SELECTIVE INTERACTION OF CYTOSKELETAL PROTEINS WITH LIPOSOMES

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1. Introduction

The initial step in cell activation by a ligand is believed to be its binding to specific receptors on the cell surface [1], and a ligand-induced redistribution or cross-linking of the receptor proteins is considered to be a step in the generation of a transmembrane signal [2]. It has been proposed that transmembrane surface proteins associate with components of the cytoskeletal network at the inner face of the cell membrane [3]. Therefore, a network of cytoskeletal structures on the inner face of membrane could interfere with the topology of receptor moieties on the outer surface of membrane [4]. In this case, it is considered that the hydrophobic interaction between membrane lipids and cytoplasmic proteins has an important role in the generation of the transmembrane signal. In attempting to understand this role, the interaction between phospholipid vesicles and cytoplasmic proteins was studied and it was found that many of the cytoskeletal proteins such as tubulin [5], actin, α-actinin and myosin associated strongly with liposomes made from dimyristoyl or dipalmitoyl phosphatidylcholine vesicles [6]. In this report, we describe the association of actomyosin complex of polymorphonuclear leukocytes (PMN) and purified muscle actin with phospholipid vesicles and the formation of protein-liposome recombinants depending on the disorder of the phospholipid matrix.

2. Materials and methods

2.1. *Actomyosin complex of PMN and muscle actin*

PMN were drawn from guinea pigs after intraperitoneal injection of 2% casein as described in [7] and the actomyosin complex of cells was extracted as in [8]. Actin was extracted from rabbit muscle and purified as in [9].

2.2. *Preparation of liposomes*

Liposomes were made with dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), cholesterol, cetylamine and dicetylphosphate as described in [6]. Unilamellar vesicles containing carboxyfluorescein (CF) were prepared by the method of Klausner et al. [10] for the measurement of phase transition release (PTR). Phospholipids were dissolved in chloroform–methanol (2:1) and the thin filmed lipids were hydrated by vortex-mixing and sonicated with a sonicator (Branson, type 185) in the presence of CF (0.1 M) at 25°C for DMPC and 45°C for DPPC. The CF-containing vesicles were centrifuged at 100 × *g* for 5 min and unilamellar vesicles were fractionated in a Sepharose 4B column (1.5 × 25 cm) with a 0.1 M NaCl–20 mM phosphate buffer (pH 6.8) eluant at 4°C.

2.3. *Interaction of liposomes and proteins*

The actomyosin complex or a supernatant of PMN, muscle actin or bovine serum albumin (BSA) was incubated with the liposomes at 4–45°C for 5–30 min and the protein-associated liposomes were separated from other proteins by discontinuous Ficoll density gradient centrifugation as described in [6]. The associated proteins were analysed by SDS–polyacrylamide gel electrophoresis as in [11].

2.4. *Phase transition release (PTR)*

Changes in fluorescence intensity following the release of CF from lipid vesicles were measured using a Shimadzu spectrophotometer (Type RF 510) at 515 nm by exciting wavelength at 470 nm as described in [12] or by agitating the materials in a cuvette equipped with a magnetic stirror and a thermostatically controlled cuvette holder for elevating the cuvette temperature.

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2.5. *Protein and phospholipid content and other chemicals*

Protein concentration was determined according to [13] using BSA as a standard. The phospholipid phosphorous was determined by using the method in [14]. DPPC, DMPC, cetylamine, dicetylphosphate and BSA (Fraction V) were purchased from Sigma Chemicals (St Louis MO). CF was purchased from Kodak and purified by the method in [15]. All other used reagents were of reagent grade.

3. Results and discussion

3.1. *Association of actomyosin complex with liposomes*

As described in [6], cytoskeletal proteins associated with phospholipid liposomes and protein-associated liposomes were separated from the other cytoplasmic proteins by Ficoll density gradient centrifugation. These data suggest that the actomyosin complex in the cytoplasm may associate with these liposomes. Most of the cytoskeletal proteins in the actomyosin complex extracted from PMN by the method in [8] associate with liposomes of DMPC as shown in fig.1. Purified muscle actin shows a similar association with liposomes.

We examined whether non-cytoskeletal proteins also associate with the liposomes using BSA as a representative and found that the extent of the association was quite small under the same experimental conditions.

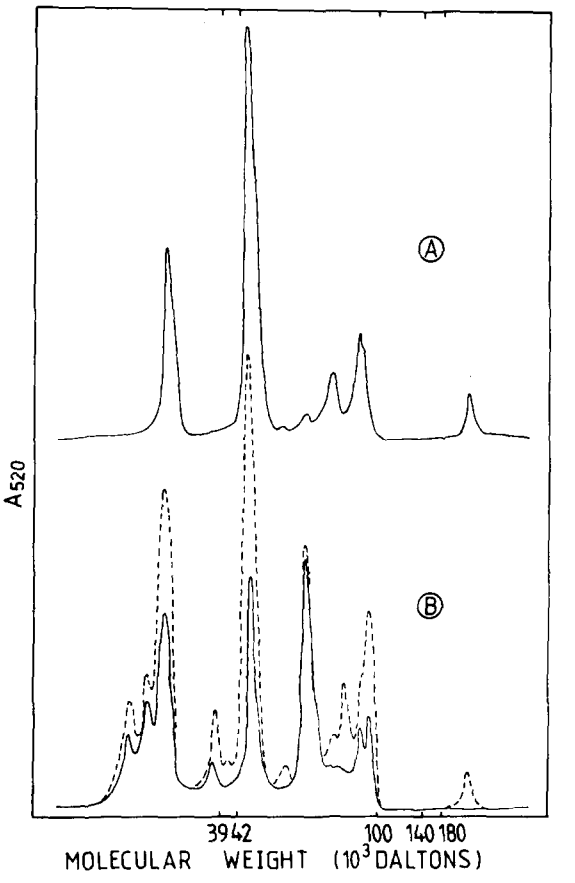

Fig.1. SDS–PAGE densitometric patterns of liposome-associated protein of actomyosin complex isolated from PMN. The actomyosin complex was incubated with DMPC liposomes in 0.1 M NaCl–20 mM phosphate buffer (pH 6.8) for 5 min at 24°C. The liposome fraction was isolated by discontinuous Ficoll density gradient centrifugation as in [6]. (A) Liposome-associated proteins; (B) solid line: non-associated proteins; dotted line: total proteins of actomyosin complex.

3.2. *Dependency of association on the lipid component and incubation temperature*

It should be emphasized that hydrophobic interaction is a main force for the association of cytoskeletal proteins with liposomes. Therefore, these particular associations were influenced by the lipid components of liposomes as shown in fig.2. The degree of association of these proteins with liposomes containing cholesterol was greatly reduced and the reduced association was overcome by the addition of cetylamine but not by dicetylphosphate. In this case non-specific association was reduced in the presence of cholesterol and cetylamine. Furthermore, of course, negligible association of BSA with liposome containing cholesterol and cetylamine was observed. None of these associations were influenced by variations in ion concentration of the incubation mixture.

A question arises whether these water-soluble proteins can be interacted with liposomes at different temperatures or not. As indicated in table 1, the association of proteins with various liposomes was extremely sensitive to temperature. Above the phase transition temperature of the liposomes, non-specific association was increased. Selective association occurred at phase transition.

3.3. *PTR of CF by actomyosin complex and actin*

A number of experiments have indicated that some of the cytoskeletal proteins are integrated into the plasma membrane [16,17]. This evidence raises the question of whether a water-soluble protein can

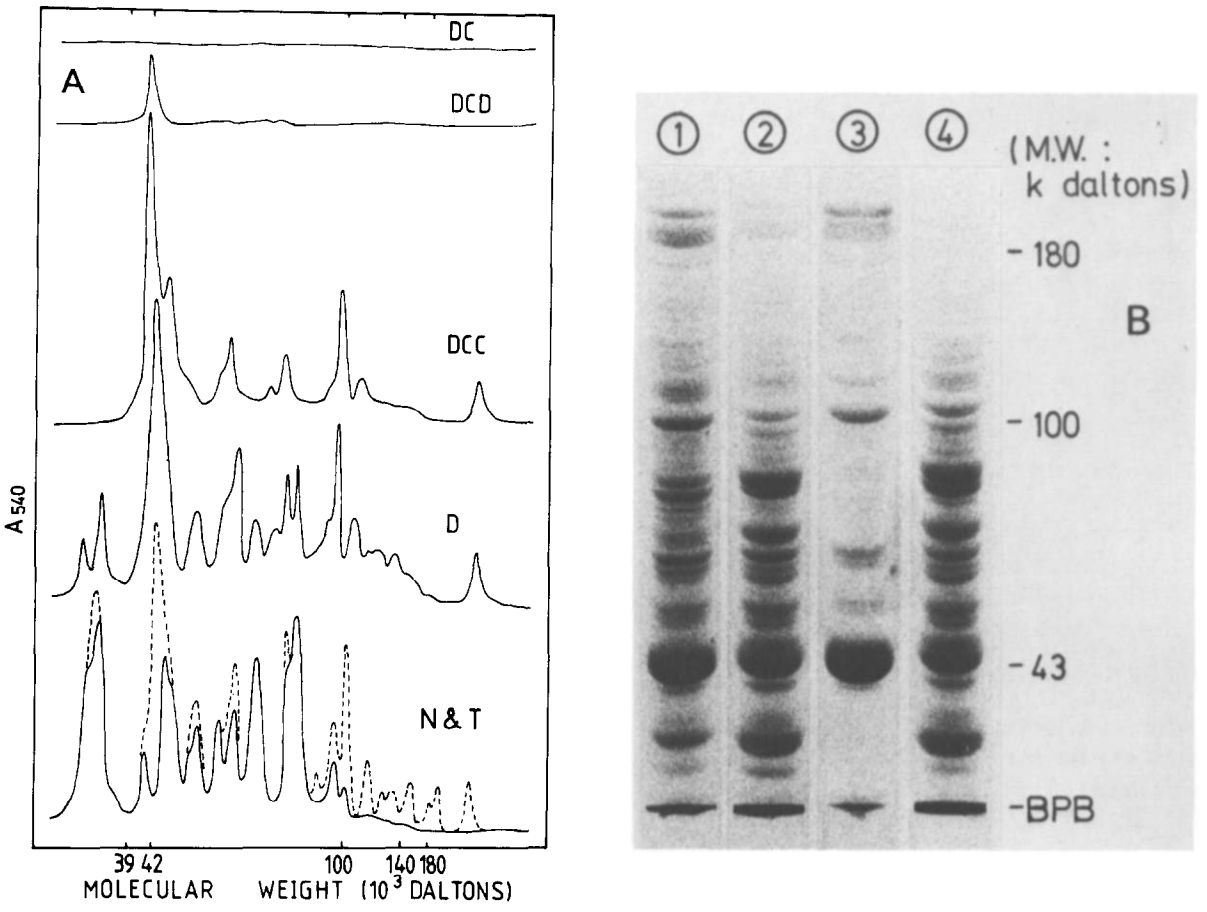

Fig.2. Effect of liposome components on the association of cytoplasmic supernatant proteins of PMN. (A) Densitometric patterns. The supernatant was incubated with liposomes at 24°C for 5 min. The lipid component of the liposomes was changed as indicated in the figure. *Abbreviations:* D, DMPC; DC, DMPC + cholesterol (molar ratio, 1:1); DCC, DMPC + cholesterol + cetylamine (1:1:0.1); DCD, DMPC + cholesterol + dicetylphosphate (1:1:0.1); N (solid line), non-associated proteins with DMPC liposome; T (dotted line), total proteins. (B) Photography of SDS–PAGE. A 7.5% polyacrylamide gel run in the presence of sodium dodecylsulfate and stained with Coomassie blue. 1 (D) and 3 (DCC) show liposome-associated proteins. 2 (D) and 4 (DCC) show liposome-non-associated proteins.

Table 1
Effect of temperature on the association of cytoplasmic proteins of PMN to various liposomes

Liposome lipid	Temperature		
	0°C	24°C	37°C
DMPC	1.40 (320.8)	1.30 (533.2)	2.05 (231.4)
DMPC + Cholesterol (1:1)	0.25 (50.1)	0 (0)	0.85 (107.8)
DMPC + Cholesterol + Cetylamine (1:1:0.1)	0.83 (205.8)	2.47 (420.5)	2.38 (278.1)
DMPC + Cholesterol + Dicetylphosphate (1:1:0.1)	0.25 (25.1)	0.15 (33.9)	1.52 (59.9)

Experimental conditions were as in fig.2. Data represent the protein (μg)/phospholipid (μg). ( ) shows the protein concentration (μg/ml) in liposome fraction

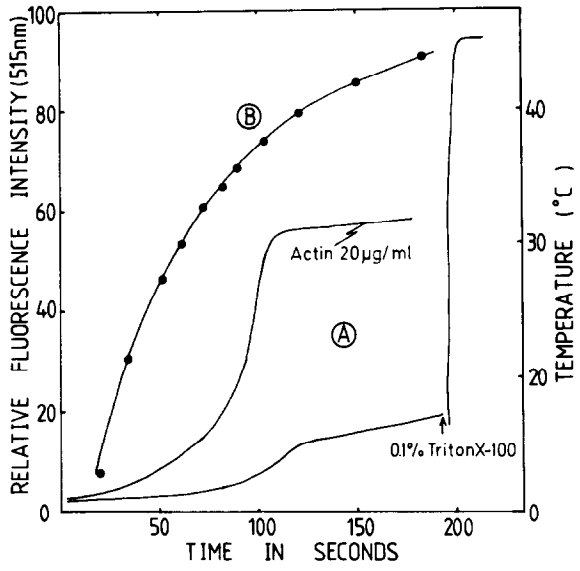

Fig.3. Effect of muscle actin on the phase transition release (PTR) curve of small unilamellar vesicles. DPPC small unilamellar vesicles (20 μM) were incubated at 0°C and phase transition release curve was obtained by the method in [12]. (A) fluorescence intensity of carboxy fluorescein (CF); (B) temperature in the cuvette. CF was excited at 470 nm and emission was read at 515 nm. Fluorescence intensity curve was obtained in the presence of 20 μg/ml of purified muscle actin.

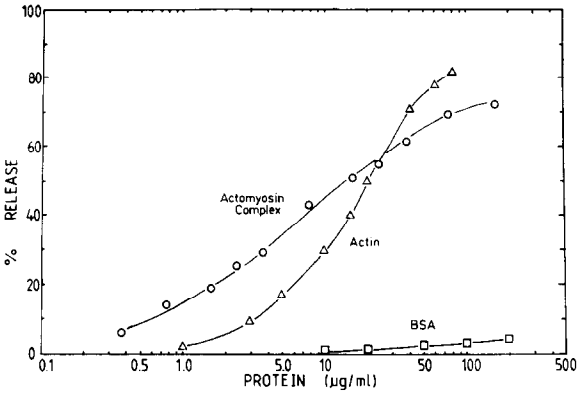

Fig.4. Dependence of protein concentration in the phase transition release from small unilamellar DPPC vesicles. Various concentrations of purified muscle actin, bovine serum albumin (Sigma) and actomyosin complex of PMN were incubated with 20 μM small unilamellar vesicles as described in section 2. Data were presented by per cent release of CF after complete release by the treatment with Triton X100.

be inserted into a lipid membrane or not. Klausner et al. [10] found a rapid, strong and stable association of tubulins with uncharged phospholipid vesicles of saturated fatty acid at the phase transition and suggested that the proteins may be integrated in the lipid membrane to form a stable tubulin-vesicles recombinant. A quite similar reaction was observed on the interaction of DPPC liposomes with other cytoskeletal proteins such as actin or actomyosin complex. The association of actin with DPPC liposomes resulted in a transient increase of permeability and CF in the internal aqueous phase of liposomes leaked out at the phase transition (fig.3). These interactions must be correlated with the extent of penetration of proteins into the bilayer as described in [18]. Below or above the phase transition, actin or actomyosin complex did not induce a rapid release of CF from DPPC liposomes at any concentration of proteins or liposomes. This PTR is also protein concentration-dependent. The concentration of protein required to induce 50% leakage for actin was approximately 20 μg/ml. The dose–response curve of leakage in the case of actin was weaker than that of actomyosin complex. This release of CF from liposomes was not influenced by bovine serum albumin (fig.4). This PTR occurs without being influenced by ionic strength, suggesting that electrostatic interaction with polar head groups of lipid is not uniquely or primarily responsible for the protein-liposome recombinant formation as indicated in [19].

In the liposomes prepared with DPPC–cholesterol (1:1), the release of CF was not detected at any temperature studied (4–45°C). No detectable leakage of the dye was observed in the presence of as high a concentration of actin as 200 μg/ml. This is due to the changes at phase transition of phospholipid vesicles by cholesterol which has a biphasic character for lipid phase [20]. In the case of microsomal lipid vesicles, a small extent of dye leakage was induced by actin or actomyosin complex.

From these data, it is considered that there are two kinds of interactions between phospholipid liposomes and cytoskeletal proteins:

(i) The association of proteins with the lipid vesicles above or below the phase transition;
(ii) The integration of proteins into the lipid phase at the phase transition.

Since a two-dimensional phase separation of phospholipids such as phosphatidylserine was detected by divalent bridging with Ca^{2+} [21,22], it is considered that the phase transition temperature of biological membranes differs in each small area depending on the lipid composition of the area. The cytoskeletal proteins therefore seem to have an important role in

transmembrane signalling in relation to these interactions with the plasma membrane depending on the various physicochemical states of membrane lipids.

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