Rapid reconstitution of a transmembrane protein into supported planar lipid membranes

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A procedure for reconstituting a transmembrane protein by the freeze-thaw method into supported planar lipid layers has been developed. A solution containing human glycophorin A was introduced between an alkylated cover glass with lipid layers from soybean phospholipids and a bare glass slide, and was then put in a glass dish which was frozen outside by liquid nitrogen. The lipid layer membranes prepared in this manner have been examined by the binding of both macrophages and wheat germ agglutinin agarose. Macrophages bound more efficiently to the membranes bearing glycophorin A and spread more rapidly than those of the control membranes.

Reconstitution *Transmembrane protein* *Freeze-thawing* *Supported lipid layer* *Planar membrane* *Glycophorin A*

1. INTRODUCTION

Supported lipid monolayer membranes have proven to be valuable models for the study of receptor-specific regulation of cell-mediated immune responses [1–7]. Although it is easy to prepare lipid monolayers containing lipid hapten, reconstitution on membrane proteins into supported lipid layers has been a topic of interest. The air–water interface method, in which spreading at an air–water interface is carried out by introducing a suspension of small unilamellar vesicles, is a useful technique for preparing supported lipid monolayers containing transmembrane protein [4,5]. The dialysis method was also used to prepare both multiple lipid monolayers containing a transmembrane protein [8,9] and the reconstitution of palmitoyl IgG prepared by acylation of antibody [10]. Both of these methods, however, take 2–3 days and need greater amounts of transmembrane proteins for reconstitution.

We describe here another method, freeze–thawing, which permits the preparation of the reconstituted membrane within half an hour. This new method may prove to be widely useful in studies involving membrane–membrane or membrane–cell interactions.

Abbreviations: WGA, wheat germ agglutinin; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PBS, phosphate-buffered saline; NBD-PE, nitrobenzoxadiazol phosphatidylethanolamine

2. MATERIALS AND METHODS

Glycophorin A was isolated from human blood in acid-citrate-dextrose and purified by a Sephadex G-100 column chromatography [11]. Alkylated glass coverslips were obtained by a slight modification of the method in [12].

Purified soybean phospholipids (150 μl) (1 mg in 1 ml of methanol) were transferred to an alkylated coverslip which was attached to a bare microscope slide (distance between coverslip and slide 250 μm). They were kept at room temperature for several seconds, taking care that the alkylated coverslip did not dry. Then they were washed with 5 ml of PBS (pH 7.4). All the methanol was washed out by this treatment. Substituting a meth-

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anol solution for PBS solution, lipid layers were prepared on the surface of an alkylated coverslip, because the polar head groups of phospholipid have affinity for water whereas the hydrocarbon tail avoids water and faces the alkylated cover glass. This was checked by observing the fluorescence of NBD-PE. After washing, 75 μl glycophorin A (8.2 μM in PBS, pH 7.4) was introduced between the alkylated cover glass and the slide. The slide with the cover glass was put in a glass dish, then the dish was set in liquid nitrogen and the solution between the cover glass and the slide allowed to freeze. After freezing, the slide was taken from the dish and kept at room temperature until the solution melted. The cover glass was washed with 5 ml PBS, then macrophages ($P388D_1$ cell line) or WGA agarose (Vector Lab.) in PBS with 1% FCS was introduced between the cover glass and the slide, and allowed to settle on the lipid layers. Reconstitution of glycophorin A into lipid layers was detected by the binding of both macrophages and WGA agarose. Following incubation at 37°C for the appropriate time, the slide was inverted and examined by Nikon VFD-R microscope with a camera. The percent of macrophages or WGA agarose bound to lipid layers containing glycophorin A was calculated by photographing several fields on a slide at random and counting both cells (or agarose) in the focal plane of the lipid layers and cells (or agarose) in the focal plane of the bare glass slide.

3. RESULTS

In the present experimental conditions (37°C and 30 min incubation) 30% of WGA agarose bound to the soybean phospholipid layers with glycophorin A. This binding percentage of WGA agarose is slightly smaller than the value for the DMPC lipid layers made by the dialysis method [8]. In the absence of glycophorin A, however, no WGA agarose bound to the lipid layers at all. Similarly, glycophorin A was found to incorporate into lipid layers made from DMPC and DPPC. The efficiency of the reconstitution was very low and less than 10% of WGA agarose was bound to DMPC or DPPC lipid layers. The different results between the lipid layers from soybean phospholipids and the lipid layers from DMPC (or DPPC) may be dependent on the membrane fluidity at 0°C (the freezing and thawing temperature of the reconstituting solution).

Then, macrophages have receptors for glycoproteins [13]. When macrophages were allowed to settle onto soybean phospholipid layer membranes bearing glycophorin A, most of cells adhered to the planar membranes, as shown in fig.1. Subsequent incubation at 37°C caused number of cells to spread and thus increase their contact area with the planar membranes. In the absence of glycophorin A, however, macrophage adherence decreased drastically, as shown in fig.2. On subsequent incubation at 37°C, fewer cells spread on these control membranes.

The results demonstrate that glycophorin A was reconstituted by the freeze–thaw technique into

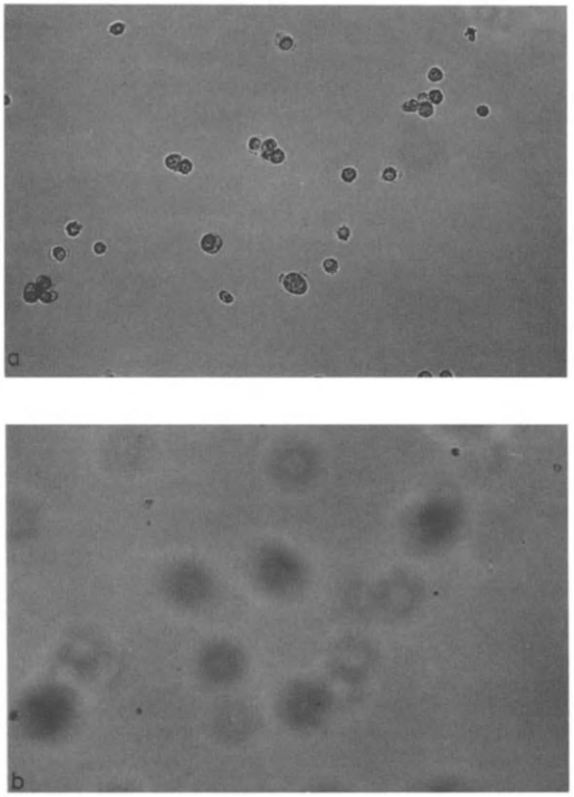

Fig.1. Phase contrast photomicrographs of macrophages. (a) Bound macrophages on the soybean phospholipid membranes with glycophorin A. (b) A few unbound macrophages on the bare glass slide. Measurements were taken after 15 min incubation at 37°C.

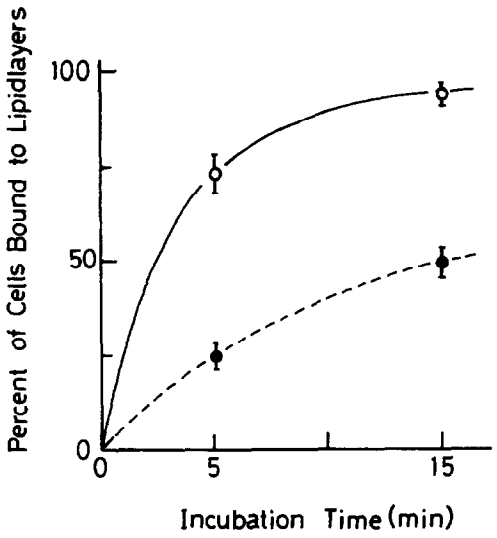

Fig.2. Binding of macrophages to the lipid membranes from soybean phospholipids. (○) Cells bound to lipid layers containing glycophorin A; (●) cells bound to the control lipid layers without glycophorin A. The percentage of cells bound to glycophorin A and the control lipid layers was calculated by photographing several fields on a slide at random and counting cells in the focal plane of lipid layers and cells in the focal plane of the bare glass slide. Bars are standard deviations.

the supported lipid membranes from soybean phospholipids.

4. DISCUSSION

Glycophorin A is a transmembrane protein with a 131-residue polypeptide chain [14]. Examination of the amino acid sequence of this molecule revealed the existence of 3 major domains: an NH_2 terminus which is exposed to the outside of the erythrocyte membrane and carries all of the sugar groups; a hydrophobic region of approx. 22 amino acids; and a carboxy terminal segment which is considered to be on the cytoplasmic face of the membrane [15,16]. As intact glycophorin A was incorporated into the planar lipid membranes it is important to consider the localization of the carboxy terminus of glycophorin A on the supported lipid membranes.

It may be most plausible that the lipid layers described here are multiple lipid monolayers, as formed by the dialysis method [8,10]. To confirm this, lipid monolayers were spread at an air–water interface by allowing a solution of soybean phospholipids in the mixtures of methanol and chloroform. The single lipid monolayers, formed at a pressure of 36–38 dyn/cm^2, were transferred to alkylated glass coverslips using techniques described previously [12]. Using the freeze–thaw method described here, glycophorin A was examined for incorporation into the single lipid monolayers. The amount WGA agarose bound to the monolayers was 5–10%. The reconstitution of the glycophorin A into the single lipid monolayers was less efficient than that of the multiple lipid monolayers described in section 3. We speculate that the carboxy-terminal segment of glycophorin A is embedded inside the multiple lipid monolayers, between the polar head groups of soybean phospholipids.

In short, the freeze–thaw method is suitable for the reconstitution of a membrane protein into supported lipid layers as well as the reconstitution of a membrane protein into liposome vesicles [16]. The method described here is very convenient and useful for the study of the interaction between membrane proteins and cells.

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