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Membrane Skeletons and Cytoskeletal-Membrane Associations 1-10

Actin-Associated Cell-Surface Glycoprotein From Ascites Cell Microvilli: A Disulfide-Linked Multimer

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Isolated microvilli of the MAT-C1 subline of the 13762 rat mammary adenocarcinoma contain a transmembrane complex composed of a cell surface, cytoskeleton-associated glycoprotein (CAG), actin, and a 58,000-dalton polypeptide (58K). The behavior of CAG has been studied by differential centrifugation and velocity sedimentation gradient centrifugation of detergent extracts of microvilli. CAG can be pelleted along with a fraction of the microvillar actin even in the presence of ionic detergents and under microfilament-depolymerizing conditions. By velocity sedimentation analysis CAG in Triton/PBS extracts sediments as a large, heterogeneous species (sedimentation coefficient > 25S). In Sarkosyl and sodium dodecyl sulfate (SDS) the size and heterogeneity are somewhat reduced. In SDS CAG sediments as a 20S species in the absence of mercaptoethanol and as a 5S species in the presence of mercaptoethanol. These results indicate that CAG is a disulfide-linked multimer in the microvillus membrane. We suggest that the stable multimeric structure of CAG permits it to act as the membrane association site for several microfilaments and plays an important role in the formation and stabilization of the microvillus structure.

Key words: cell surface, glycoprotein, actin, disulfide

Microfilament-plasma membrane associations are believed to be important to the organization of cell-surface molecules in animal cells [1,2]. However, the molecular details of such interactions are unclear [1,2]. To approach this question, we have been studying microvilli isolated from ascites sublines of the 13762 rat mammary adenocarcinoma [3,4]. From the microvillar membranes we have isolated a stable complex containing actin and a cell-surface glycoprotein (CAG, cytoskeleton-associated glycoprotein) [5]. An intriguing aspect of this work is that these transmembrane complexes differ depending on whether they are isolated from cells with mobile or immobile cell-surface receptors [5]. Complex isolated from the branched microvilli of cells with immobile receptors (MAT-C1 cells) contains three components: CAG,

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actin, and a 58,000-dalton polypeptide (58K). The last two are present at the cytoplasmic surface of the membrane [4]. Complex isolated from the unbranched microvilli of cells with mobile receptors (MAT-B1 cells) has only CAG and actin [5]. These results suggest that the transmembrane complex is involved in controlling the redistribution of cell-surface receptors on the ascites tumor cells. Further evidence for this involvement was obtained by concanavalin A (Con A) treatments of cells or microvilli, which induced anchorage of the major Con A receptors to the cytoskeleton by a bridge between the receptors and CAG of the transmembrane complex [6].

We have proposed that the transmembrane complex is an association site for microfilaments at the microvillus membrane. Recently, we have demonstrated that transmembrane complex is pelleted with the microvillar microfilament core during low-speed sedimentation [7]. More significantly, CAG and 58K cosediment with microfilaments on velocity sedimentation sucrose gradients in the presence and absence of phalloidin, the microfilament-stabilizing agent. Since phalloidin increases the sedimentation rate of the microfilaments by specifically stabilizing the microfilaments, the transmembrane complexes must be associated with the microfilaments [8].

The most important element of the transmembrane complex is CAG. An understanding of its structure is essential to understanding the behavior of the putative microfilament-membrane interaction site. In this report we show that CAG is present in the microvillus membrane as a disulfide-linked multimer. This structure would allow it to act as the interaction site for multiple microfilaments at the microvillus membrane and could play an important role in formation and stabilization of the microvilli.

EXPERIMENTAL PROCEDURES

Extraction and Fractionation Procedures

Microvilli were prepared from leucine-labeled ascites cells (13762 rat mammary adenocarcinoma MAT-C1 subline) as previously described [3,4]. The microvilli (approx. 0.5 mg protein per ml) were extracted for 15 minutes at room temperature with the indicated detergent/buffer. For fractionation by differential centrifugation the extracts were immediately chilled on ice and centrifuged at 10,000g for 15 minutes at 4°C to give a low-speed pellet (LSP) and low-speed supernate. The supernate was further centrifuged at 100,000g for one hour to give a high-speed pellet (HSP) and high-speed supernate (HSS).

For velocity sedimentation sucrose density gradient centrifugation extracts or supernates (approx. 1 ml) were chilled on ice, applied to the tops of 7–25% sucrose gradients prepared in the same buffer and detergent mixture used for the extraction, and centrifuged at 25,000 rpm for 16 hours using an SW 40 rotor. Fractions were collected by extrusion from the tops of the gradients, dialyzed against 0.1% sodium dodecyl sulfate (SDS) and 0.1 mM EGTA, lyophilized, and solubilized in SDS electrophoresis buffer [4].

Extraction Media

Triton/PIPES. Triton X-100 (0.2%), 20 mM PIPES (piperazine-N,N′-bis[2-ethane sulfonic acid]), 100 mM KCl, 50 mM NaCl, 2 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM dithioerythritol, 0.1 mM PMSF (phenylmethysulfonyl fluoride), pH 6.8.

Triton/GEM. Triton X-100 (0.2%), 5 mM glycine, 1 mM EDTA, and 5 mM mercaptoethanol, pH 9.5.

Triton/PBS. Triton (0.2%), 120 mM NaCl, 5 mM KCl, 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.4.

Deoxycholate/phosphate, Zwittergent/phosphate and Sarkosyl/phosphate. Detergent (0.25%) in 30 mM sodium phosphate, pH 7.4.

Analytical Methods

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed on 8% acrylamide slab gels according to the procedure of King and Laemmli [9]. For quantitative comparisons of isotopically labeled proteins on gels the Coomassie-stained bands were excised, incubated with 0.5 ml of Soluene-350 at 40° for five hours, and mixed with 3 ml of Instagel. Delume (100 μl) was added and the samples were shaken for at least 24 hours prior to counting on a Beckman LS 2800 liquid scintillation counter equipped with a random coincidence monitor for detection of chemiluminescence. The sedimentation coefficients were estimated by comparison with values for an acetylcholinesterase preparation from chicken muscle [10], kindly donated by Dr. Richard Rotundo.

RESULTS

Effects of Protein Solubilizing and Denaturing Agents on the Extraction and Behavior of Transmembrane Complex

Transmembrane complex was originally isolated from microvillar membranes prepared under conditions which depolymerize actin microfilaments [5]. Subsequently, we found that a preparation enriched in transmembrane complex could be prepared by differential centrifugation of detergent extracts of isolated microvilli [11]. Figure 1 shows the fractions obtained by differential centrifugation of microvilli treated with Triton/Pipes buffer. The microfilament core was obtained as a low-speed (10,000g) pellet (LSP). The low-speed supernate was centrifuged at 100,000g to give a high-speed pellet (HSP) enriched in transmembrane complex. The differential centrifugation procedure for preparing transmembrane complex eliminates the membrane preparation step, which is time-consuming and sacrifices material.

Because of its simplicity the differential centrifugation procedure was used to investigate the effects of various membrane extraction and protein-denaturing agents on the behavior of CAG. Since microvillar membranes are prepared under conditions for depolymerizing microfilaments, we examined by differential centrifugation the effect on the microvilli of glycine-EDTA-mercaptoethanol (GEM), the buffer we used for the preparation of microvillar membranes [4,5]. When microvilli were extracted with Triton/GEM and subjected to differential centrifugation, most of the actin was solubilized (Fig. 2). Both 10,000g and 100,000g pellets were enriched in the three components of the transmembrane complex. Quantification of glycoproteins by staining gels is difficult because of low staining density and poor reproducibility. Therefore the actin, CAG, and 58K were quantified on extracted microvilli from leucine-labeled cells. Gel bands were excised, extracted, and counted as described previously [5]. The ratios of actin:CAG and actin:58K in both the 10,000g and 100,000g Triton/GEM pellets were found to be approximately one (0.6–1.2, two experiments, four determinations), similar to those in isolated transmembrane complex [5]. In contrast these ratios were found to be in the range of five to eight in the 100,000g differential centrifugation pellets of microvilli extracted with Triton/PBS or other nondepolymerizing buffers. The results with GEM indicate that transmembrane-complex-containing actin can be obtained directly from microvilli under conditions in which microfila-

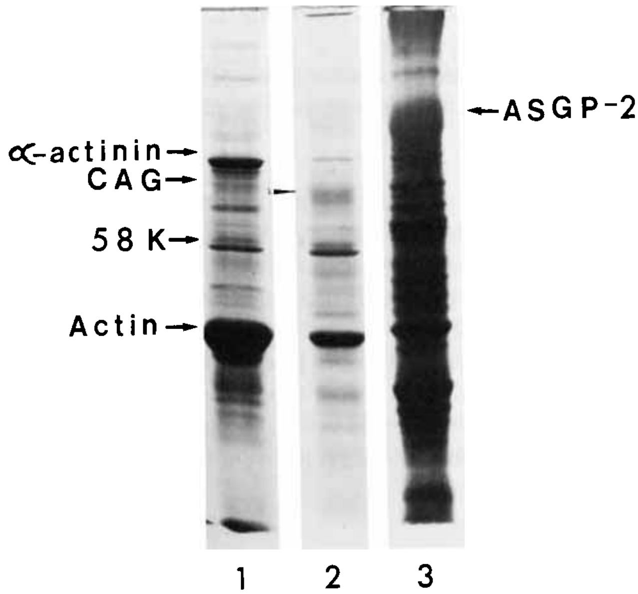

Fig. 1. SDS PAGE of differential centrifugation fractions of Triton/PIPES extracts of MAT-C1 microvilli. Extracts were sedimented at 10,000g to give a low-speed pellet containing the microfilament core (lane 1) and supernate. The low-speed supernate was subsequently centrifuged at 100,000g to give a high-speed pellet enriched in the three components of the transmembrane complex (lane 2) and a high-speed supernate containing solubilized microvillus components (lane 3). The migration positions for actin, 58K, CAG, α-actinin, and ASGP-2 are noted.

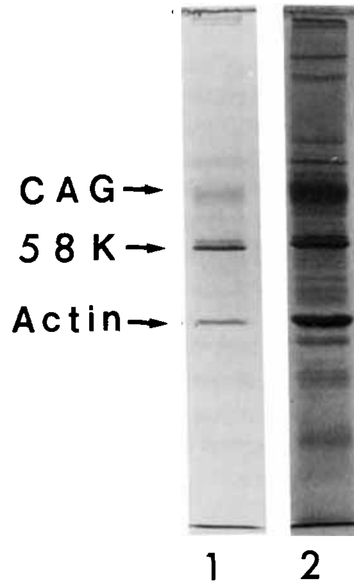

Fig. 2. SDS PAGE of differential centrifugation fractions of Triton/GEM extracts of MAT-C1 microvilli. Extracts were sedimented at 10,000g to give low-speed pellets (lane 1) and the supernates were subsequently centrifuged at 100,000g to give high-speed pellets (lane 2).

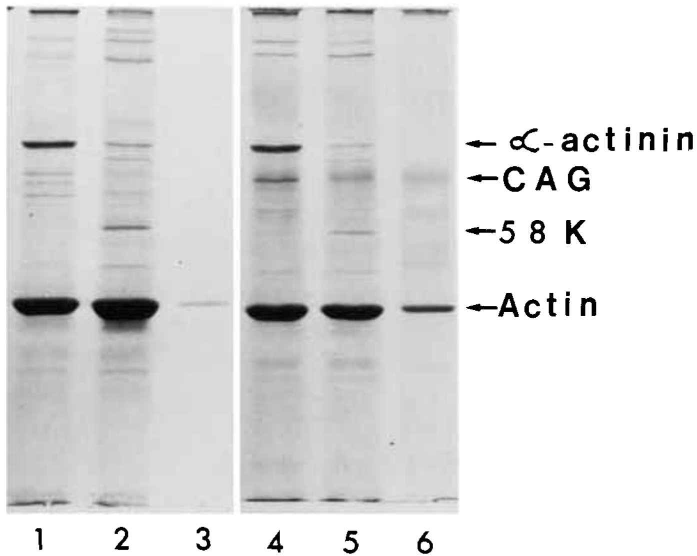

Fig. 3. SDS PAGE of differential centrifugation fractions of deoxycholate/phosphate (lanes 1 and 4), Zwittergent/phosphate (lanes 2 and 5), and Sarkosyl/phosphate (lanes 3 and 6) extracts of MAT-C1 microvilli. Extracts were sedimented at 10,000g to give low-speed pellets (lanes 1–3), and the low-speed supernates were subsequently centrifuged at 100,000g to give high-speed pellets (lanes 4–6).

ments are essentially completely depolymerized, but the preparation contains more contaminating proteins than complex prepared from microvillar membranes.

Because ionic detergents are generally better membrane-solubilizing and protein-dissociating agents, the effects of several of these on microvilli were examined by the detergent lysis and differential centrifugation procedure. The extractions were performed under low ionic-strength conditions which facilitate microfilament depolymerization. Both deoxycholate, a mild anionic detergent, and Zwittergent, a zwitterionic detergent, under these conditions caused breakdown of the microfilament core and the appearance of increased amounts of actin in the high-speed pellet (Fig. 3). Moreover, deoxycholate released 58K, which was found in the soluble fraction (data not shown). In contrast, cetyltrimethylammonium bromide, a mild cationic detergent, caused very little breakdown of the microfilament core or release of microfilament-associated proteins (data not shown). Sarkosyl, a stronger anionic detergent, almost completely depolymerized and solubilized the microfilament core, leaving essentially no low-speed pellet (Fig. 3). The high-speed pellet from Sarkosyl contained primarily CAG and actin.

The results show that CAG is sedimented with a fraction of the microvillar actin under conditions which solubilize most ($> 90\%$) of the actin and other microvillus proteins.

Velocity Sedimentation Analysis of Detergent Lysates of Microvilli

Further evidence for the behavior of CAG in detergent lysates was obtained by velocity sedimentation sucrose density gradient centrifugation. When Triton/PBS lysates of microvilli were fractionated, CAG was distributed over the bottom half of

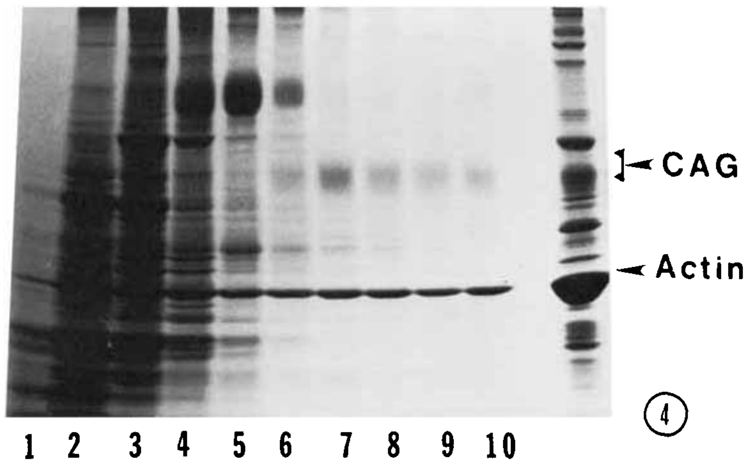

Fig. 4. SDS PAGE of fractions from velocity sedimentation gradient of 10,000g supernate from Triton/PBS extract of MAT-C1 microvilli. The top of the gradient is to the left. Lane on right is the Triton/PBS 10,000g pellet.

the gradient (Fig. 4, sedimentation coefficient > 25S). In contrast, when Sarkosyl lysates were similarly fractionated the CAG fraction was primarily found near the center of the gradient with a sedimentation coefficient near 20S (Fig. 5). These results indicate that the more highly dissociating detergent, Sarkosyl, is breaking down protein-protein interactions present in the Triton extracts, but is not able to convert CAG to a monomeric form.

The apparent large size of CAG in Sarkosyl suggested unusually stable CAG-CAG interactions. Therefore microvilli were solubilized in SDS, a strong anionic detergent and protein denaturant, and subjected to velocity sedimentation fractionation (Fig. 6). Even in the presence of SDS CAG sediments as a large molecule (sedimentation coefficient 20S), as it did in Sarkosyl. These results establish that CAG itself is a large, highly stable species in the extracts, since essentially all of the actin has been dissociated from the CAG in SDS.

Effect of Disulfide Reduction on the Behavior of CAG

The apparent molecular weight observed for CAG under reducing conditions by SDS PAGE is about 75–80,000. In contrast velocity sedimentation under nonreducing conditions in the presence of SDS shows that CAG is a large species with an apparent sedimentation coefficient of about 20S. To determine whether the reducing conditions were responsible for the size difference, three experiments were performed. First, microvilli were solubilized in SDS/PBS with or without 50 mM mercaptoethanol and fractionated by gradient centrifugation. In the presence of mercaptoethanol the CAG peak at 20S was not observed, apparently shifting on the gradient to the fractions containing soluble proteins, which obscured it. It can be detected in the soluble gradient fractions by fluorography of gels from glucosamine-labeled microvillar extracts. Second, we prepared Triton/PBS 10,000g pellets, enriched in transmembrane complex, solubilized them in SDS in the presence or absence of mercaptoethanol, and fractionated them by velocity sedimentation (Fig. 7). The results show that

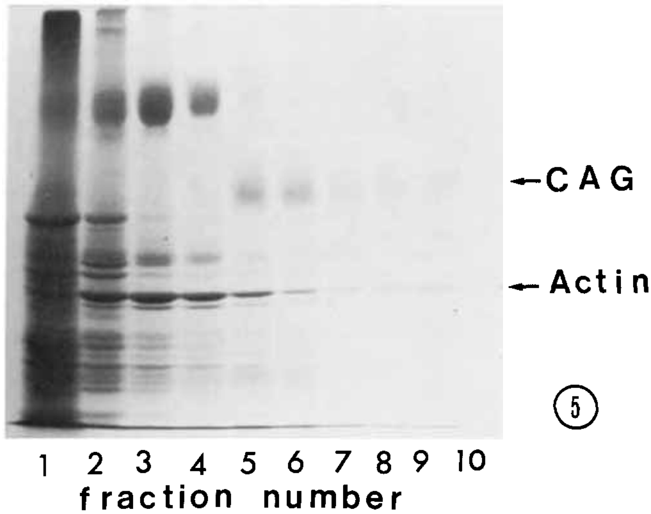

Fig. 5. SDS PAGE of fractions from velocity sedimentation gradient of Sarkosyl/phosphate extract of MAT-C1 microvilli. The top of the gradient is to the left.

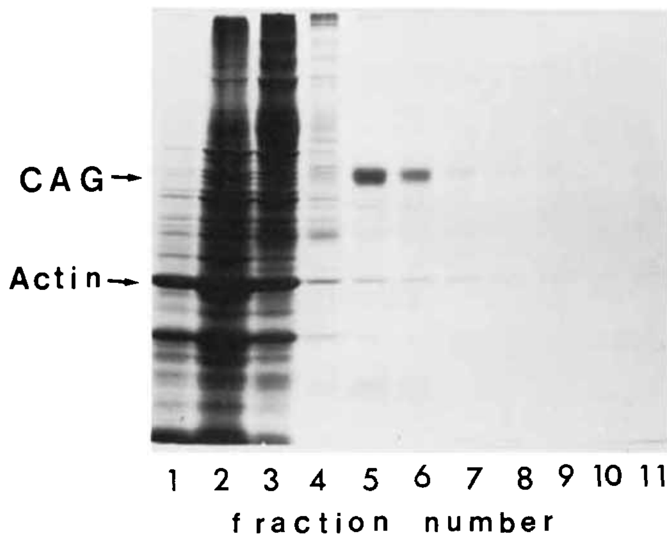

Fig. 6. SDS PAGE of fractions from velocity sedimentation gradient of SDS/PBS extract of MAT-C1 microvilli. Note the heavy CAG band in fractions 5 and 6.

mercaptoethanol reduces the sedimentation rate (sedimentation coefficient 5S) of the CAG to that expected for a monomer of the size indicated by its behavior on SDS PAGE (75–80,000 daltons). Third, the CAG fraction from an SDS gradient run under nonreducing conditions, as in Figure 6, was recentrifuged under velocity sedimentation conditions in the presence and absence of mercaptoethanol (Fig. 8). These results clearly show the shift of CAG in the presence of reducing agent and indicate tht CAG is a disulfide-linked multimer. This reductive cleavage of the CAG multimer can also

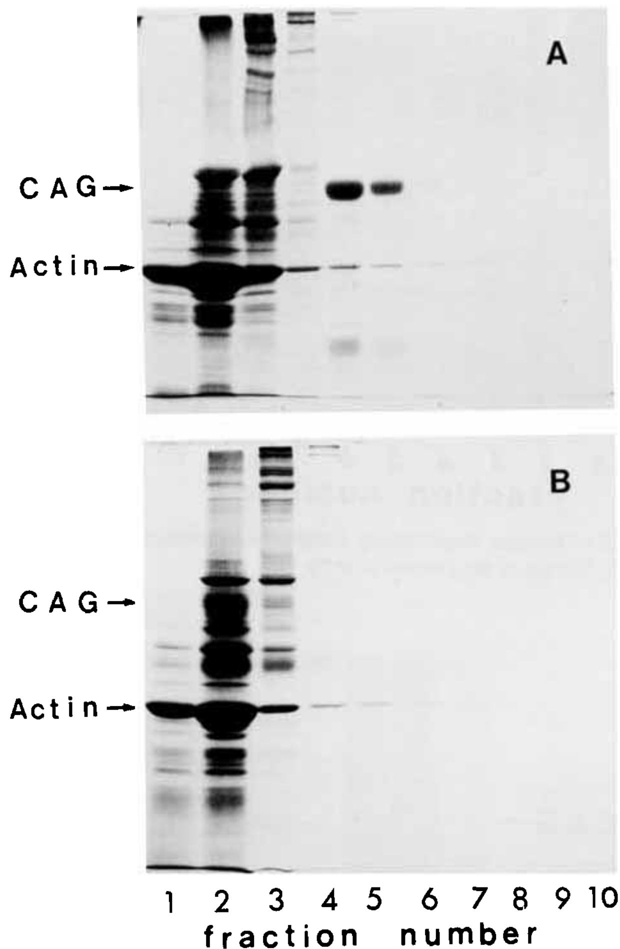

Fig. 7. SDS PAGE of fractions from velocity sedimentation gradients of 10,000g pellets of Triton/PBS extracts of microvilli solubilized in SDS/PBS without (A) and with (B) 50 mM mercaptoethanol. Microvilli were extracted with Triton/PBS and sedimented at 10,000g. The pellets were solubilized in SDS/PBS, applied to a gradient and fractionated as described in Experimental Procedures.

be accomplished with 5 mM dithioerythritol (data not shown). Since the binding of SDS to proteins during membrane solubilization prevents disulfide formation, these results also indicate that CAG is present as a disulfide-linked multimer in the microvilli.

DISCUSSION

These studies provide us with important insights regarding the structure of microvillar transmembrane complexes and their putative role in the organization of the cell surface. The key element in the structure of the transmembrane complex is clearly the disulfide-linked oligomer of CAG. Disulfide linkages have been reported in other cell-surface components. Intramolecular disulfide bonds are found in platelet

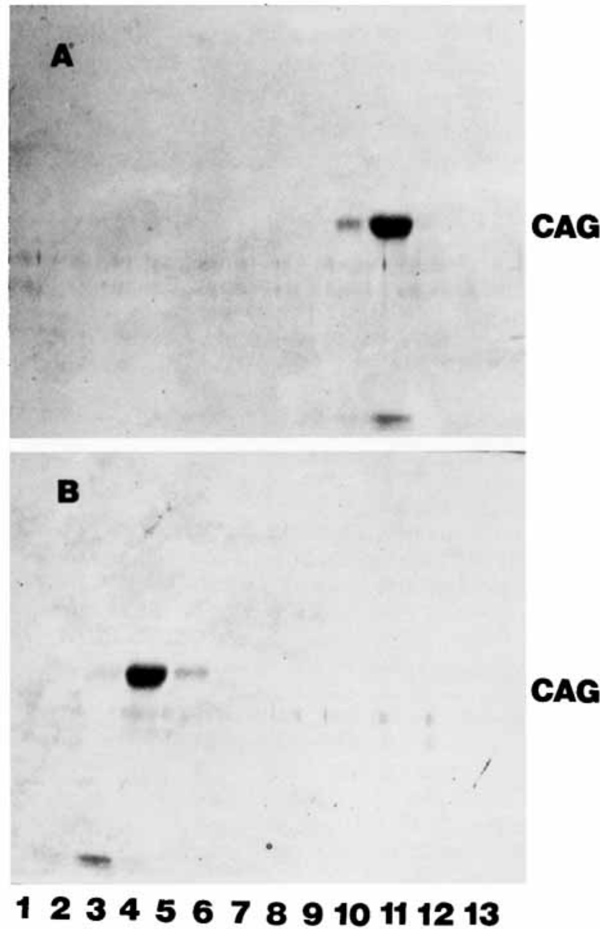

Fig. 8. SDS PAGE of fractions from velocity sedimentation gradients of CAG, partially purified as in Figure 6, without (A) and with (B) 50 mM mercaptoethanol.

cell-surface glycoproteins [12]. Intermolecular disulfide bonds have been reported for some cell-surface receptors, such as the insulin receptor [13] and membrane immunoglobulin [14]. However, these molecules are smaller and contain subunits which are considerably different in size. Disulfides also link components of the extracellular matrix, such as fibronectin [15] and a 140,000-dalton protein [16]. Both of these are disulfide-linked polymers as opposed to the more discrete multimers of CAG. Moreover, both of these are extracellular matrix proteins associated with the cell surface, while CAG behaves as an integral membrane protein.

We feel that the structure of the transmembrane complex outlined here provides additional support for our hypothesis that the complex is a site for a direct membrane-microfilament interaction. The size of the CAG multimer could provide an attachment site for one or more microfilaments. Moreover, the tendency of the complex to aggregate could provide additional sites in close proximity which might be used in

forming the loose bundles or arrays of microfilaments found in the ascites microvilli [7]. We have previously proposed that the complexes act as polymerization sites for actin [11]. One intriguing possibility is that the transmembrane complexes initiate the formation of microvilli by nucleating actin polymerization at a site which then becomes the tip of the growing microvillus. In this case a major function of the CAG and transmembrane complex would be in the formation of microvilli.

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