

Mechanism of Concanavalin A-induced Anchorage of the Major Cell Surface Glycoproteins to the Submembrane Cytoskeleton in 13762 Ascites Mammary Adenocarcinoma Cells

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ABSTRACT Concanavalin A (Con A)-induced anchorage of the major cell surface sialoglycoprotein component complex (ASGP-1/ASGP-2) was studied in 13762 rat mammary adenocarcinoma sublines with mobile (MAT-B1 subline) and immobile (MAT-C1 subline) cell surface Con A receptors. Treatment of cells, isolated microvilli, or 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 Triton X-100 in PBS. When Con A-treated microvillar membranes were extracted with a buffer containing Triton X-100, the sialoglycoprotein complex was found associated in the residues with a transmembrane complex composed of actin, a 58,000-dalton polypeptide, and a cytoskeleton-associated glycoprotein (CAG), also a Con A-binding protein, in MAT-C1 membranes, and of actin and CAG in MAT-B1 membranes. Untreated membrane Triton residues retained very little ASGP-1/ASGP-2 complex. Association of the sialoglycomembrane complex and the transmembrane complex was also demonstrated in Con A-treated, but not untreated, microvilli by their comigration on CsCl gradients. Association of both complexes with the cytoskeleton 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 transmembrane complex and the sialoglycoprotein 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, the major cell surface Con A-binding component, to CAG of the transmembrane complex, which is natively linked to the cytoskeleton via its actin component. Since Con A-induced anchorage occurs in sublines with mobile and immobile receptors, the anchorage process cannot be responsible for the differences in receptor mobility between the sublines.

The organization and reorganization of cell surface components are widely believed to be controlled by a submembrane actin-containing cytoskeleton (1). Although anchorage to the cytoskeleton must be an important part of the mechanism for determining this organization, little is known of the molecular details of anchorage mechanisms. In the erythrocyte, a fraction of the cell surface anion transport (band 3) molecules is bound to the spectrin-actin cytoskeletal matrix via ankyrin (2). In more complex cells, apparent associations of cell

surface glycoproteins with cytoskeletal residues prepared by nonionic detergent extractions have been induced by the lectin Concanavalin A (Con A)¹ (3, 4). However, no infor-

¹ *Abbreviations used in this paper:* ASGP-1, a peanut agglutinin-binding sialoglycoprotein; ASGP-2, a Concanavalin A-binding sialoglycoprotein; CAG, cytoskeleton-associated glycoprotein; Con A, Concanavalin A; DPBS, Dulbecco's phosphate-buffered saline; α -MM, α -methylmannoside.

mation on the linkage mechanism has been presented.

Ascites sublines of the 13762 rat mammary adenocarcinoma provide a useful system for investigating these questions. These cells have a relatively simple major cell surface component composition. More than 90% of labeled glucosamine incorporated into glycoproteins in these cells is found in two cell surface glycoprotein components, ASGP-1, a peanut agglutinin-binding sialomucin, and ASGP-2, a Con A-binding protein (5). These are present as a complex at the cell surface (6, 7), and are termed the sialoglycoprotein complex (ASGP-1/ASGP-2). Capping studies with fluorescent Con A and peanut agglutinin demonstrated the association of the two components, and have shown that the glycoprotein complex is immobile in one subline (MAT-C1) and mobile in other sublines, e.g., MAT-B1 (8, 9). These observations suggest a Con A-mediated linkage of the sialoglycoprotein complex to the cytoskeleton. The availability of sublines with mobile and immobile receptors make this a particularly attractive system for investigating receptor anchorage mechanisms.

Microvilli and microvillar membranes can be isolated from both of these sublines for investigation of membrane-cytoskeleton interactions (10–12). By detergent extractions and fractionations we have been able to isolate a second type of complex from microvillar membranes, termed the transmembrane complex (13, 14). This complex contains actin associated with a third glycoprotein, cytoskeleton-associated glycoprotein (CAG). Although CAG binds Con A, lectin binding is not necessary for the association of CAG with actin (14). These results imply a direct association in the native state of a cell surface molecule with the cytoskeleton (14) and suggest a mechanism whereby other cell surface components may become linked to the cytoskeleton. These observations raise two questions concerning the control of the organization of the major cell surface components of these ascites cells. (a) Does the linkage of the sialoglycoprotein complex (ASGP-1/ASGP-2) occur by direct attachment to the cytoskeleton, or does it occur indirectly by a Con A bridge between ASGP-2 and CAG? (b) Can the difference in Con A receptor mobility between the MAT-B1 and MAT-C1 sublines be explained by differences in anchorage of the Con A receptors, e.g., ASGP-2?

In the present investigation we have demonstrated that Con A-induced anchorage of the ASGP-1/ASGP-2 complex occurs via its linkage to the CAG-actin complex, with Con A itself apparently serving as the linking agent. Moreover, the anchorage mechanism appears to be the same in sublines with mobile and immobile Con A receptors, indicating that the difference in receptor mobility is not due to a difference in anchorage mechanisms.

MATERIALS AND METHODS

Materials: Con A was purchased from E. Y. Laboratories, Inc. (San Mateo, CA). Buffers were prepared from reagent-grade salts; Triton X-100, α -methylmannoside (α -MM), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO). D-[1-¹⁴C]Glucosamine (50–60 μ Ci/mmol), L-[³⁵S]methionine (1,300 Ci/mmol), and [³²P]phosphate (8 mCi/ml) were obtained from Amersham Corp. (Arlington Heights, IL). L-[¹⁴C]leucine (30 μ Ci/mmol) was obtained from ICN K & K Laboratories Inc. (Plainview, NY) and Instagel from Packard Instruments Co., Inc. (Downers Grove, IL).

Cell Passage and Metabolic Labeling: MAT-B1 and MAT-C1 ascites sublines of the 13762 rat mammary adenocarcinoma tumor were maintained by weekly intraperitoneal injection of 1.0 – 1.5×10^6 cells in 0.1 – 0.3 ml of 0.9% NaCl into 60–90-d-old female Fischer 344 rats (15). Cells used

for these studies were recovered from the peritoneal cavity after 7 d, washed with ice-cold Dulbecco's phosphate-buffered saline (DPBS), and resuspended in DPBS. Metabolic labeling with [¹⁴C]glucosamine (15–25 μ Ci), [¹⁴C]leucine (20 μ Ci), [³⁵S]methionine (25 μ Ci), or [³²P]phosphate (25 μ Ci) was accomplished by injection of the label in 0.1 ml of 0.9% NaCl into the peritoneal cavity of tumor-bearing rats ~16–18 h before sacrifice and recovery of the cells.

Con A Treatments and Detergent Extractions: Microvilli were prepared as described previously (11, 12). Microvillar membranes were prepared in glycine-EDTA-mercaptoethanol, pH 9.5 (16), to break down polymerized actin and yield vesicles (11, 12).

Cells, microvilli, or microvillar membrane fractions were incubated in the absence or presence of Con A for 15 min at 37°C in a gyratory shaking water bath at 120 rpm. The addition of α -MM as a competitive inhibitor of Con A served as a control for specific Con A binding. In the two-step incubation protocols, e.g., the addition of α -MM to Con A-treated preparations, excess primary reagents were removed by centrifugation. In all cases the unbound reagents were removed by centrifugation. The preparations were then incubated in 1.0 ml of 0.1% Triton X-100 in DPBS containing 0.5 mM PMSF for 15 min at 37°C in the shaking water-bath. Aliquots of Triton X-100-treated preparations were centrifuged in the Beckman Airfuge (Beckman Instruments, Inc., Palo Alto, CA) for 20 min at 30 psi at 4°C for separation of Triton-insoluble material (Triton residue).

Microvillar membrane fractions incubated in the absence or presence of Con A were also treated with S buffer (17; 0.5% Triton X-100, 5 mM Tris, 0.15 M NaCl, 2 mM MgCl₂, 0.2 mM ATP, 0.2 mM dithioerythritol and 0.1 mM PMSF, pH 7.6) and centrifuged as above to give S buffer residues.

Gel Electrophoresis: SDS PAGE was performed as described by King and Laemmli (18) on 4.5–12% acrylamide gradient or 7.5 or 8% separating gels with a 2-cm 3.5% stacking gel. Aliquots of cells, microvilli, or microvillar membrane fractions were solubilized by the addition of an equal volume of electrophoresis sample buffer (12) before electrophoresis. Gels were stained and destained (12) and photographed with 35-mm Kodak Pan-X film. Fluorography was performed as described previously (6).

Radioactivity Determination: For quantification of radioactivity, aliquots of samples from the Triton X-100 extraction experiments were taken before and after centrifugation for the isolation of Triton residues, mixed with 3 ml of Instagel, and counted. Analysis of radioactivity in slab gels was performed on excised slices of ASGP-2, CAG, 58-kd polypeptide, and actin incubated with Soluene 350 (14), mixed with 3.0 ml Instagel, and counted.

Density Gradient Centrifugation: Preformed CsCl gradients in Triton/PBS (0.2% Triton in DPBS containing no calcium) buffer were prepared by gently under-layering 2.0 ml each of buffer containing 1.60, 2.00, 2.40, 2.80 and 3.20 M CsCl in centrifuge tubes (5/8 \times 3 in). Approximately 1.0 ml of Triton/PBS-solubilized microvillar membrane extract was gently layered onto each gradient. Centrifugation was performed at 55,000 rpm for 18 h in a 75 Ti rotor at 4°C. Fractions (1.0 ml) were collected, dialyzed against three changes of 0.2% SDS, 0.1 mM PMSF, 1.0 mM EGTA over 48 h, and lyophilized for SDS PAGE analysis.

Sucrose density gradient centrifugation was performed on Triton PBS-solubilized microvilli as previously described (14).

RESULTS

Triton X-100 Extraction of MAT-B1 and MAT-C1 Cells in the Presence and Absence of Con A

MAT-B1 cells have highly mobile cell surface Con A receptors, while the MAT-C1 receptors appear immobile (8, 9). To compare the anchorage mechanisms in these sublines, we performed Triton X-100 extractions of the cells under conditions used to investigate receptor mobility in these cells (8). Since >90% of glucosamine label incorporated into cell glycoprotein is found in ASGP-1/ASGP-2 (5), the release of label from the cells was used as a measure of the ability of Con A to induce association of the ASGP-1/ASGP-2 complex with the cytoskeleton. Under the conditions used, 60–85% of the label was extracted from the untreated cells (Table I). Pretreatment with Con A caused retention of about half of the released label in MAT-B1 cells and three-fourths in MAT-C1 cells. The same effects were observed under conditions in which more of the label (>90%) was released. The retention of label by Con A could be largely prevented by inclusion of α -MM with the Con A (Table I), or the label could be released by

subsequent treatments with α -MM (data not shown).

To discover which glycoproteins were being retained, we examined the residues by SDS PAGE (Fig. 1). Since ASGP-1 and ASGP-2 stain poorly with Coomassie Blue, the gels on cells labeled with glucosamine were subjected to fluorography. Although several new Coomassie Blue-staining bands appeared in the residues from Con A-treated cells, none of them contained detectable radioactivity. However, the fluorograms showed convincingly that ASGP-1 and ASGP-2 were markedly retained in residues of the Con A-treated samples compared with the controls. The apparently greater amount of ASGP-2 in the fluorograms of Figs. 1 and 2 reflects the greater amount of glucosamine label in MAT-C1 cells and microvilli (5).

These results clearly show that Con A induces the retention of the ASGP-1/ASGP-2 complex in the cell cytoskeleton of both MAT-B1 and MAT-C1 cells. Since the complexity of the residues limits further analyses, the effects of Con A on the extractability of ASGP-1/ASGP-2 complex in isolated microvilli, which show a simple electrophoretic pattern for Triton residues (12, 13, 20), were determined.

Extraction of MAT-B1 and MAT-C1 Microvilli in the Presence and Absence of Con A

When isolated microvilli from glucosamine-labeled cells were extracted with Triton X-100 under the conditions noted previously, 70–85% of the label was released from treated

samples. Table II gives values for a typical experiment. About two-thirds of this released label was retained in Con A-treated MAT-B1 microvilli, while >90% of released label was retained in Con A-treated MAT-C1 microvilli. SDS PAGE together with fluorography showed that the primary retained components were ASGP-1 and ASGP-2 (Fig. 2). To demonstrate that there is no 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, we quantified ASGP-2 by gel band excision and counting (14). MAT-B1 and MAT-C1 residues showed 77 and 84%, respectively. Thus the proportion of ASGP-2 anchored to the cytoskeleton does not differ substantially between the two sublines, even though they differ qualitatively in their receptor mobilities.

It is possible that the retention of ASGP-1 and ASGP-2 does not reflect their association with the cytoskeleton, but rather a general stabilization of the membrane, including the lipid. Therefore extraction studies were conducted on microvilli isolated from MAT-C1 cells dual-labeled with [¹⁴C]glucosamine and either [³²P]phosphate, [³⁵S]methionine, or [³H]leucine. Under conditions in which ~90% of the released glucosamine was retained after Con A treatment, only ~20–25% of the phosphate, incorporated into protein as well as phospholipid (unpublished observations), was retained (Table III). These results, together with the SDS PAGE analyses, tend to exclude a general membrane or lipid stabilization by Con A.

Extraction of Untreated and Con A-treated Microvillar Membranes

Microvillar membranes, prepared by homogenization in glycine-EDTA-mercaptoethanol (pH 9.5) (11, 12), contain few if any microfilaments, but contain a fraction of actin which remains as an insoluble residue on extraction with Triton X-100 (12, 13). As shown in Fig. 3 for MAT-C1 microvillar membranes, ASGP-1 and ASGP-2 are the predominant components retained in residues remaining after Triton extraction of Con A-treated membranes.

TABLE I
Effect of Con A on the Triton Extraction of Glucosamine Label from MAT-B1 and MAT-C1 Cells

Treatment	% Extracted	
	MAT-B1	MAT-C1
None	67	83
Con A	36	25
Con A plus α -MM	65	77

Results are shown for a typical experiment. 2×10^6 cells were treated with 150 μ g Con A and 200 mM α -MM, as noted, before Triton extraction.

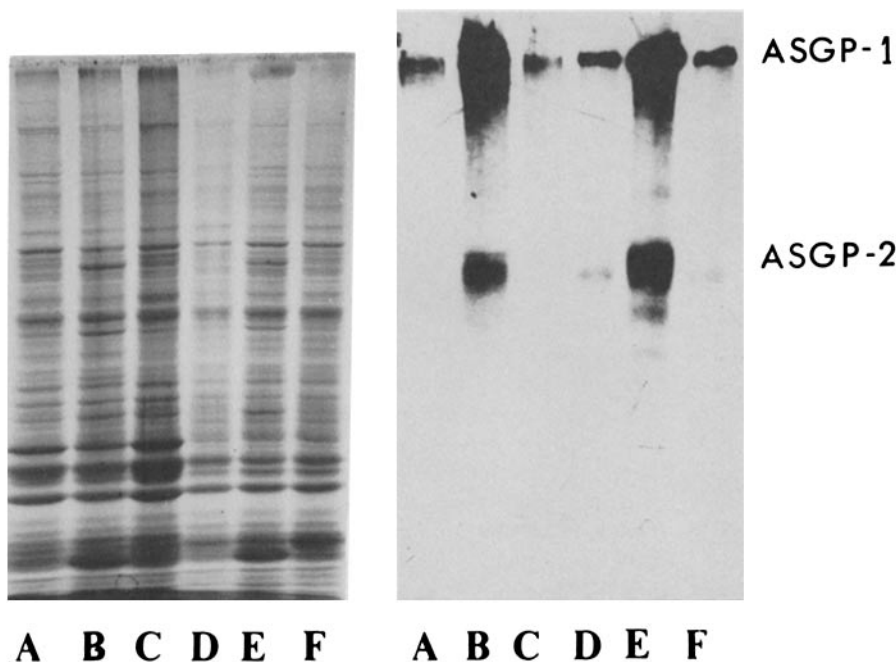


FIGURE 1 SDS PAGE (4.5–12% acrylamide gradient) of Triton X-100/DPBS cytoskeletal residues of MAT-B1 (A–C) and MAT-C1 (D–F) cells. (A and D) Untreated controls; (B and E) Con A-treated cells; (C and F) cells treated with Con A and α -MM. Left panel, Coomassie Blue-stained gels; right panel, fluorogram.

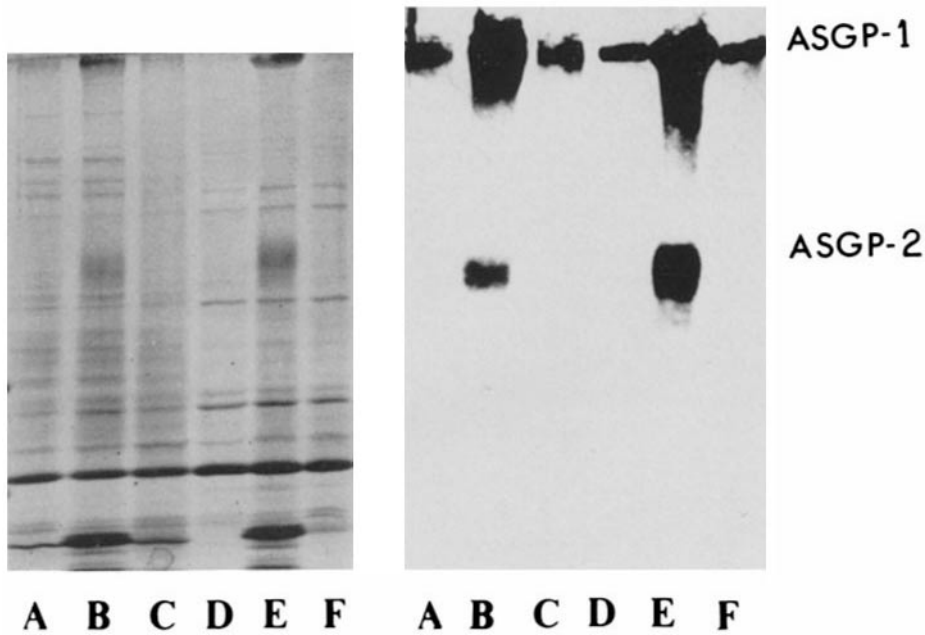


FIGURE 2 SDS PAGE (4.5–12% gradient) of Triton X-100/DPBS cytoskeletal residues of MAT-B1 (A–C) and MAT-C1 (D–F) microvilli. (A and D) Untreated controls; (B and E) Con A-treated microvilli; (C and F) microvilli treated with Con A and α -MM. Left panel, Coomassie Blue-stained gel; right panel, fluorogram.

TABLE II

Effect of Con A on the Triton Extraction of Glucosamine Label from MAT-B1 and MAT-C1 Microvilli

Incubation 1	Incubation 2	% Extracted	
		MAT-B1	MAT-C1
Buffer	Buffer	75	80
Con A	Buffer	24	7
Con A	α -MM	76	62
Con A plus α -MM	α -MM	75	63

Results are shown for a typical experiment. Microvilli (0.2 mg protein) were incubated with 150 μ g Con A and/or 0.2 M α -MM, as noted, before extraction.

TABLE III

Effect of Con A on the Triton Extraction of Radiolabeled Components of Microvilli Isolated from Biosynthetically Labeled MAT-C1 Cells

Treatment	% Extracted			
	[¹⁴ C]- Gluco- samine	[³⁵ S]- Methi- onine	[¹⁴ C]- Leucine	[³² P]- Phos- phate
None	73	80	80	88
Con A	7	54	58	70
Con A plus α -MM	73	73	75	75

Results are shown for typical experiments. Microvilli (0.1 mg protein) were incubated with 250 μ g Con A and/or 0.5 M α -MM, as noted. Chloroform/methanol extracted 72% of the ³²P in the control preparation.

Extraction of Untreated and Con A-treated Microvillar Membranes with S Buffer

There are two likely ways in which the ASGP-1/ASGP-2 complex could be attached to a submembrane cytoskeleton. (a) The ASGP-1/ASGP-2 complex could become linked directly to the cytoskeleton as a result of the interaction of ASGP-2, the major Con A-binding component (6), with Con A. (b) Alternatively, the ASGP-1/ASGP-2 complex could become linked to a second cell surface molecule, which is already attached to the submembrane cytoskeleton. In previous studies we have shown that treatments of microvillar membranes with S buffer solubilized most of the membrane

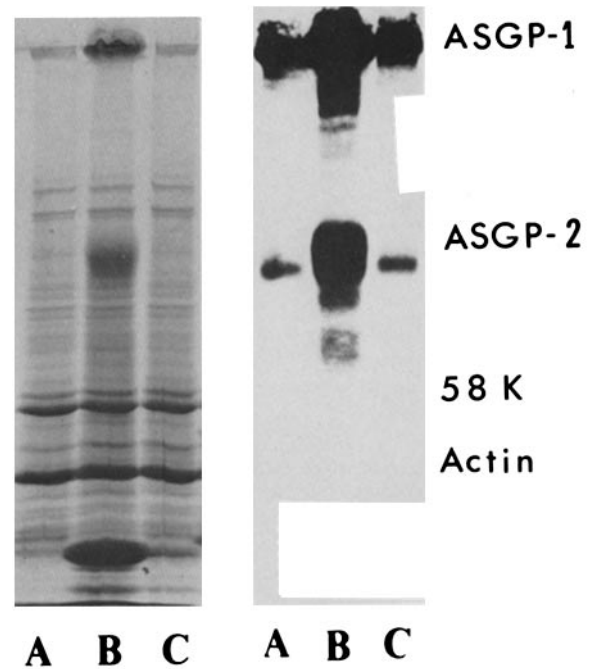


FIGURE 3 SDS PAGE (4.5–12% gradient) of Triton X-100/DPBS cytoskeletal residues of MAT-C1 (A) microvillar membranes; (B) Con A-treated membranes; (C) membranes treated with Con A plus α -MM. 58K, 58-kd polypeptide. Left panel, Coomassie Blue-stained gel; right panel, fluorogram.

proteins and membrane-associated actin (11, 14). A sedimentable residue composed primarily of actin, the 58-kd polypeptide, and CAG from MAT-C1 membranes or actin and CAG from MAT-B1 membranes could be obtained after the S-buffer extraction (14). Stable transmembrane complexes containing only these components in approximately equimolar ratios were isolated from both sublines by either gel filtration or sucrose density gradient centrifugation. We proposed that these transmembrane complexes are the linkage sites between the submembrane cytoskeleton and the membrane (13, 14).

To determine whether 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. In the Con A-treated samples the residues from membranes of both cell types have ASGP-1 and ASGP-2 (Fig. 4, B and D) in addition to the actin, CAG, and 58-kd polypeptide (Fig. 4C, MAT-C1) and actin and CAG (Fig. 4A, MAT-B1) found in the 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 the 58-kd polypeptide are not increased in the Con A-treated samples compared with the untreated controls (Table IV). The most reasonable explanation for these results is that the ASGP-1/ASGP-2 complex is linked by Con A 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 untreated microvillar membranes (data not shown).

CsCl Gradient Centrifugation of Triton Extracts of Con A-treated and Untreated Microvilli

To demonstrate conclusively that there is an association between the transmembrane complex and the ASGP-1/ASGP-2 complex and that they are not simply co-sedimenting, we examined microvilli from leucine-labeled cells after Triton X-100 treatment by CsCl density gradient centrifugation. The extracted microvilli were centrifuged for only 5 h on a preformed gradient to retard dissociation of the 58-kd 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 (5). If the sialoglycoprotein and

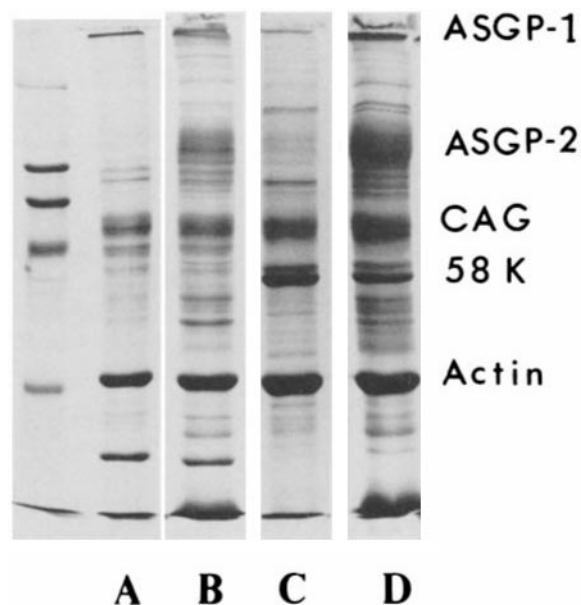


FIGURE 4 SDS PAGE (7.5% acrylamide) of S-buffer residues of Con A-treated (B and D) and untreated (A and C) MAT-B1 (A and B) and MAT-C1 (C and D) microvillar membranes. The unlettered lane on the left contains molecular weight standards myosin, β -galactosidase, phosphorylase a, BSA, and ovalbumin.

TABLE IV

Ratios of dpm 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

Residues were obtained as described in Materials and Methods. (58K, 58-kd polypeptide.)

transmembrane complexes are linked by Con A, the components of the transmembrane complex should be shifted to a higher density in the presence of Con A, reflecting the greater carbohydrate content (70%) contributed by ASGP-1 (5, 6). That this occurs is demonstrated in Figs. 5 and 6. The sialoglycoprotein complex is found at densities 1.3–1.4 (fractions 1–4) in the Con A-treated and control samples; CAG and actin are observed to be coincident with ASGP-2 only in the presence of Con A, as shown in Fig. 6. Since the only means of shifting the actin and CAG to a higher density is through interaction with a highly glycosylated component, these results provide strong evidence for a Con A-mediated association between the transmembrane complex and the sialoglycoprotein complex.

The 58-kd polypeptide is slightly displaced from CAG on the gradient, indicating that it is slowly being dissociated from the transmembrane complex on the gradient. If centrifugations are performed for 18 h, the 58-kd polypeptide is completely displaced to lower densities, but actin and CAG remain coincident. These results confirm previous observations on the stability of the actin-CAG interaction (14).

Sucrose Density Gradient Centrifugation of Triton Extracts of Con A-treated and Untreated Microvilli

Because CsCl depolymerizes actin filaments, the CsCl density gradients do not indicate whether the complexes are associated with additional microvillar cytoskeletal elements. To examine this question, we extracted Con A-treated and control (without Con A or with Con A plus α -MM) microvilli with Triton/PBS and fractionated the extracts by differential centrifugation. Centrifugation at 10,000 g for 15 min gave a microfilament core containing actin and α -actinin as the major components (Fig. 7). Recentrifugation of the supernate at 100,000 g for 1 h gave a pellet containing actin, CAG, and the 58-kd polypeptide as major components. Con A and ASGP-2 were associated with both pellet fractions of the Con A-treated microvilli (Fig. 7, lanes A and C). Somewhat surprisingly, the amount of actin in the low speed pellet was decreased by Con A treatment (Table V). This shift in actin from the low speed pellet to the high speed pellet suggests that Con A treatments may disrupt filament cross-linking. 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 complexes and polymerized actin, the 10,000-g supernates were fractionated by rate-zonal sucrose density gradient centrifugation to determine whether an association between the

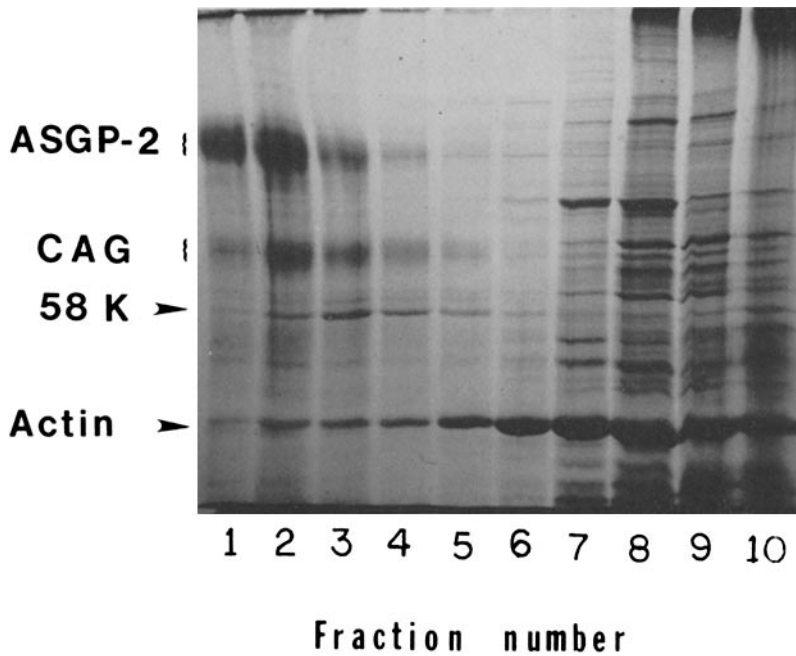


FIGURE 5 SDS PAGE (7.5%) of fractions from CsCl gradient of Triton/PBS extracts of Con A-treated microvilli. Microvilli were treated with Con A as described in Materials and Methods, washed, and extracted with 0.2% Triton X-100 in DPBS containing no calcium for 15 min at 37°. The extract was centrifuged as described in Materials and Methods and fractions were collected for SDS PAGE. The top of the gradient is on the right.

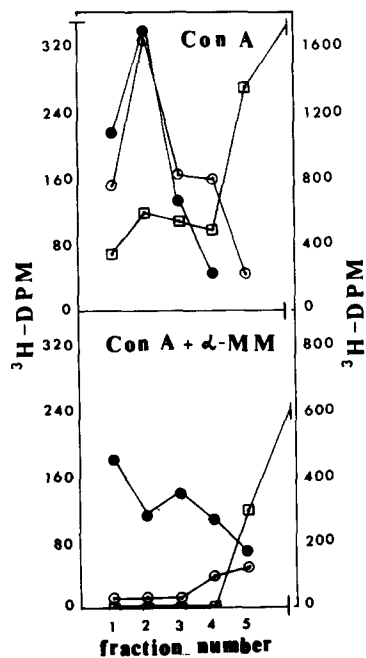


FIGURE 6 CsCl gradient profiles for actin (\square), CAG (\circ) and ASGP-2 (\bullet) for Triton extracts of [^3H]leucine-labeled microvilli treated with Con A or Con A plus 0.2 M α -MM. The samples and gels were prepared as in Fig. 5, and the appropriate bands were excised and counted. The gradient was approximately linear from fraction 1 (density 1.4) to fraction 5 (density 1.3).

polymerized actin and transmembrane complexes 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 polymerized actin. Since sialoglycoprotein complexes are smaller than transmembrane complexes (14), Con A-mediated association of the two complexes should result in a dramatic shift of ASGP-2 further into the gradient. Gradient fractions were analyzed by SDS PAGE, as shown in Fig. 8, and the bands for actin, CAG, and ASGP-2 were excised for counting. The profiles of these components are shown in Fig. 9. 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 fractions 8 and 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 cross-linking 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 cross-linking of ASGP-2 to CAG causes the shift of all three species on the gradient. Since the ratio of actin to CAG in the peaks from the sucrose gradients is about 20 times that found for the transmembrane complexes (c.f., Fig. 6), 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 gradient in the presence of Con A (Fig. 9A) indicates the association of ASGP-2 with polymerized actin, demonstrating the association of the sialoglycoproteins with the cytoskeleton. In conjunction with the CsCl gradient results (Figs. 5 and 6), 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.

Further studies are needed to determine the structural properties of the polymerized actin, how it is associated with transmembrane complexes, and whether it is linked to the microvillus microfilament core.

DISCUSSION

Although membrane-cytoskeleton interactions play an important role in many models describing the control of the redistribution of cell surface receptors (19, 21), little is known about the mode(s) of anchorage of receptors to the cytoskeleton. We have investigated this question in ascites sublines of the 13762 rat mammary adenocarcinoma, which have mobile (MAT-B1) and immobile (MAT-C1) Con A receptors. In previous studies we have isolated stable transmembrane complexes containing a cell surface glycoprotein (CAG) and

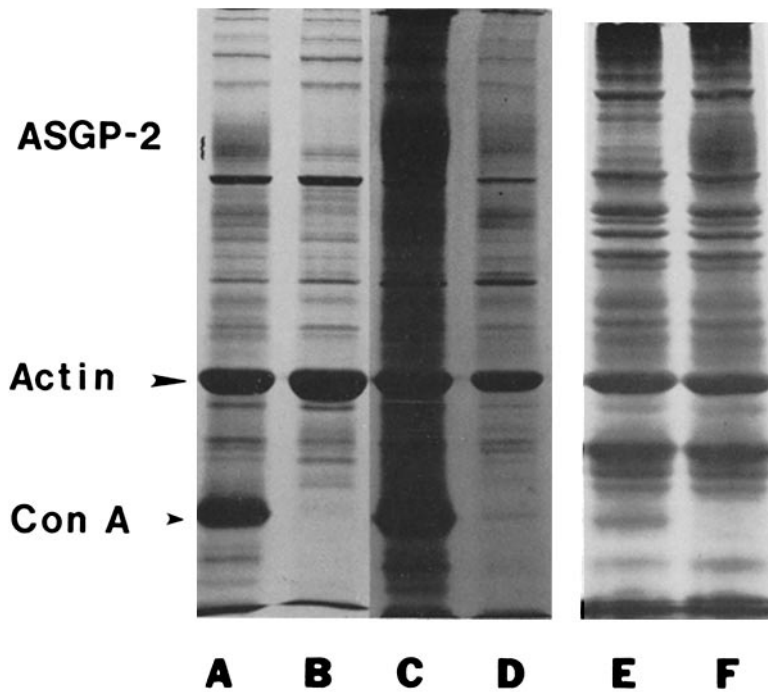


FIGURE 7 SDS PAGE (8% acrylamide) of sedimented fractions of Triton-extracted microvilli treated with Con A (lanes A, C, and E) or Con A plus α -MM (lanes B, D, and F). Samples were equivalently loaded so that protein and glycoprotein differences are readily apparent. The extracts were centrifuged at 10,000 g for 15 min to give pellets (lanes A and B) and supernates. The supernates were further centrifuged at 100,000 g for 1 h to give pellets (lanes C and D) and supernates (lanes E and F).

TABLE V
Con A Effect on Actin Distribution in Triton Extracts by
Differential Centrifugation

	% of total actin		
	10-kg pellet	100-kg pellet	100-kg supernate
Control (no Con A)	51	15	34
Con A	30	33	37
Con A plus α -MM	49	16	35

Extracts were prepared as described for Fig. 7.

actin from these sublines (14). These transmembrane complexes provide a putative link between the cell surface and cytoskeleton (14, 20).

In the present studies we have investigated the anchorage of the sialoglycoprotein complex containing the major Con A-binding protein (6) of these ascites cells. Previous studies have shown that this complex is mobile in MAT-B1 and immobile in MAT-C1 cells upon Con A treatment (7). We now provide evidence that anchorage of the 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. (a) ASGP-1/ASGP-2 complex is readily sedimented with cytoskeletal residues from Con A-treated whole cells, microvilli, and microvillar membranes, but not from untreated samples. (b) ASGP-1/ASGP-2 is associated with transmembrane complexes sedimented from microvillar membranes treated with Con A. (c) Con A treatment causes a shift of the transmembrane complex to a higher density on CsCl gradients as a result of its association with the heavily glycosylated sialoglycoprotein complex. (d) Sucrose density gradient centrifugation shows that the sialoglycoprotein complex is associated with transmembrane complexes linked to polymerized actin in Con A-treated, but not untreated, samples. The sucrose gradient studies provide additional evidence for the association of transmembrane com-

plexes with polymerized actin. We had previously demonstrated the association of CAG with F-actin by myosin affinity precipitation analysis and by DNase-induced sedimentation changes (20). These three lines of evidence support this glycoprotein-cytoskeleton interaction.

Since no additional actin is found in the Con A-treated transmembrane complexes with associated ASGP-1/ASGP-2 by sedimentation (Fig. 4) or CsCl gradient centrifugation, the most plausible 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. However, antibody-induced capping of cell surface components (22) cannot be explained by the indirect bridge mechanism. Moreover, co-capping of different antigens by a single antibody (23) cannot be explained by either of these mechanisms unless the antigens are associated. Some proposed mechanisms for capping, e.g., the sliding filament model (19), require anchorage to the cytoskeleton; others (21) do not. To explain antibody-induced co-capping by an anchorage-requiring process requires that mechanisms for anchorage other than those described in this work be involved. Even without knowing the details of those mechanisms, it may be feasible to distinguish between anchorage-dependent and -independent models for capping by extraction and affinity fractionation of transient or permanent complexes found during antibody-induced capping.

Another important conclusion from our studies is that Con A-induced anchorage of ASGP-1/ASGP-2 is observed in sub-

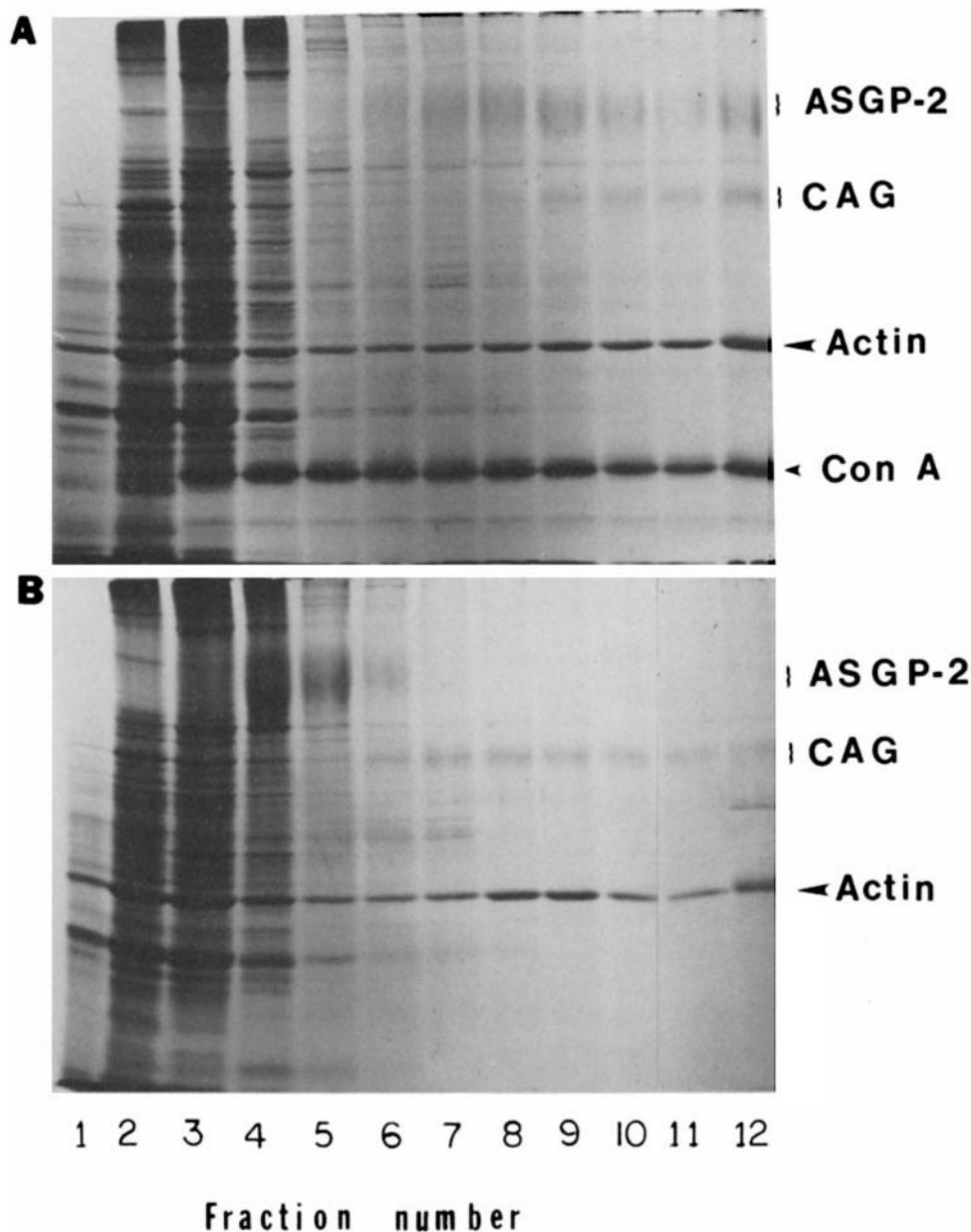


FIGURE 8 SDS PAGE (8% acrylamide) of sucrose density gradient fractionations of the 10,000 g supernates of Triton-extracted microvilli pretreated with 100 μ g Con A (A) or Con A plus 0.2 M α -MM (B). The sucrose gradients were run as described previously (14), except that DPBS containing no calcium was used in the gradients as a buffer. The fractions from the top of the gradient are on the left.

lines with mobile or immobile Con A receptors. Thus anchorage is not sufficient to confer either mobility or immobility on the Con A receptors of these ascites cells. We propose that the difference in receptor mobility between the two sublines resides in the relative stabilities of the membrane-cytoskeleton interaction. In previous studies we have shown that the MAT-B1 and MAT-C1 sublines differ in their cell surface morphologies (8), cell surface receptor mobilities (8, 9), and in the presence of the 58-kd polypeptide in their microvilli, microvillar membranes (12), and transmembrane complexes (14). We have postulated that the 58-kd polypeptide acts to retard breakdown of the submembrane cytoskeleton by stabilizing the actin microfilament-transmembrane complex interactions and preventing microfilament and microvillus breakdown (14).

Our view of Con A-induced capping in these cells is as follows. Binding of Con A to MAT-B1 cells cross-links the cell surface Con A receptors, including ASGP-2 and CAG. The cross-linking 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 (9). 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 (Fig. 7 and Table V). This breakdown of subsurface actin structures is followed by a reorganization of the actin and the 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 cross-linking and micro-aggregation. However, the subsequent depolymerization of the submembrane actin structures does not occur in MAT-C1 microvilli (Table V), because the membrane-cytoskeleton linkage has been stabilized by the presence of the 58-kd polypeptide. This premise is consistent with the greater stability of the MAT-C1 microvilli to cytochalasins and hypotonic treatments (9). Failure to depolymerize submembrane actin leads to a "freezing" of the cell surface with the sialoglycoprotein complexes cross-linked to immobile CAG molecules.

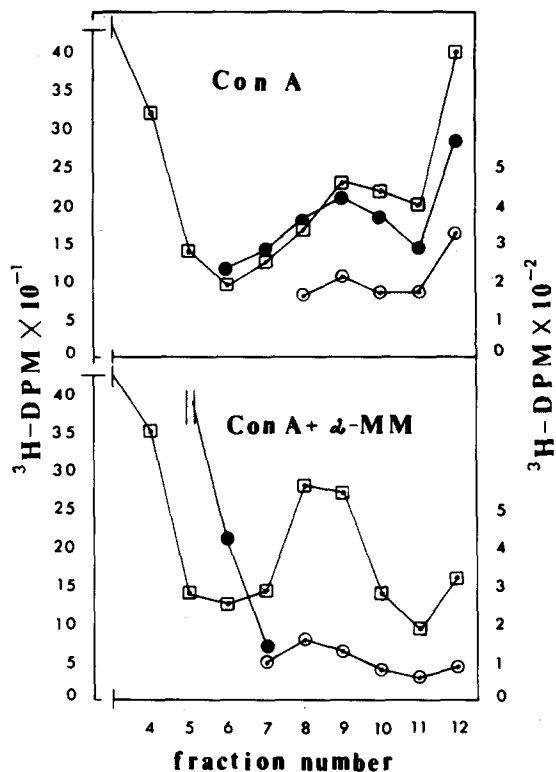


FIGURE 9 Sucrose gradient profiles for actin (□), CAG (○), and ASGP-2 (●) for the 10,000 g supernates of [^3H]leucine-labeled microvilli treated with 100 μg Con A (top) or Con A plus 0.2 M α -MM (bottom).

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 (25). At least some of these are attached (2) to a stable submembrane cytoskeleton that has stable actin oligomers (24). In contrast, the submembrane cytoskeleton of more complex cells (e.g., lymphoid cells) appears more dynamic (21). One problem with this type of analysis is that Con A at high concentrations inhibits Con A receptor redistribution in lymphoid cells (25). Since microtubule-disrupting agents, such as colchicine, can reverse this inhibition (26), one explanation is that high concentrations of Con A stabilize the submembrane cytoskeleton via microtubule-microfilament interactions (8, 21). Moreover, differences in receptor redistribution between transformed and untransformed fibroblasts (27) might be explained by differences in organization of submembrane actin due to changes in actin binding proteins such as vinculin (28, 29) and tropomyosin (30). 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, 21, 22, 31) remains to be determined.

This research was supported by grants from the United States Public Health Service (GM 30295 and CA 31695) and the National Science

Foundation (PCM 810969) to K. L. Carraway and by CA 14395 to the Comprehensive Cancer Center of the State of Florida.

Received for publication 14 October 1982, and in revised form 17 June 1983.

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