Concanavalin A-induced cap formation in rat ascites hepatoma cells (AH 7974) and the interaction of cytoplasmic proteins with plasma membranes.

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Abstract

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KEYWORDS: concanavalin A, actins, liposomes, phase transition release

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Concanavalin A-induced Cap Formation in Rat Ascites Hepatoma Cells (AH 7974) and the Interaction of Cytoplasmic Proteins with Plasma Membranes

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Concanavalin A (Con A) forms a tetramer at neutral pH which has four mannose binding sites. When the tetramers bind to mannosides of cell surface glycoproteins, the ligand-receptor complexes accumulate at one pole of the cell at 37°C, a phenomenon known as capping. Cytoskeletal elements have been thought to be involved either directly (1) or indirectly (2) in the mobility of surface receptors.

In the preceding papers (3,4), the authors reported that cap formation occurred in Ehrlich ascites tumor cells treated with Con A, and discussed the role of microfilaments in the capping phenomenon. The interaction of cytoplasmic proteins, including cytoskeletal proteins, with the cell membrane seemed to play an important role in capping. In the present study, capping of ascites hepatoma (AH) cells (AH7974) was observed by electron microscopy after detergent treatment, and the association of cytoplasmic proteins with phospholipid membranes was examined to clarify the role of the protein-membrane interaction in capping.
Materials and Methods

Cells. AH cells were harvested 7-9 days after inoculation into the peritoneal cavities of rats and suspended in Krebs-Ringer-phosphate solution (pH 7.0).

Cytoplasmic proteins. Several kinds of cytoplasmic proteins were prepared for use in the protein-liposome interaction experiments. AH cells 7-9 days after inoculation or rat liver tissue samples were washed with isotonic saline solution and homogenized with hypotonic solution containing 10 mM NaCl, 1 mM MgCl₂, 1 mM phenylmethylsulphonyl fluoride (PMSF), a serine-proteinase inhibitor, 1 mM ethylene glycol bis(β-aminoethyl ether)-N, N', N'-tetraacetic acid (EGTA) and 15 mM phosphate buffer (pH 6.8) at 4°C using a homogenizer equipped with a glass or a Teflon pestle. The homogenate was centrifuged at 100,000 × g for 60 min, and the supernatant was used as the cytoplasmic protein fraction. The actomyosin complex from guinea pig polymorphonuclear leukocytes was prepared by the method of Nishikawa et al. (5), and actin from rabbit skeletal muscle was prepared by the method of Mommaerts (6).

Preparation of liposomes. Liposomes were prepared by the method of Klausner et al. (7). Dipalmitoyl phosphatidylcholine (DPPC) (15 mM) was dissolved in 0.5 ml of chloroform-methanol(2:1, v/v) in the presence or absence of cholesterol (15 mM) and/or phosphatidylserine (15 mM). A thin film of lipid was obtained by evaporating the lipid solution in test tubes (1 × 15 cm) using a Taiyo concentrator (TC-8). After adding 0.5 ml of 0.1 M NaCl-20 mM Tris-HCl buffer (pH 6.8) to the test tubes, the contents were sonicated using a Sonifier (Branson, type 186) at 50°C for 60 min under nitrogen gas. Sonication was performed in the presence of 0.1 M carboxyfluorescein (CF) when liposomes containing CF were needed for the phase transition release experiment described below. After allowing these liposomes swell at room temperature for 2 h, unilamellar liposomes were fractionated by Sepharose 4B column chromatography at 4°C.

Reagents. DPPC, cholesterol, and phosphatidylserine were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Con A was purified from urease (Sigma Chemical Co.).(8), and fluorescein isothiocyanate (FITC)-Con A (FITC/protein ratio, 1.66) was prepared as reported (3). CF

was obtained from Eastman-Kodak Co., Rochester, N.Y., U.S.A. and purified by the method of Ralston et al. (9). DNase I was purchased from Worthington Biochem. Corp., Freehold, N.J., U.S.A. and DNA (type I, thymus-derived) was purchased from Sigma Chemical Co. Other chemicals were of reagent grade.

Cap formation. AH cells were incubated with FITC-Con A at 37°C for 5 min and observed by fluorescence microscopy as described elsewhere (3). Photos were taken with Kodak Tri-X film.

Scanning electron microscopy. AH cells were fixed with 2.5% glutaraldehyde solution and placed on poly-L-lysine coated slide glasses (10). The cells were dehydrated in ethanol, immersed in isoamyl acetate and dried by critical point drying method. The specimens were coated with Pt-Pd and observed under a Hitachi electron microscope (HHS-2R).

Transmission electron microscopy. AH cells were treated with a mixture of 0.1% glutaraldehyde and 0.2% Triton X-100 for 5-10 min, fixed with 2.5% glutaraldehyde for 1 h and postfixed with 1% OsO₄ solution for 1 h (4). The cells were embedded in Spurr’s low viscosity medium. Thin sections were stained with uranyl acetate and lead citrate and observed under a JEOL transmission electron microscope (100 CX).

The isolation of protein-bound liposomes. The association of cytoplasmic or purified proteins with liposomes was examined by a method modified from that of Caron et al. (11). The liposome preparation containing 15 µmol each of lipids/ml (0.25 ml) was incubated with cytoplasmic protein fraction (10 mg/ml, 0.25 ml) at 20°C for 10 min. This mixture was suspended in an equal volume of Ficoll solution (1.12 g/ml, 0.5 ml) containing 0.1 M NaCl-20 mM Tris-HCl buffer (pH 6.8, density 1.10) such that the density was between 1.06 and 1.07. A discontinuous Ficoll density gradient was made with densities, from the bottom of the centrifuge tube, of: 1.07, 1.05, 1.04, 1.03 and 1.01. The sample solution was layered between the solutions of densities, 1.07 and 1.05 (Fig. 5a). After centrifugation at 100,000 × g for 60 min, the original cytoplasmic protein fraction, liposome-associated fraction and non-liposome-associated fraction were collected and separated by SDS-PAGE. The SDS-PAGE gels were analyzed densitometrically using a densitometer (Shimadzu CS 900).
Phase transition release (PTR). The phase transition release curve was obtained by the method of Weinstein et al. (12) using an altered fluorospectrophotometer (RF-510, Shimadzu). Liposomes containing CF in an ice-cold cuvette in the presence or absence of cytoplasmic or purified proteins were transferred to a heated cuvette whose holder was maintained at 45°C which is higher than the phase transition temperature of dipalmitoyl phosphatidylcholine. The temperature change in the reaction mixture was recorded continuously using a thermometer. The change of fluorescence intensity following the release of CF from liposomes which passed through the phase transition temperature was continuously measured at 515 nm with excitation at 470 nm.

Determination of G-actin content by DNase I inhibition method (13). G-Actin binds to DNase I at the molar ratio of 1:1 and inhibits its enzyme activity. DNase I (0.2 mg/ml) was dissolved in 0.2 mM CaCl₂, 5 mM imidazole buffer (pH 7.0) and mixed with 1-25 μl actin or cytoplasmic proteins in the cuvette of a spectrophotometer, followed by the addition of 1.5 ml DNA solution (40 μg/ml) dissolved in 10 mM imidazole buffer containing 2 mM MgCl₂, 1 mM CaCl₂ and 1 mM NaCl (pH 7.0). The change in optical density at 260 nm was recorded, and the amount of G-actin was determined from the extent of the inhibition of DNase activity. The amount of total actin was determined after treatment of cytoplasmic proteins with 1 M guanidine hydrochloride. The amount of F-actin was calculated from the amount of G-actin and total actin, and expressed as a percent of total actin.

Determination and analysis of proteins. Protein concentrations were determined according to Lowry et al. (14) using bovine serum albumin as a standard. Cytoplasmic proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and molecular weights were determined by comparison with standard markers (180, 140, 100, 42, 39 kDa).

Results

Con A-induced cap formation in AH cells. After cells were fixed and stained with FITC-Con A, fluorescence was observed as a ring (Fig. 1a). When cells were incubated in Krebs-Ringer-phosphate solution with FITC-Con A at 37°C for 5 min, cap formation was caused in 60% of the cells (Fig. 1b). These cells tended to aggregate together. By scanning electron microscopy, non-treated AH cells appeared to be covered with short microvilli (Fig. 2a). When these cells were treated with Con A, some microvilli gathered and formed patches (Fig. 2b). Figs. 3a and b are transmission electron micrographs of the Triton shells of normal and Con A-treated AH cells. In normal cells, microvilli were distributed equally around the cell and the cell membrane was disrupted by the detergent treatment. Submembranous regions were relatively translucent. In Con A-treated cells (Fig. 3b), an amorphous mass was seen in the submembranous region of patched microvilli around the cell. It was also seen densely at one pole of the cell.

The analysis of the liposome-associated proteins. Fig. 4a illustrates the change in the test tubes before and after centrifugation. The protein-bound liposomes floated upward to the interface between 1.03 and 1.01. Each of the protein fractions (the original cytoplasmic proteins (Total), liposome-associated cytoplasmic proteins (L, + prot.)
and non-associated cytoplasmic proteins (P)) was separated by SDS-PAGE (Fig. 4b) and the densitometric curves of the SDS-PAGE gels of L (+ prot.) and P are shown in Figs. 5 a and b, respectively. The cytoplasmic proteins included cytoskeletal elements, actin (45 kDa), alpha- and beta-tubulin (52, 55 kDa), and high molecular weight proteins such as

Fig. 2 Scanning electron micrographs of the cell surface. (a) Non-treated cell. (b) Con A-treated cell. Microvilli gather and form patches.

Fig. 3 Transmission electron micrographs of Triton shells. (a) Non-treated cell. Microvilli were preserved. (b) Con A-treated cell. Amorphous masses were observed in the submembranous region of gathered microvilli around the cell and densely at one pole of the cell showing the close association between the cell membrane and these elements.
myosin and actin binding proteins (more than 200 kDa) which showed affinity toward the liposomal membrane. The association of cytoplasmic proteins with liposomes was affected by the lipid composition of the liposome membrane. Fig. 5e shows the pattern of protein associated with liposomes prepared with a DPPC/cholesterol molar ratio of 1:1. The association of proteins decreased markedly when cholesterol was included in the lipid membrane (Compare it with Fig. 5a.). The decreased association of cytoplasmic protein

Fig. 5 The effect of the addition of cholesterol to the liposomal membrane on the association of cytoplasmic proteins with liposomes. Figs. 5a and b show the densitometric curves of the SDS-PAGE gels of the L and P fractions respectively shown in Fig. 4b. Both liposomes were composed of dipalmitoyl phosphatidylcholine (DPPC). The dotted line in Fig. 5b shows the densitometric curve of the gel of the original cytoplasmic proteins (Total). Fig. 5c shows the densitometric curve of the SDS-PAGE gel of cytoplasmic proteins associated with cholesterol-containing liposomes (DPPC: Chol. 1:1).

Fig. 6 The effect of 10 mM Ca²⁺ on the association of cytoplasmic proteins of AH7874 cells with liposomes. Liposomes consisted of DPPC : Chol : PS at a molar ratio of 1 : 0.5 : 0.5.
due to the addition of cholesterol to DPPC liposomes was negated by the addition of phosphatidylserine (PS) (Fig. 6a). The protein-liposome association was inhibited by a high concentration of Ca\(^{2+}\) ions (Fig. 6b).

**Qualitative and quantitative analysis of actin.** All actin which bound to the liposome membrane was F-actin; G-actin was not detected in the bound fraction (Table 1).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Percent</th>
<th>G-actin</th>
<th>F-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Associated protein</td>
<td></td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Non-associated protein</td>
<td></td>
<td>54</td>
<td>46</td>
</tr>
</tbody>
</table>

a: The amount of total actin was expressed as 100 percent.

In the fraction of non-liposome associated proteins, both G- and F-actin were detected when the protein/liposome ratio was high. However, a large proportion of F-actin bound to the liposomes when the protein/lipid ratio was low (data not shown).

**Phase transition release (PTR).** A strong affinity of cytoplasmic proteins of AH cells to liposomes was observed. When these proteins bound to CF-containing liposomes, the permeability of the liposomal membrane was affected, and CF release occurred. Fig. 7 shows the phase transition release of CF from CF-containing DPPC-cholesterol-PS liposomes with or without cytoplasmic proteins. By raising the temperature of the liposome-containing medium from 0°C to 45°C through the phase transition temperature of DPPC, a small amount of CF release was noted at the phase transition temperature (Control). Marked CF release was observed in the presence of cytoplasmic proteins (100

**Fig. 7** The effect of cytoplasmic proteins of AH7974 cells and rat liver cells on phase transition release of carboxyfluorescein (CF) from liposomes. CF-containing liposome (DPPC: Chol : PS=1:0.5:0.5) were prepared as described under Materials and Methods. They were incubated in 0.1 M NaCl and 20 mM Tris-HCl buffer (pH 6.8) at 0°C and placed on a heated cuvette holder with or without the cytoplasmic proteins (0.1 mg/ml). The effect of 1 mM Ca\(^{2+}\) was also examined. Non-specific release which occurred by Triton X-100 at a concentration of 0.1% is indicated by a horizontal bar.

**Fig. 8** The effect of the actomyosin complex on phase transition release of carboxyfluorescein from liposomes. Actomyosin was added at the concentrations indicated in the figure.
μg/ml) within 1 min at a temperature 5°C lower than the phase transition temperature. Calcium partially inhibited CF release. The actomyosin complex extracted from polymorphonuclear leukocytes and actin from rabbit skeletal muscle were tested for PTR (Figs. 8-9). High PTR was observed when the actomyosin complex obtained from polymorphonuclear leukocytes was added even at low concentrations (Fig. 8). Muscle actin also induced an increase in PTR in a dose dependent manner (Fig. 9). However, the action of actin was weaker than that of the actomyosin complex.

![Graph](image1)

**Fig. 9** The effect of rabbit muscle actin on phase transition release of carboxyfluorescein from liposomes. Actin was added at the concentrations indicated in the figure. The change in the temperature in the cuvette is shown at the abscissa.

*Change in the proportion of G-actin in the cytoplasmic proteins of AH cells.* Based on the findings that an amorphous mass (Triton X-100 insoluble mass in Fig. 3b) associated with the AH cell membrane after Con A treatment and that only F-actin bound to liposomes, it was expected that G-actin in cytoplasmic proteins would decrease with the increase in F-actin after Con A treatment. Fig. 10 shows the time course of the changes in G-actin content in AH cells after treatment with Con A (1 mg/ml) in Krebs-Ringer-phosphate solution containing 1 mM ATP and 1 mM Ca²⁺ at 37°C. The content of G-actin decreased with longer incubation with Con A. The decrease in G-actin due to Con A occurred in a dose dependent man-

![Graph](image2)

**Fig. 10** Time course of changes in intracellular G-actin content in Con A-treated AH cells. AH cells (3 x 10⁴ cells/ml) were pre-incubated in the presence of 1 mM Ca²⁺ and 2 mM ATP at 37°C for 5 min. After adding 400 μg/ml Con A, the cells were further incubated at 37°C for 10, 20, 30 and 40 min, at which times the G-actin content was determined.

![Graph](image3)

**Fig. 11** Dose dependent effect of Con A on the intracellular G-actin content in AH cells in the presence of 1 mM Ca²⁺. The incubation time with Con A was 20 min.
Discussion

Con A-induced cap formation has been observed in many cell types. Previously, we reported cap formation in Ehrlich ascites tumor cells and observed the grouping of microvilli and accumulation of microfilaments at the cap region. Shimizu et al. (15) reported that in a free-cell line of rat hepatoma cells (AH7974F), Con A as well as wheat germ agglutinin and Ricinus communis agglutinin induced cluster formation of their receptors concomitant with the agglutination of cells. In our study, Con A treatment was shown by fluorescence microscopy to induce cap formation in AH7974 cells. Although the grouping of microvilli, seen in Ehrlich ascites tumor cells (3, 4), was not observed, the morphology of microvilli changed drastically. The extraction of cytoplasmic materials by Triton X-100 revealed the presence of an amorphous mass in the submembranous region of Con A-treated cells. This mass was not appeared as filamentous in AH cells. However, this kind of mass was also observed in Ehrlich ascites tumor cells and identified as actin filaments (4). Cytoskeletal elements belong to Triton-insoluble fraction, so, this mass may contain cytoskeletal elements. We have concluded that the presence of an amorphous mass in submembranous region indicates the presence of a strong interaction between cytoskeletal elements and cell membrane in Con A-treated AH cells.

Cytoplasmic proteins usually face toward the inner surface of the cell membrane and may interact with membrane lipid and/or membrane-integrated proteins. In the present study, the interaction of membrane lipid and cytoplasmic proteins extracted from AH cells was analyzed using two experimental liposome systems: flotation method of protein-bound liposomes and phase transition release. The protein-bound liposomes (50 nm in diameter) were isolated by Ficoll density gradient centrifugation, and the protein fractions were separated by SDS-PAGE. The liposome-associated proteins included many cytoskeletal elements including F-actin. The association of proteins and liposomes changed according to the composition of lipid components of the liposomes and Ca$^{2+}$ in the incubation mixture. The addition of cholesterol diminished the association of the proteins with the liposomes and the addition of phosphatidylserine restored the association. High concentrations of Ca$^{2+}$ in the medium inhibited the protein-liposome association.

The cell membrane contains 20–30% cholesterol and 9% phosphatidylserine. However, phosphatidylserine is distributed only in the inner leaflet of the plasma membrane of liver cells, so its content therein is 18%. A low content of cholesterol is known to correlate with high membrane fluidity (16). But, this is not always the case. For example, the plasma membrane of AH cells contains more cholesterol (0.372 μmole/mg protein) than that of normal adult liver cells (0.224 μmole/mg protein) (17). However, the former has higher membrane fluidity than the latter as observed by receptor redistribution (15). A freeze-fracture study using filipin-sterol complexes showed that areas low in cholesterol content were located in coated pits (18), in the presynaptic membrane of the active zone, in the post-synaptic membrane where acetylcholine receptors aggregate (19), and at the site of micropinocytosis (20). Among these areas low in cholesterol, the presynaptic membrane of the active zone does not seem to represent high membrane fluidity. Because, in this zone, membrane particles are not randomly distributed, but form double rows and do not seem to move laterally. Therefore, other factors may be also responsible for moving or fixing membrane receptors in the plane of high or low cholesterol membranes, although cholesterol plays an important role.
in determining the distribution and function of membrane protein molecules (19). One of the candidates for these factors is cytoplasmic protein which underlines the membrane of surface receptors.

Con A which increases receptor redistribution may alter the local membrane fluidity and cause a partial phase separation of membrane lipids. The ligand also change the content of F-actin (Figs. 10-11). Therefore, wherever an area free of cholesterol appears, the interaction of lipid-cytoplasmic proteins may increase in Con A-treated cells.

Phase transition release is a useful technique for studying the association between membrane lipid and cytoplasmic proteins (7, 12, 21). This method was applied to the analysis of membrane-bound enzymatic activity, PLA$_2$ (22, 23), and to the estimation of the effect of a membrane stabilizing agent on this enzyme activity (24). The association of cytoplasmic proteins with liposomes was established by flotation method (11, 25, 26) and confirmed by the PTR method (7, 12, 21). It is very interesting that actomyosin complexes associate with liposomal membrane more intensely than actin.

Many models to explain surface receptor redistribution have been advanced (1, 2). Bourguignon et al. (1) proposed the existence of X-proteins that may link microfilaments to surface receptors. According to this model, microfilaments which accumulate beneath the membrane directly move surface receptors. Oliver et al. (2) presented a model in which microfilaments do not directly move receptors. According to this model, the first event leading to capping-related phenomena is the recruitment of microfilaments. These filaments organize into bundles and interact with the membrane to generate tension. This interaction has several consequences. First, it produces a characteristic change in cell shape. Second, this asymmetric tension exerted inward and along the cell surface initiates wave motion over the cell surface which in turn leads to receptors moving into the capped region (surfboard mechanism). In our present study, the recruitment of F-actin was observed in Con A-treated cells (Figs. 12-13), and, the association of actin or actomyosin with liposomes was clearly demonstrated (Figs. 8-9). Recently, a close relationship between integral membrane proteins and cytoskeletal elements was established in erythrocytes and other cells (27). However, model experiments of the interaction between membrane proteins and cytoskeletal elements were not performed. Though Oliver did not specify the association of microfilaments with membrane lipid as the cause of surface tension, our data indicate that the membrane wave may occur as a result of the lipid-cytoskeleton association as well as the membrane protein-cytoskeletal protein interaction. From the evidence of this study, it was considered that the association of cytoskeletal proteins with the inner surface of the plasma membrane is an important process in the Con A-induced cap formation in AH cells.

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