evidence for the association of the transmembrane complexes with the polymerized actin of the cytoskeleton. Coincidence of the ASGP-2 and actin on the sucrose gradient in the presence of Con A indicates the association of ASGP-2 with polymerized actin, demon-

Figure 29. SDS PAGE of Sucrose Density Gradient Fractions of the 10 K g Supernates of Triton-Extracts of Con A-Treated Microvilli.

The 7-25% sucrose gradients were run as described in the experimental procedures. The fractions from the top of the gradient are on the left.

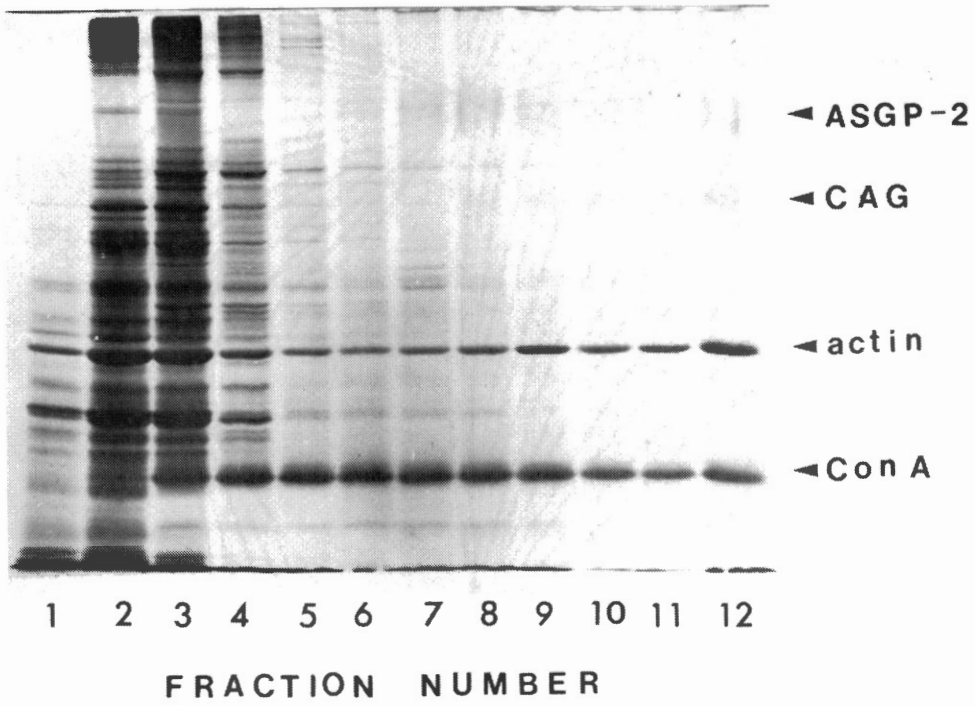

ASGP-2
CAG
actin
Con A
1 2 3 4 5 6 7 8 9 10 11 12
FRACTION NUMBER

Figure 30. SDS PAGE of Sucrose Density Gradient Fractions of the 10 K g Supernate of Triton Extracts of Con A Plus α-MM-Treated Microvilli.

The 7-25% sucrose gradients were run as described in methods. The fractions from the top of the gradient are on the left.

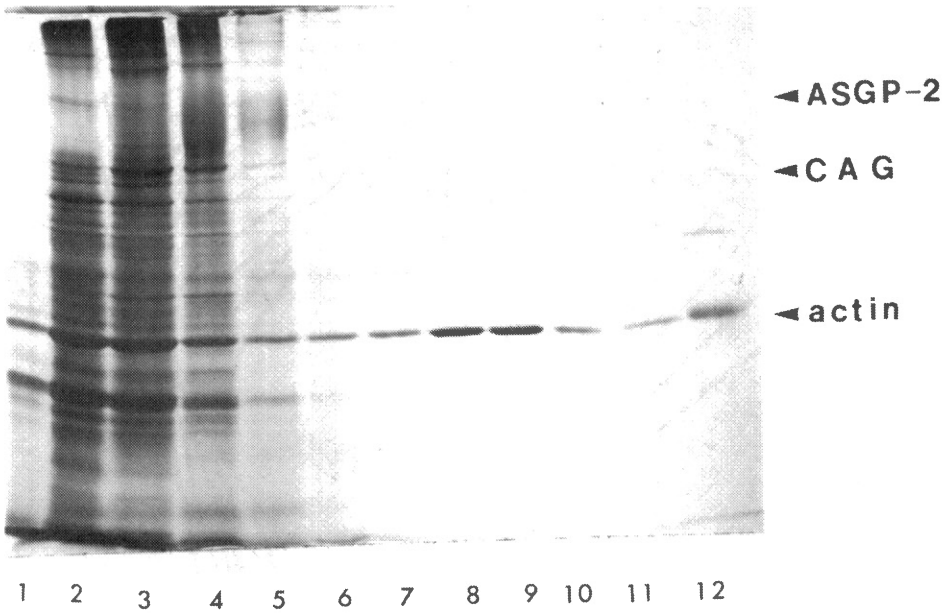
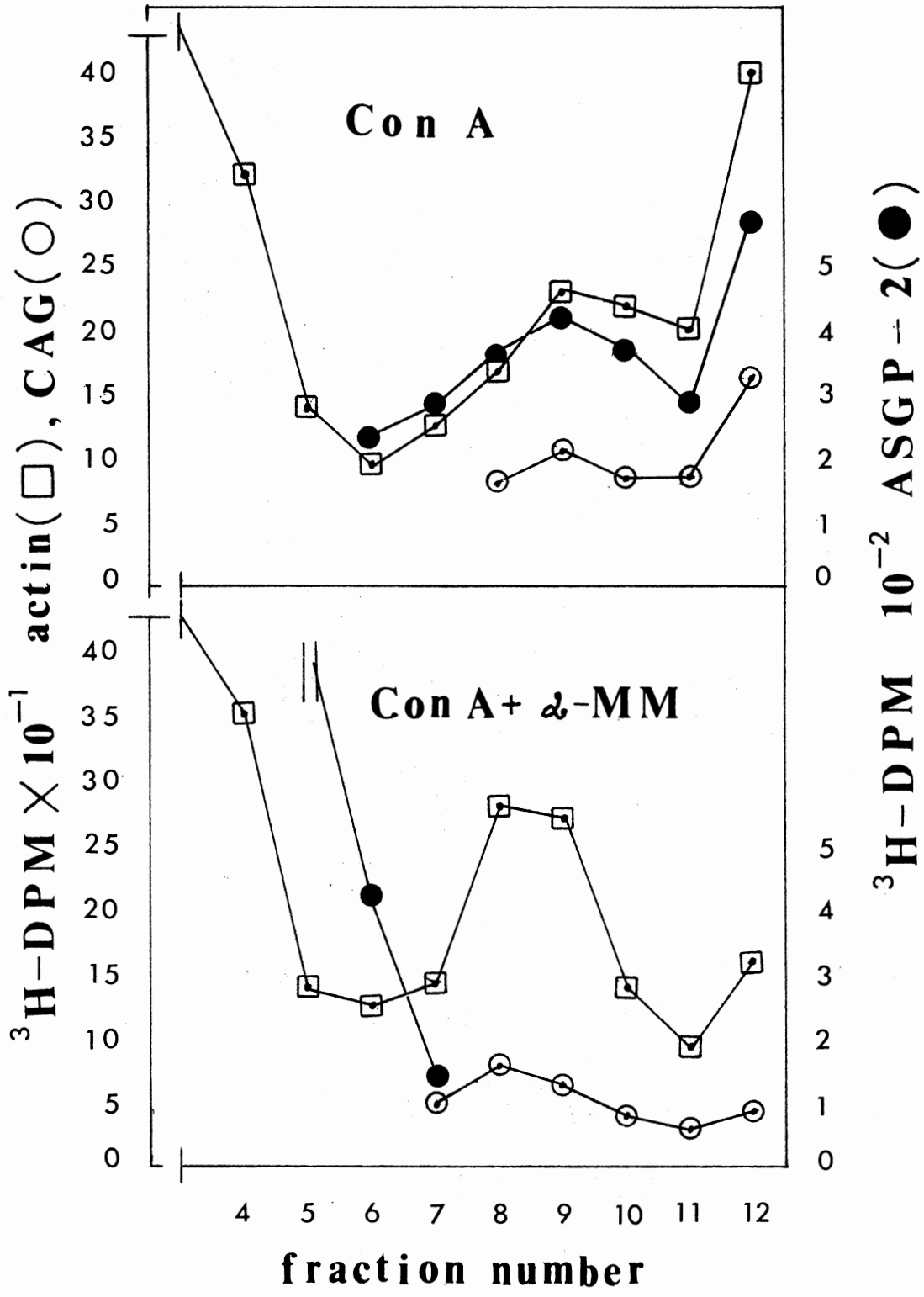

ASGP-2
CAG
actin
1 2 3 4 5 6 7 8 9 10 11 12
FRACTION NUMBER

Figure 31. Sucrose Gradient Profiles of the Fractions from Figures 29 and 30.

The appropriate bands of SDS PAGE gels of Figures 29 and 30 were excised and counted for radioactivity.


Con A
Con A+ α-MM
^{3}H-DPM $\times 10^{-1}$ actin(□), CAG(○)
^{3}H-DPM 10^{-2} ASGP-2(●)
0
5
10
15
20
25
30
35
40
0
1
2
3
4
5
4
5
6
7
8
9
10
11
12
fraction number

strating the association of the sialoglycoproteins with the cytoskeleton.

In conjunction with the CsCl gradient results, these data provide strong support for an indirect interaction between the sialoglycoprotein complex and the cytoskeleton, which is mediated by CAG of the transmembrane complex and Con A.

Separation of Actin Forms by Fractionation of Microvilli

Because the preparation of microvillar membranes may cause changes in the native microvillar actin, we have applied the procedures described above to the direct fractionation of microvilli. When MAT-C1 microvilli are treated with S buffer at room temperature and centrifuged at 10,000 X g for 15 min, an insoluble residue is obtained which contains about 40% of the microvilli actin. By SDS PAGE the predominant components are actin and α-actinin. Only small amounts of the 58 K polypeptide and CAG are present in this residue. Electron microscopy of negatively stained preparations shows an abundance of crosslinked actin microfilaments. When the 10,000 X g supernates are centrifuged at 100,000 X g for 1 hr, a residue is obtained which contains primarily the 58 K polypeptide, CAG and 10-15% of the microvillar actin. The 100,000 X g supernate contains 45-50% of the microvillar actin. As noted earlier (22), the soluble actin from microvilli contains the oligomers, which are present in about a 3:1 ratio with G-actin. These results indicate that the actin distribution in the microvillar membranes differs from that in the microvilli only by the absence of the filamentous actin.

Preparation and Characterization of Microfilament Core

MAT-C1 microvilli were extracted with 30 mM sodium phosphate buffer (pH 7.4) containing 0.25% DOC and centrifuged at 10,000 X g for 15 min at $4^{o}C$. The pellet consists of actin and α-actinin as the major components. The pellet is termed a microfilament core which is composed of the crosslinked actin filaments. However, unlike the microfilament core found in the brush border, the MAT-C1 microfilament core does not contain significant quantities of the highly specialized actin binding proteins (e.g., villin, fimbrin, and 110 K). The fact that the microfilament does not have significant proteins besides actin and α-actinin raises the question of whether the α-actinin plays a role in crosslinking the actin filaments (or stabilizing the microfilament).

It has been reported that calcium inhibits the binding of α-actinin to the microfilament (121). To study this question, the samples of microfilament core obtained by DOC extraction and 10,000 X g centrifugation were treated with various concentrations of Ca^{2+}. Figure 32 shows the extraction profile for α-actinin. At concentrations of greater than 100 μM Ca^{2+}, approximately 80% of α-actinin is released from the microfilament but most of actin (> 85%) is still sedimented at 10,000 X g (Table X). This observation suggests that α-actinin is not the only cross-linker of microfilaments in the core.

Furthermore, to see if other crosslinking proteins are involved in stabilizing the microfilament, the microvilli were extracted with Triton-PIPES (pH 6.8) in the absence and presence of an exogenous

Figure 32. Extraction of Actin and α-Actinin from DOC Cores by Ca^{2+}.

● : α-actinin; ○ : Actin.

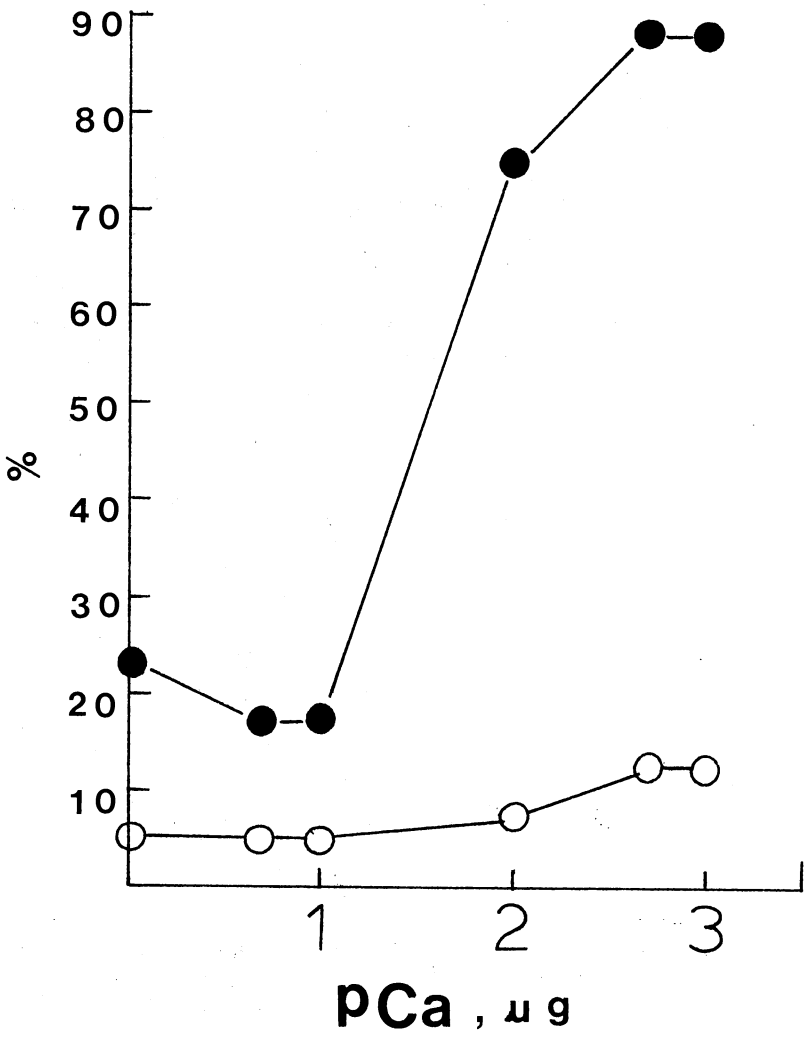

90
80
70
60
50
%
40
30
20
10
1
2
3
pCa , μg

TABLE X

EXTRACTION OF ACTIN AND α-ACTININ FROM DOC CORES BY Ca^{2+}

Ca^{2+} (μg/ml)	% of Total	
	Actin	α-Actinin
0	<5	23
5	<5	17
10	<5	17
100	5–10	75
500	10–15	88
1000	10–15	88

protease, trypsin. The effect of the protease on stabilizing the microfilament was examined by SDS PAGE. Figure 33 shows that essentially all of the higher molecular weight proteins and more than 70% of α-actinin are degradated by trypsin. Table XI shows there is a greater percentage of actin in low speed pellet in the absence of trypsin than that found in the presence of trypsin, and further the percentage of actin of the high speed pellet in the presence of trypsin is higher than that found in the absence of trypsin. The percentage of soluble actin in high speed supernates remains constant in both trypsin-treated and untreated samples. These observations imply that some microfilaments are dissociated into individual uncrosslinked filaments because of degradation of crosslinkers by protease. However, the amount of actin in the low speed pellet of the trypsin-treated sample approaches 75% of the amount of actin in low speed pellet of the untreated control. These observations may suggest that factor(s) other than crosslinking is (are) also involved in stabilizing the microfilament core. It has been reported that actin does not require auxiliary proteins to form bundles (64, 102).

To study the effect of microvillar protein concentration on the microfilament core, various amount of MAT-C1 microvilli were extracted with Triton-PBS, and actin forms were fractionated by differential centrifugation (Figure 34). It can be seen from Table XII that as the protein concentration of the microvilli decreases, the percentage of actin found in the low speed pellet also decreases. In contrast, the percentage of actin in the high speed pellet tends to increase with decreasing concentrations of microvillar protein. Since the

Figure 33. Trypsin Effect on Actin Distribution.

MAT-C1 microvilli were extracted with Triton-PIPES for 15 min at 37°C both in the absence and presence of trypsin. The extracts were fractionated by sequential differential centrifugations. A, D, and G: control; B, E, and H: 1 µg trypsin per ml. A, B, and C: 10,000 g pellet; D, E, and F: 100,000 g pellet; G, H, and I: 100,000 g supernate.

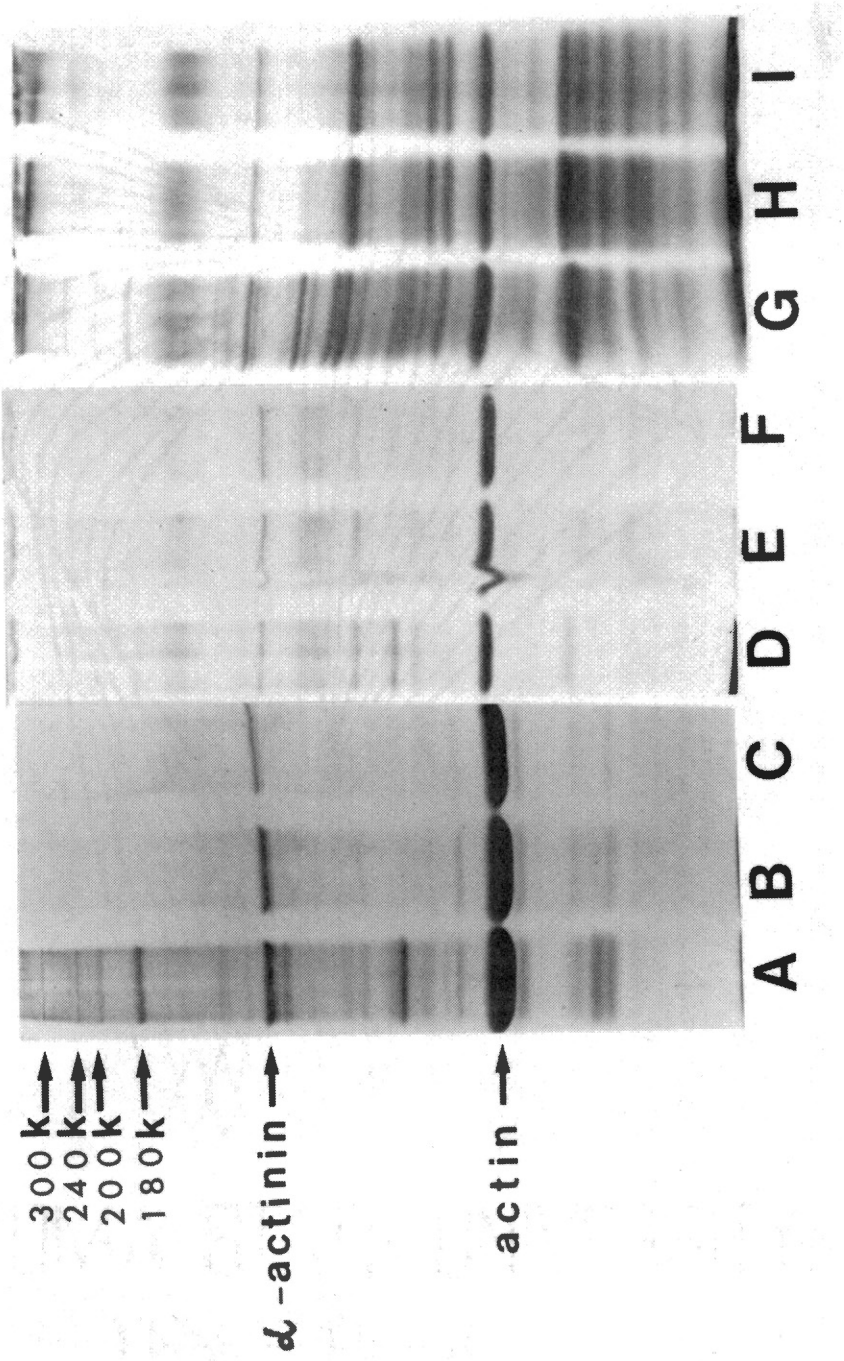

A B C D E F G H I
300k
240k
200k
180k
α-actinin
actin

TABLE XI

THE EFFECT OF TRYPSIN ON ACTIN DISTRIBUTION

Trypsin, μg/ml	% actin of total		
	10 K g pellet	100 K g pellet	100 K g supernate
0	53	19	28
1	45	29	26
5	38	34	28

Figure 34. Protein Concentration Dependence on Actin Distribution.

1) 750 µg protein per ml; 2) 500 µg; 3) 250 µg;
4) 125 µg.

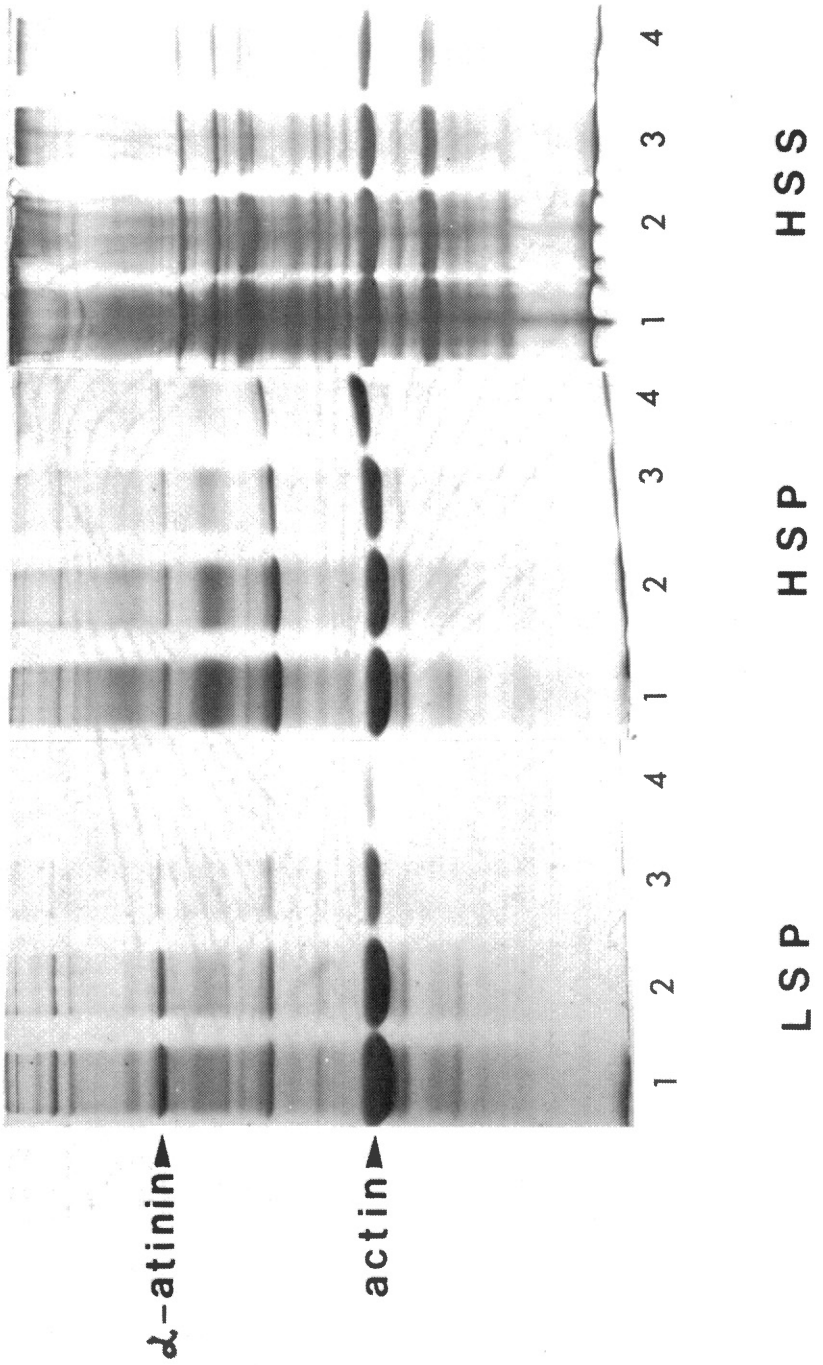

α-atinin
actin
1 2 3 4
LSP
1 2 3 4
HSP
1 2 3 4
HSS

TABLE XII

THE PROTEIN CONCENTRATION DEPENDENCE ON ACTIN DISTRIBUTION

Microvilli, µg/ml	% actin of total		
	10 K g pellet	100 K g pellet	100 K g supernate
750	36	21	43
500	30	25	45
250	21	34	45
125	17	37	46

percentage of actin found in the high speed supernate tended to remain relatively constant over the range of concentrations studied, it may be concluded that as microvillar protein concentration is increased, smaller amounts of actin are transferred from the low speed pellet to the high speed pellet. These observations lend support to the finding (35) that bundle assembly can occur as a result of thermodynamic effects when polymers became sufficiently concentrated.

The combined observations suggest that the factors involved in stabilizing microfilament core are contributed not only by crosslinking protein(s) but also by physical contact.

CHAPTER IV

DISCUSSION

Microvilli can be prepared from both MAT-B1 and MAT-C1 cells by a gentle shearing procedure, followed by differential centrifugation. SDS PAGE comparisons of two sublines showed a single observable qualitative polypeptide difference. The polypeptide has an apparent molecular weight of 58,000-daltons and is associated with submembrane cytoskeleton.

SDS PAGE pattern of the non-ionic detergent insoluble cytoskeletal residues from both MAT-B1 and MAT-C1 sublines shows a broad band at 75,000-80,000-daltons, along with actin and the 58,000 polypeptide only in case of MAT-C1 subline. By IEF/SDS PAGE this component was observed as a series of spots with increasing apparent molecular weight at decreasing pI values. This behavior is typical of sialoglycoproteins. The presence of carbohydrate (sialic acid residues) was demonstrated by glucosamine labeling and neuraminidase treatments. In addition the association of this glycoprotein with actin-containing structures was demonstrated by myosin affinity precipitation and by the change in sedimentability upon DNase treatments that release soluble actin (22). Based on these results, the glycoprotein is designated a cytoskeleton-associated glycoprotein (CAG). The results described above imply that the 58,000 polypeptide (58 K) and CAG are associated with the actin-containing cytoskeletal residues of the microvilli. Moreover,

lactoperoxidase iodination and neuraminidase treatment of the sealed microvilli suggest that CAG is at the cell surface.

To investigate the organization of CAG, 58 K, and actin in the microvilli, the microvillar membranes are prepared under conditions that depolymerize filamentous actin. Although microfilaments were not observed in either the microvillar membranes or their Triton residues (18), actin is the major protein. CAG and 58 K in MAT-C1 and CAG in MAT-B1 are also abundantly represented, but less prominent than actin. Moreover, both CAG and 58 K of Triton-solubilized MAT-C1 microvillar membranes co-sedimented with actin at 100,000 X g.

As determined by scintillation counting of bands from gels of ^{3}H-leucine-labeled microvillar membranes, the ratios of these three components in Triton X-100-insoluble residues are approximately equimolar, suggesting a specific complex. Because we do not know the relative amounts of leucine in each protein or the rates of incorporation of label, it is not possible to predict the exact ratios. However, the fact that the ratios are reproducibly near one suggests that this is not simply a nonspecific aggregate.

Further evidence for a complex was obtained from the observations that the three components co-fractionated on gel filtration and sucrose density gradients. If they were present as three homopolymers, one would expect separation by these techniques unless they are fortuitously all the same size and shape. The sucrose density gradient results indicate that the complex is large and heterogeneous. Estimates using calibration markers on the gradients or gel filtration column indicate that the molecular mass values are greater than 10^{6} (data not shown). Other membrane components extracted with non-ionic detergents show

similar behavior: the acetylcholine receptor (52) and the galactose-specific lectin of liver membranes (89). There are two possible explanations for this type of behavior. One possibility is that the three-membered complexes are present in the membrane as multimolecular aggregates, as is the acetylcholine receptors. The second possibility is that the complexes are present as discrete units in the membrane, which aggregate upon detergent extraction. Experiments must be undertaken to attempt to distinguish between these alternatives. These results suggest a membrane-associated three-membered complex from MAT-C1 microvilli, but do not indicate how the three members are associated.

Evidence for a direct linkage between actin and CAG is obtained by isolating, from MAT-B1 microvillar membrane, a complex containing only CAG and actin. Additional evidence for a direct actin-CAG interaction is obtained by denaturing treatments of the MAT-C1 complex. 58 K could be released by 0.5 M urea or 1 M guanidine-HCl, but release of CAG and actin requires 6 M urea or 3-4 M guanidine-HCl.

Since CAG can be localized at the mcirovillar surface and actin and 58 K are found inside microvillar membranes, the putative complexes must be transmembrane. The simplest explanation is that CAG is a transmembrane glycoprotein.

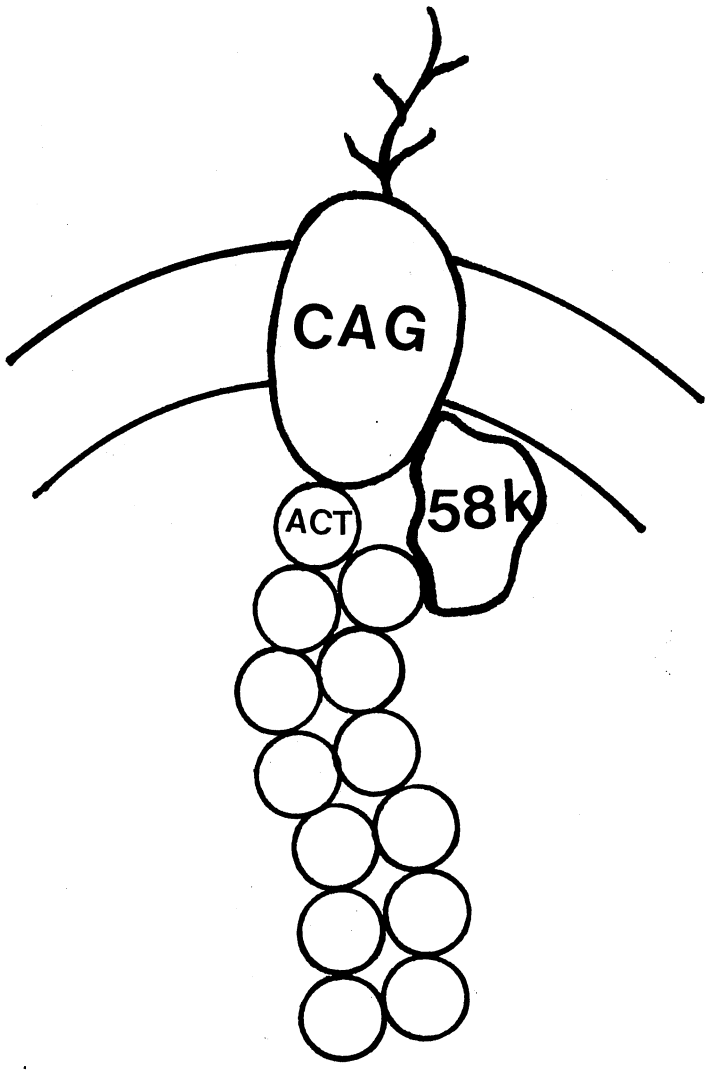
Carraway et al. (20) have previously proposed that the restriction of cell surface receptor mobility and the branched structure of the MAT-C1 microvilli are a consequence of the greater stability of MAT-C1 microvilli. The present results provide an explanation for this stability if it is assumed that anchorage of the actin to the membrane determines the stability of the microfilaments, which would be necessary

to maintain the microvillus structure. We propose that microfilaments are linked to the membrane through the actin-CAG association, possibly with an oligomeric actin (22) structure as a linker. We further suggest that in vivo the 58 K polypeptide stabilizes the association of the actin-CAG complex with the microfilaments in the cells to retard disruption of the filament-membrane interaction and thus stabilize the microfilaments. Figure 35 shows a simple feasible model for attachment of transmembrane complexes to microfilaments.

Much more needs to be learned about the interaction of CAG and actin and how this interaction is controlled in the cells. Whether this type of interaction is a general phenomenon is unknown. However, there have been other recent reports of cell surface glycoprotein associations with cytoskeletal residues. H-2 histcompatibility antigen (66) and 5'-nucleotidase (80) remain with a cytoskeletal fraction after nonionic detergent extraction of cell surface membrane fractions. In other examples, the association of cell surface glycoproteins with cytoskeletal residues is enhanced by treatment with Concanavalin A (96, 111). The mode of association with the cytoskeleton has not been described for any of these examples. Perhaps a direct glycoprotein linkage also occurs in these cases. It is intriguing to speculate that cells may contain several transmembrane glycoproteins with specific cytoplasmic side polypeptide sequences that can bind to actin, thus providing anchorage sites on the cytoskeleton for selected cell surface molecules.

The anchorage of the sialoglycoprotein complex (ASGP-1/ASGP-2) containing the major Con A-binding protein has been investigated. Previous studies have shown that this complex is mobile in MAT-B1 and

Figure 35. Model for Association of MAT-C1 Microvillar Microfilaments with Transmembrane Complexes and Stabilization of the Association by 58 K.


CAG
ACT
58k

immobile in MAT-C1 sublines on Con A treatment (53). We now provide evidence that anchorage of this sialoglycoprotein complex to the cytoskeleton results from its linkage via a Con A bridge to CAG of the transmembrane complex.

Four lines of evidence support this proposal. 1) ASGP-1/ASGP-2 complex is readily sedimented with cytoskeletal residues from Con A-treated microvilli and microvillar membranes, but not from untreated samples. 2) ASGP-1/ASGP-2 is associated with transmembrane complexes sedimented from microvillar membranes treated with Con A. 3) Con A treatment causes a shift of the transmembrane complex to a higher density on CsCl gradient as a result of its association with the heavily glycosylated sialoglycoprotein complex. 4) Sucrose density gradient centrifugation shows that the sialoglycoprotein complex is associated with transmembrane complex linked to polymerized actin in Con A-treated samples, but not untreated controls. The sucrose gradient studies provide additional evidence for the association of transmembrane complex with polymerized actin.

Since no additional actin is found in the Con A-treated transmembrane complex with associated ASGP-1/ASGP-2 by sedimentation or CsCl gradient centrifugation, the most likely mechanism for the association of the two complexes is that the sialoglycoprotein ASGP-2, the major Con A-binding protein, is linked by Con A to CAG, the transmembrane complex Con A-binding protein. Moreover, the association of polymerized actin with ASGP-2 and CAG, as shown on sucrose gradients, provides a mechanism whereby the glycoproteins can be linked to the rest of the microvillus cytoskeleton. These results provide evidence for two types of mechanisms of anchorage to the cytoskeleton, direct

(CAG) and indirect (ASGP-2), which could be involved in restricting or aiding receptor redistribution. Either mechanism could be involved in determining the organization of Con A receptors.

Another important conclusion from these studies is that Con A-induced anchorage of ASGP-1/ASGP-2 is observed in the sublines with mobile or immobile Con A receptors. Thus anchorage of the sialoglycoprotein complex to the cytoskeleton would not be sufficient to confer either mobility or immobility on the Con A receptors of their ascites cells. We propose that the difference in receptor mobility between the two sublines residues in the relative stabilities of the membrane-cytoskeleton interaction.

Our view of Con A-induced capping in these cells is as follows. Binding of Con A to MAT-B1 cells crosslinks the cell surface Con A receptors, including ASGP-2 and CAG. The crosslinking and aggregation lead, by some as yet unknown mechanism(s), to a breakdown of the submembrane cytoskeleton and its associated microfilaments. This premise is consistent with morphological changes, i.e., loss of microvilli, of MAT-B1 cells treated with Con A (60). It is also consistent with the change in sedimentation observed for MAT-C1 microfilament core actin of Con A-treated microvilli without depolymerization of actin. This breakdown of surface actin structures is followed by a reorganization of the actin and cell surface glycoproteins to give cap structures, again by an undefined mechanism(s). We propose that in MAT-C1 cells binding of Con A to the cells also leads to crosslinking and aggregation. However, the subsequent depolymerization of the submembrane actin structures does not occur in MAT-C1 microvilli, because the membrane-cytoskeleton linkage has been stabilized by the

presence of the 58 K polypeptide. This premise is consistent with the greater stability of the MAT-C1 microvilli to cytochalasins and hypotonic treatments (60). Failure to depolymerize submembrane actin leads to a freezing of the cell surface with the sialoglycoprotein complexes crosslinked to immobile CAG molecules.

According to this scheme, it is the control of the mobilization of actin or the movement of actin microfilaments that is critical for receptor mobility. This hypothesis is consistent with observations in other systems. For example, cell surface components in the erythrocyte are relatively immobile (30). At least some of these are attached (2) to a stable submembrane cytoskeleton which has stable actin oligomers (11). In contrast the submembrane cytoskeleton of more complex cells (e.g., lymphoid cells) appears more dynamic (94). One problem with this type of analysis is that Con A at high concentrations inhibits Con A receptor distribution in lymphoid cells (30). Since microtubule-disrupting agents, such as colchicine, can reverse this inhibition (31), one explanation is that high concentrations of Con A stabilize the submembrane cytoskeleton via microtubule-microfilament interactions (20, 94). Moreover, differences in receptor redistribution between transformed and untransformed fibroblasts (6) might be explained by differences in organization of submembrane actin due to changes in actin binding proteins such as vinculin (7, 39) and tropomyosin (70). Thus the behavior of submembrane actin may provide a unifying paradigm for understanding the control of the organization of cell surface components. Whether this paradigm can explain all of the different observations on cell surface organizational changes in different cell types (1, 91, 94, 112) remains to be determined.

The reported results indicate the presence of as many as four forms of actin in the microvilli. The microfilamentous actin is sedimented at low speed after S buffer solubilization and must be crosslinked into larger structures than individual microfilaments. Because of its abundances α-actinin may be a prime candidate for the crosslinker. However, it may not be the only crosslinker involved, and the other factor, e.g., physical contact, also may be involved in bundling the filamentous actin. Previous studies (22) showed that about 10% of the microvillar actin is released by S buffer extraction in a form of the size of G-actin. However, whether this represents the actual amount of G-actin in the microvilli or results from breakdown of the other actin forms during extraction remains to be determined. The membrane-associated actin of the microvilli exists in two forms. Less than 10% of this actin is part of the large transmembrane complexes. Most of the membrane-associated actin appears in S buffer as oligomers.

These accumulated results permit us to develop a model of the organization of the actin in the microvillus and its role in controlling cell surface organization. The key element in this model is the actin-containing transmembrane complex. We envision that it acts as an attachment site for actin microfilaments as well as a site for actin polymerization at the cytoplasmic surface of the plasma membrane (Figure 35). Because of the stoichiometry of actin to CAG in the microvillus, it is unlikely that most of the transmembrane complexes are occupied by microfilaments. Instead, we envision it as being attached to oligomeric segments of actin.

CHAPTER V

SUMMARY

To investigate the molecular nature of the cell surface-cytoskeleton interaction, microvilli and microfilament-depleted microvillar membranes from MAT-B1 and MAT-C1 sublines have been prepared and studied by biochemical fractionations and detergent extractions. The qualitative comparisons of MAT-B1 and MAT-C1 microvilli and microvillar membranes by SDS PAGE showed that they differed primarily by the presnece of 58,000 dalton polypeptide (58 K) which was found in MAT-C1 but not in MAT-B1 subline. A glycoprotein of an apparent molecular weight of 75,000-80,000 daltons has been found in an insoluble cytoskeleton of nonionic detergent-solubilized microvilli and microvillar membranes from the two sublines. By the two-dimensional isoelectrofocusing/sodium dodecyl sulfate polyacrylamide gel electrophoresis, this component was observed as a series of spots. This behavior is typical of sialoglycoproteins. Glucosamine labeling and neuraminidase treatments indicated that this component was a sialoglycoprotein. Lactoperoxidase iodination and neuraminidase treatments showed its presence at the microvillar surface.

Extraction of MAT-C1 microvillar membranes with Triton X-100-containing buffers gave insoluble residues showing three major components by SDS PAGE: actin, the 58,000 dalton polypeptide, and the cell surface glycoprotein of 75,000-80,000 daltons. As determined by

scintillation counting of bands from gels of ^{3}H-leucine-labeled microvillar membranes, the stoichiometry among the three components approached equimolar, suggesting a defined three-membered complex. The three components of the putative complex in the Triton-solubilized microvillar membranes comigrated on rate-zonal sucrose density gradient centrifugation and Sepharose CL-2B gel filtration. Its glycoprotein-actin interaction could be disrupted only by under strongly denaturing conditions (e.g., 3-4M guanidine hydrochloride). The complex prepared from MAT-B1 microvillar membranes by Sepharose CL-2B gel filtration contained actin and the glycoprotein but not the 58,000-dalton polypeptide. In addition, DNase treatments, myosin affinity analysis, and sucrose density gradient centrifugation suggested an association between the complex and the microvillar microfilaments. In light of these results the glycoprotein was termed a cytoskeleton-associated glycoprotein (CAG), which is a transmembrane glycoprotein. The complex was named the transmembrane complex. We propose that the cell surface-cytoskeleton interactions in the 13762 tumor cell microvilli involve direct association of submembrane cytoskeleton (actin) with the cell surface glycoprotein.

Treatment of the microvilli and the microvillar membranes with Con A resulted in marked retention of ASGP-1 and ASGP-2, a Con A-binding protein, in cytoskeletal residues of both sublines obtained by extraction with phosphate-buffered saline containing Triton X-100. When the Con A-treated microvillar membranes were extracted with Triton X-100-containing buffer, the sialoglycoprotein complex (ASGP-1/ASGP-2) was found associated in residues with a transmembrane complex. The untreated membrane Triton insoluble residues retained

very little ASGP-1/ASGP-2 complex. Association of the sialoglycoprotein complex and the transmembrane complex was also demonstrated in Con A-treated, but not in untreated microvilli, by their comigration on CsCl gradients. Association of both complexes with the polymerized actin of microvilli was shown by sucrose density gradient centrifugation. A fraction of the polymerized actin comigrated with the transmembrane complex alone in the absence of Con A and with both the sialoglycoprotein complex and the transmembrane complex in the presence of Con A. From these results we propose that anchorage of the sialoglycoprotein complex to the cytoskeleton on Con A treatment occurs by cross-linking ASGP-2 to CAG, also a Con A-binding protein, of the transmembrane complex which is natively linked to the cytoskeleton via its actin component. Since Con A-induced anchorage occurs in the sublines with mobile and immobile receptors, the anchorage process cannot be responsible for the differences in receptor mobility between the sublines.

By extraction with actin-stabilizing buffers (isotonic Triton-Mg^{2+}-ATP) microvillar actin could be fractionated into four forms. Approximately 40-50% of the actin was sedimented by low speed centrifugation. The pellets contained the microfilaments, and actin and α-actinin were the predominant proteins. Less than 10% of actin was in the transmembrane complex. About 30-40% of the actin was found as small oligomers and about 10% as G-actin, implying the actin oligomers are present in about a 3-4:1 ratio with G-actin. These results suggest that the actin-containing transmembrane complexes may serve as membrane association sites for oligomeric actin segments and microfilaments and possibly as initiation sites for actin poly-

merization.

In view of the results we propose a model (Figure 35) which might explain the differences in microvillus stability and a receptor mobility of the MAT-B1 and MAT-C1 cells: the 58,000-dalton polypeptide might stabilize the attachment of the microfilaments to the transmembrane complex in MAT-C1 cells by its interaction with actin of the attached microfilaments as well as actin of the transmembrane complex. Stabilization of the microfilament-transmembrane complex interaction by the 58 K would retard microfilament breakdown and act to retard receptor mobility by some as yet unknown mechanism.

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