The reconstitution of the membrane cytoskeleton using a lipid layer

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Abstract:

Actin filaments play central roles in shape determination, cytokinesis, and cell motility. Recently, we found a new type of actin filament that is seemed to contact tightly membrane surface and be buried into membrane under electron microscope. However, the function of this actin filament is not obvious. Then, we have reproduced of such actin filaments using a lipid layer instead of the cell membrane cytoskeleton. We confirmed that the several actin filaments extended on the lipid layer while contacting firmly. The appearance of actin filaments on the lipid layer is quite similar to the new type of actin filaments buried in the cell membrane.

1. INTRODUCTION

The membrane cytoskeleton is also called as membrane undercoat and viewed as a complex network array of actin filaments, microtubules and intermediate filaments[1, 2, 3]. In particular, actin filaments play central roles in shape determination, cytokinesis, and cell motility. Many actin-binding regulatory proteins have been identified and classified according to their effects on actin filaments. According to accumulated biochemical evidences, Rho-family small GTPase such as Rho, Rac and Cdc42 regulate the formation of stress fibers, lamellipodia and filopodia respectively. However, these proteins do not function alone and independently, but rather do so in harmony with effecter proteins such as WAVE, N-WASP, IQGAP1, VASP and so on, working in down stream of small GTPase. Remodeling of actin filaments has been elucidated well in conjunction with the functions of Arp2/3, profilin, cofilin etc following above effecter and regulator proteins. However, morphological counterparts of such biochemical events have been poorly understood. We investigate spatial architecture of actin filaments just beneath the membrane with reference to spatial distribution of their effecter and regulator proteins. In course of study, we found that actin filaments were able to be classified into three types at least according to spatial distribution. One is so called stress fibers spanning between focal contacts. The second one is the actin filament branching and making complicated network just beneath the membrane. This type of actin filaments is thought to be involved intimately in lamellipodia formation. Here, we noted the third type of actin filaments that are crowing on the membrane while contacting tightly with the surface (looks like almost buried into membrane) (Fig. 2A). Such actin filaments are branched poorly and has clear striation derived from series of actin monomer. Indeed, this type of actin filament was not decorated with any particles and also never labeled with anti-VASP, N-WASP and so on. Generally, actin filaments are considered to be unstable without regulatory actin binding proteins such as toropomyosin. This morphological observation was suggested that lipid membrane stabilizes bare actin filaments. Then we examined whether the lipid membrane absorb pure actin filaments and stabilize or not using reconstitution system. For this purpose, we designed an experimental system that mimicks the membrane cytoskeleton, as shown in Fig. 1.

2. MATERIALS AND METHODS

2.1 Protein preparation

Actin was prepared from the acetone powders of rabbit skeletal muscle[4]. An acetone powder was extracted at 0 ℃ for 30min with 60ml of Solution A (1 mM Tris-HCl (pH=8.0), 0.2 mM $MgCl_2$, 0.1 mM EGTA and 0.1 mM ATP) and filtered with Büchner funnel. G-actin (the monomeric form) was polymerized for 1 h at room temperature by the addition of KCl and $MgCl_2$ into the filtrate to the final concentrations of 50 mM and 1 mM, respectively. KCl was added to 0.5 M and the solution was stirred for 0.5 h at room temperature. The F-actin (the filamentous form) was collected by centrifugation at 415,000 $\times g$ for 1.5 h. The pellet was dissolved in G-buffer (10 mM Tris-HCl (pH=8.0), 0.2 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM ATP and 1 mM DTT) and dialyzed against G-buffer. The dialyzed solution was used as purified G-actin. The concentration of G-actin was determined from the absorbance at 290 nm, using an extinction coefficient of E_{290}=0.63 ml mg^{-1} cm^{-1} [(5)].

We used the recombinant tropomyosin of chicken skeletal α-tropomyosin. Tropomyosin expressed in *E. coli* binds poorly to actin and does not polymerize in head-to-tail manner because the first methionine residue is not acetylated[6]. The fusion tropomyosin which has two residues A and S fused at the N-terminus of chicken skeletal a-tropomyosin was used. This fusion tropomyosin restored actin binding and head-to-tail polymerization[7]. The protein was expressed in BL21(DE3) cells and was purified as described by Nitanai *et al.*[8], with minor modifications.

2.2 Electron microscopy

Lipid (1, 2-Dipalmitoyl-*sn*-glycero-3-phosphocholine) was dissolved in chloroform at a concentration of 0.4 mg/ml. The lipid solution was spread on the surface of the buffer (10 mM Tris-HCl (pH=8.0), 0.2 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM ATP and 1 mM DTT) in a Teflon trough and left 1 h to allow the chloroform to evaporate, leaving a lipid monolayer. Purified monomeric actin was injected into the buffer covered with the lipid layer (Fig. 1). The actin was polymerized by the addition of KCl to 100 mM and $MgCl_2$ to 2 mM, respectively. After 2h incubation at room temperature, a carbon-coated grid was placed onto the surface and then lifted up. The grid was negatively stained by 2 % uranyl acetate solution and examined under a Hitachi H-7600 electron microscope at 100kV.

In the case of adding tropomyosin, after actin polymerization, the excess of tropomyosin was injected so that actin and tropomyosin were in the ration of 5:1.

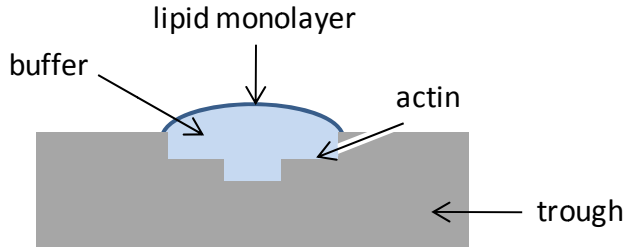


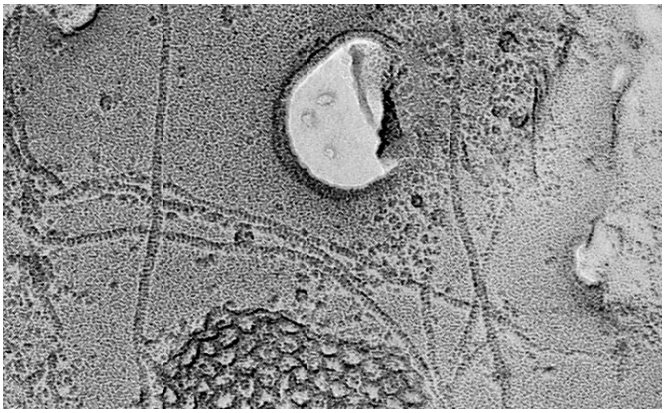
Fig. 1. The scheme of the experimental system that mimicks the membrane cytoskeleton.

3. RESULTS AND DISCUSSION

We observed the several actin filaments extended on the lipid layer (Fig. 2B, 2C). These actin filaments are seemed to contact the membrane surface widely and buried into membrane. If actin filaments exist on the membrane surface and are not buried into membrane, the clearer outlines of actin filaments must be observed because the stain reaches to the space between the molecule and the lipid layer. In order to examine whether actin filaments are buried into the lipid layer or not, we prepared the specimen as filaments on the membrane. A drop containing actin filaments was applied to a copper grid, stained and observed under electron microscope. Actually, the outlines of actin filament on the cupper grid without the lipid layer were seems to be clearer at a glance.

New type of actin filament was not decorated with any particles. To confirm whether unstable bare actin filaments are stabilized by lipid membrane, we tried to examine the distinction between actin filaments with or without tropomyosins. Unexpectedly, in the presence of tropomyosins, the filaments extended on the lipid layer were observed under electron microscope (Fig. 2C). However, it is difficult to judge whether tropomyosins wind aroud actin filaments from electron micrographs. Possibly bare actin filaments may be observed. It is necessary to examine in detail and improved.

A.



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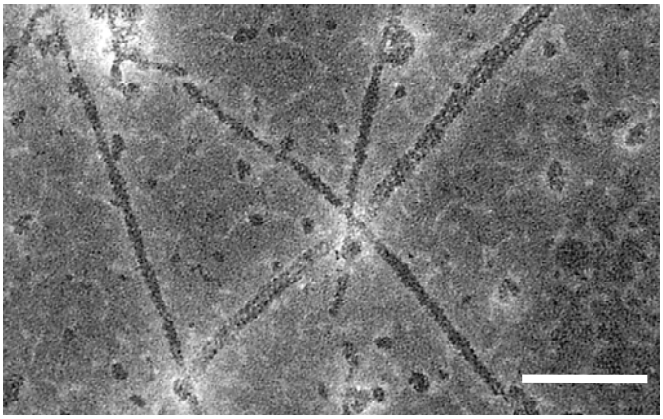

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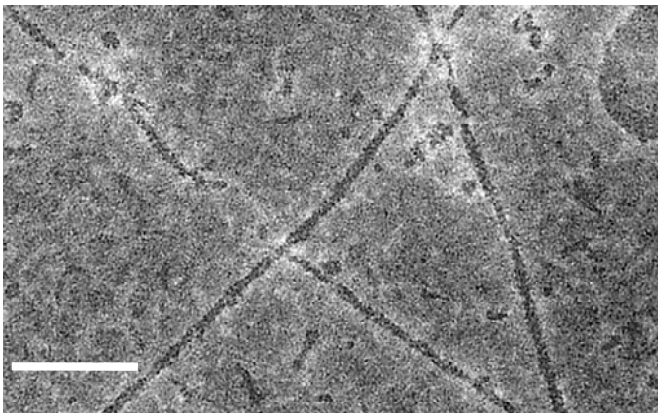

Fig. 2. Electron micrographs of the actin filaments.
(A) Electron micrograph of the surface of the cell membrane.
(B) Electron micrograph of the surface of the lipid layer when actin was injected. Bar, 100 nm.
(C) Electron micrograph of the surface of the lipid layer when actin and tropomyosin were injected. Bar, 100 nm.

Next, we would like to reveal the characteristic of this actin filament on the lipid layer.

The interpretation of the interaction between the cell membrane and the cytoskeleton *in situ* is difficult due to the complicated interaction among cytoskeletal filaments, many related regulatory proteins and effecter proteins. This reconstitution system may be useful to understand the function of the actin filament and the interaction between the other proteins and the membrane.

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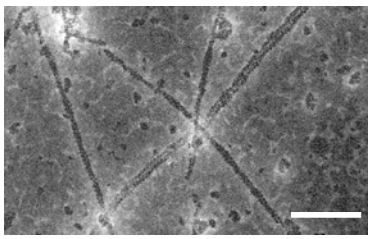

Fig. Electron micrograph of the surface of the lipid layer. Bar, 100 nm