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Biochimica et Biophysica Acta 1194 (1994) 335–340

BBA Biochimica et Biophysica Acta

Transfer of membrane proteins from human platelets to liposomal fraction by interaction with liposomes containing an artificial boundary lipid

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Received 25 February 1994; revised 15 June 1994

Abstract

The direct transfer of membrane proteins from human platelets to the liposomal fraction was examined, particularly in relation to platelet activation during the process. The incorporation of an artificial boundary lipid, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC), in the interacting liposome considerably enhanced the efficiency of the protein transfer. The transfer proceeded with neither significant activation nor lysis of the platelet, and the activation of the platelet with thrombin did not affect the amount of the transferred proteins. A wide range of platelet membrane proteins was transferred, and they were almost comparable to those in a sample prepared by glycerol lysis/centrifugation. In addition, they included the major surface glycoproteins GPIIb and GPIIIa without noticeable contamination of soluble cytosol proteins. The protein transfer method is a one-pot process and clearly more convenient than the conventional 'extract and reconstitute' approach. These results strongly support the use of the transfer process, especially with DDPC, as an alternative to the conventional detergent-solubilization or the solvent-extraction methods for preparation of samples of platelet membrane proteins.

Key words: Membrane protein; Protein transfer; Platelet; Liposome; Platelet activation; Artificial boundary lipid

1. Introduction

The process of platelet activation and aggregation is one of the crucial steps in the blood clotting process, in which numerous proteins on the platelet membrane are involved. The structure and the function of those platelet membrane proteins are the key to understanding the mechanism of the platelet coagulation process. Some of the proteins have been identified although the detail of the process is yet to be elucidated [1].

Heparin blocks the platelet aggregation indirectly by inhibiting the stimulatory effect of thrombin on the aggregation [2–4]. However, recent studies [5–7] revealed that heparin also directly promote the aggregation of platelet, possibly as the results of its binding to platelet, though the detail of the promotion process has not been clear yet. Sobel and his co-workers have determined [8] the affinity and kinetic parameters in the heparin binding to platelet and are currently trying [9,10] to identify a platelet protein that is responsible for binding heparin.

In studies of platelet membrane proteins, such as the search for the heparin binding protein, it is often desirable to use the platelet membrane proteins on an isolated plasma membrane or in a reconstituted system, in order to avoid possible interference or complication from other platelet cell components or platelet activities, particularly, the activation. Platelets can be activated by chemical, mechanical or physiological stimulations. The activation triggers various responses

Abbreviations: DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DDPC, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine; PAGE, polyacrylamide gel electrophoresis; LDH, lactate dehydrogenase (EC 1.1.1.27).

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SSDI 0005-2736(94)00160-Q

from platelet such as morphological transformation, aggregation, release of chemical substances including serotonin and so on. Different techniques have been employed for the isolation of the platelet plasma membrane without causing activation, though those techniques generally require a complex and careful processing because of the susceptibility of platelet to exogenous stimuli [11].

It has been shown [12–22] that the coincubation of liposomes with cells transfers proteins from the cell plasma membrane to the liposomal fraction that was obtained after centrifugal separation from the cell. Kobayashi et al. reported [20] that coincubation of rabbit platelets with an aqueous suspension of dilauroylphosphatidylcholine (DLPC) yielded vesicles containing the platelet membrane proteins. On the other hand, Huestis and her co-workers observed [15,16,18] transfer of membrane proteins from erythrocytes and lymphoma cells directly to DMPC liposome. Recently, Sunamoto and his co-workers found that, in erythrocytes [21] and B16 melanoma cells [22], incorporation of an artificial boundary lipid, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC) [23], in the interacting liposome considerably increased the amount of the transferred protein.

In our quest for the heparin binding proteins, we have needed a simpler and more convenient method to obtain a platelet membrane protein sample than the conventional procedures. This protein transfer with DDPC containing liposome seems to be an ideal option for obtaining a membrane protein sample directly from cells. However, the applicability of the transfer process to platelets is not clear yet. In particular, with platelets, effect of the transfer process on platelet activation has to be first evaluated. In this paper, we examined the protein transfer from human platelets with DDPC containing liposomes in relation to platelet activation, and then we evaluated the feasibility of the process for a sample preparation of platelet membrane proteins.

2. Materials and methods

2.1. Materials

DMPC and thrombin were commercially available from Sigma (St. Louis, MO, USA). DDPC [23] was prepared by Dojindo (Kumamoto, Japan). Concentrated platelets were a generous donation from Dr. Manabu Oki of Kyoto Red Cross Blood Center. Fluorescamine was purchased from Funakoshi (Tokyo, Japan). All other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan). Purified platelet membranes were prepared by the glycerol lysis technique of Barber and Jamieson [24].

2.2. Liposome preparation

Typically, 72.0 μmol of DMPC or the same amount of a mixture of DMPC and DDPC was dissolved in 3.0 ml of chloroform, and the solvent was removed under reduced pressure. The thin film of the lipid on the flask wall was further dried under vacuum overnight. The lipid film thus made was suspended in 6 ml of a Hepes buffer solution (10 mM; with EDTA (1 mM), glucose (5 mM) and sodium chloride (150 mM); pH 7.4). The lipid suspension was passed through a series of Nucleopore polycarbonate membrane filters of subsequently decreasing pore sizes (in the order of 1 μm, 0.6 μm, and 0.4 μm, five times each, and then 0.2 μm, ten times) set in an extruder (Lipex Biomembranes, Vancouver, Canada) by using nitrogen pressure of $1.5 \cdot 10^6$ Pa [25]. The liposome suspension was employed to centrifugation ($35000 \times g$, 3 h), and the liposome pellet thus obtained was re-suspended in 3.0 ml of the Hepes buffer solution. The size distribution of the liposome was determined by a dynamic laser light scattering measurement (Photal DLS-700, Hirakata, Japan). All the liposome preparations had their mean diameters in the range of 130–180 nm and rather mono-dispersive.

2.3. Interaction of the liposome with human platelet

A washed human platelet suspension was prepared from concentrated platelets. Contaminating erythrocytes were removed by repeated centrifugation ($70 \times g$, 10 min) from the platelet rich plasma. Then, the platelets were sedimentated at $1300 \times g$ for 10 min and washed three times with the Hepes buffer ($1300 \times g$, 10 min). The washed platelet suspension thus obtained ($3.0 \cdot 10^9$ cells) was mixed with a liposome suspension (final volume, 3.0 ml; total liposomal lipid concentration, 3.0 mM) and incubated at 37°C for 1 h. After the incubation, the platelet cell pellet was removed by centrifugation ($4000 \times g$, 10 min, twice). For the vesicles in the supernatant the size distribution was determined by dynamic laser light scattering. Then, the supernatant was submitted to further centrifugation ($35000 \times g$, 8 h). The supernatant thus obtained, which contained water soluble substances released from the platelet, was used for LDH and serotonin assay (see below). The vesicle pellet was washed twice by re-suspending in 1.0 ml of water and following centrifugation ($35000 \times g$, 3 h). After the washing, the pellet was re-suspended in 2.5 ml of water and submitted to electrophoresis and assay for proteins and lipids (see below).

2.4. Assay for lactate dehydrogenase and serotonin

For the assay of the extracellular LDH [26], 100 μl of β-NADH (2.5 mg/ml in the Hepes buffer) was

added to 100 μl of the soluble protein fraction, and the mixture was diluted with 1.7 ml of the Hepes buffer solution. After incubation for 15 min at 25°C, 100 μl of sodium pyruvate (2.5 mg/ml in the Hepes buffer) was added to the mixture, and the decreasing rate of absorbance at 340 nm was measured (Hitachi U-3400).

The amount of serotonin was assayed by using the *o*-phthalaldehyde method [27,28]. Briefly, 0.50 ml of the soluble protein fraction was mixed with 1.0 ml of a 10% aqueous zinc sulfate solution and then with 0.50 ml of an aqueous sodium hydroxide solution (1 M). After proteins were removed by centrifugation (12000 $\times g$, 5 min), 2.0 ml of 0.01% *o*-phthalaldehyde in an aqueous hydrochloric acid solution (10 M) was added to 1.5 ml of the supernatant, and the mixture was incubated at 75°C for 10 min. After cooling to room temperature, the fluorescence intensity of the mixture was measured (ex. 345 nm, em. 485 nm; Hitachi 650–10S). The total amount of LDH or serotonin in whole platelet was determined by using a platelet sample disrupted by sonication (Tomy UR-200RP, probe-type, 40 W, 50–50 duty, 30 s).

2.5. Assays for proteins and lipids

The protein content in the vesicle fraction was determined by the fluorescamine procedure [29]. To 200 μl of the vesicle fraction was added 700 μl of the Hepes buffer solution and 100 μl of a 1% SDS dissolved in the Hepes buffer. After the mixture was vortexed for 20 s, 500 μl of a 0.03% dioxane solution of fluorescamine was added under continuing vortexing. After 10 min, the fluorescence intensity at 475 nm was measured (ex. 390 nm). The amount of proteins in the sample was determined using a calibration curve obtained in advance for bovine serum albumin.

The amount of phospholipids in the samples was determined by using a phospholipid assay kit (Phospholipid Test C Wako, Wako Pure Chemicals).

2.6. Electrophoresis of the transferred proteins

Two samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained by basically the same procedure as described above except the scale and the ratio of liposomes and platelets interacted. A rinsed platelet pellet ($6 \cdot 10^9$ cells or $1.5 \cdot 10^{10}$ cells) was incubated with 3.0 ml of a liposomal suspension (DDPC/DMPC = 40:60 by mol, total lipid concentration = 3.0 mM) at 37°C for 1 h. The ratios of the platelet cells to the liposomal lipid were chosen based on results from preliminary experiments. Also, in order to keep the lysis of the platelet minimal, 40 mol% DDPC liposome, which did not cause measurable leakage of LDH, was used. The vesicle fraction was lyophilized and submitted to SDS-PAGE on a 7–12% gradient gel under reducing conditions, and the proteins were detected either by Coomassie brilliant blue staining or by silver staining.

3. Results

3.1. Protein transfer from platelet cell to the liposomal fraction

The transfer of platelet membrane proteins started without a noticeable lag period and reached to its completion after 1 h. In the case of the DDPC 60 mol% liposome, the half time for the transfer was less than 10 min. Table 1 summarizes the amount of proteins and lipids found in the liposomal fraction after the 1 h incubation of platelets with liposomes of various compositions. The amount of the transferred proteins increased as more DDPC was added in the DMPC liposome. The transfer by using the DDPC 80 mol% liposome yielded approximately 4 times more proteins than that by using the pure DMPC liposome. According to Sixma [11], $3 \cdot 10^9$ cells of platelet contain 1.3 mg of membrane proteins. Therefore, the amount of the membrane protein in the liposome fraction after the interaction with the 40 mol% DDPC liposome corresponds to 4.6% of the total membrane protein, and this efficiency was comparable to the value (5%) found by Kobayashi et al. [20] for a DLPC suspension.

During the whole procedure, the vesicle fraction lost 30–40% of the original liposomal lipids. The loss of the lipid in the vesicle fraction by the interaction is probably due to adsorption of the liposome to the platelet or transfer of the liposomal lipids into the platelet membrane as is the case of other systems [13,19,20].

The mean diameter of the vesicles, which was measured by using dynamic laser scattering procedure,

Table 1
Amounts of proteins and lipids in the liposome fractions after the interaction with human platelets

DDPC in DMPC liposome (mol%)	Proteins transferred (μg)	Phospholipids recovered [a] (μmol)
0	28.2 (4.8) [b]	6.24 (0.52)
40	59.2 (7.4)	5.76 (0.43)
80	108.2 (18.1)	5.41 (1.47)

Human platelets ($3.0 \cdot 10^9$ cells) were incubated with liposome suspensions (final volume, 3.0 ml; total liposomal lipid concentration, 3.0 mM) at 37°C for 1 h. After the incubation, the liposomal fraction was separated by centrifugation, and the protein and the lipid contents were determined.

[a] Amount of phospholipids in the liposome before the coincubation with platelet was 9 μmol.

[b] Mean of three experiments with the standard deviation in round brackets.

changed only less than 9 nm during the coincubation when the liposomes containing DDPC less than 60 mol% was used. Meanwhile, in the case of the 80 mol% DDPC liposome, a distortion of the distribution toward the larger mean diameter (sometimes a distortion larger than 600 nm) was observed.

3.2. Integrity of the platelet cell during the transfer

The transfer was accompanied by little release of LDH, a cytosol enzyme, and serotonin to the exterior of the platelet. Leakage of LDH from the cell reveals a loss of integrity in platelet plasma membrane [30], and the amount of extracellular serotonin indicates extent of possible activation of platelet [31]. After 1 h incubation with the liposomes, 4–8% of whole platelet LDH and less than 8% of whole platelet serotonin were found at the exterior of the platelet cell, while the release of those without the liposomal treatment was 3% and 5%, respectively. These results indicate that the transfer of the platelet membrane proteins causes neither significant lysis nor activation of platelet.

3.3. Effect of the platelet activation

The effect of platelet activation by thrombin on the transfer of the membrane protein was examined. In Fig. 1, the amount of extracellular serotonin increased as the concentration of the thrombin rose, indicating higher extent of platelet activation. However, the amount of the transferred protein was almost irrespective of the extent of the activation. This result suggests that the thrombin-induced activation of the platelet does not alter the transfer process significantly.

3.4. Electrophoresis of the transferred proteins

In Fig. 2, the proteins transferred to the liposome were analyzed by SDS-PAGE and compared with a platelet membrane sample prepared by glycerol lysis and differential centrifugation [24]. No significant difference was observed in the protein band patterns between the two samples from the transfer in the different platelet/liposome ratios. More than twenty proteins bands observed in the platelet membrane sample were seen also in the liposomal fraction, including four densely stained bands for actin (40 kDa), GPIIIa (90 kDa), GPIIb (120 kDa) and myosin (200 kDa). No extra bands were detected in the liposomal fraction, and this indicates that the transfer process is free from noticeable contamination from soluble cytosol proteins or decomposition of the platelet proteins. No noticeable effect of the DDPC content on the kinds of the transferred proteins was observed.

A more careful analysis of the protein bands revealed that the relative amount of the transferred membrane proteins differed between the two methods. For example, in the membranes prepared by lysis/centrifugation, the two bands for actin and GPIIIa were of comparable density, while in the liposome-transferred proteins the latter band was clearly less dense than the former. The transfer process had some selectivity on the kind of proteins that transferred. Meanwhile, there was no noticeable difference in the composition between the proteins transferred from non-activated platelets and the ones transferred from the pre-activated platelets on a silver-stained SDS-PAGE gel.

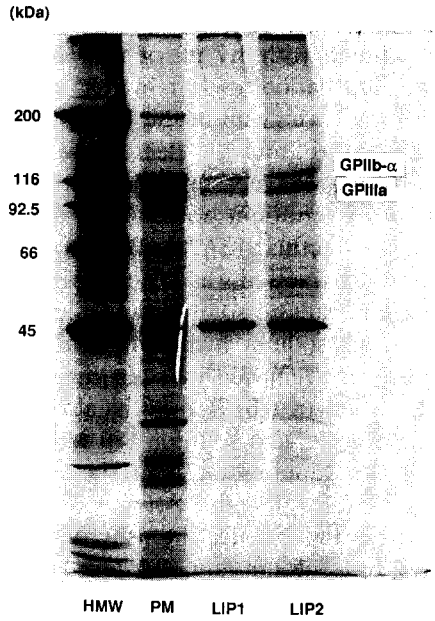

Fig. 2. SDS-PAGE of platelet proteins. HMW, molecular weight markers; PM, a platelet membrane sample prepared by glycerol lysis/centrifugation; LIP1 and LIP2, liposomal fractions (DDPC/DMPC = 40/60 by mol) after interaction with platelets (LIP1, $6 \cdot 10^9$ cells and LIP2, $1.5 \cdot 10^{10}$ cells per 9 μmol liposomal lipid) for 1 h at 37°C.

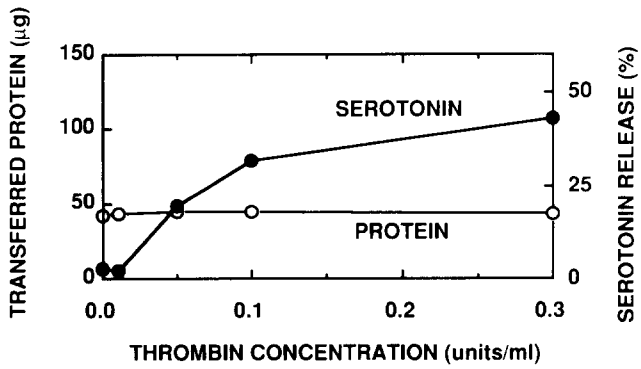

Fig. 1. Platelet activation and the protein transfer. Platelet was treated by thrombin at 37°C for 5 min, washed with the Hepes buffer twice and interacted with a DDPC(40)/DMPC(60) liposome at 37°C for 1 h as described in Section 2.

4. Discussion

An ideal method for obtaining platelet membrane proteins should meet the following criteria; it is (1) free from lysis or uncontrolled activation of the platelet (2) non-destructive to the proteins, and (3) simple. By using the DDPC containing liposome, we have shown that one can effectively extract platelet membrane proteins under controlled platelet activation. Recently, Brunauer and Huestis [31] reported inhibition of platelet activation by pre-treatment of platelets with DMPC, and this observation is consistent with our results that, even in the presence of DDPC, the amount of serotonin released from the platelet was negligibly small. The control of the activation is one of the most important points in dealing with the platelet membrane because of possible modification of the membrane status by the activation. For example, it has been known that an adhesion protein GMP-140 appears on the membrane of activated platelet [32,33].

In addition, in order to carry out an effective screening of a certain protein, it is crucial for a screening sample to contain as many kinds of original membrane proteins as possible. The protein transfer with the DDPC-containing liposome is a one-pot process and clearly much more convenient than the conventional 'extract and reconstitute' procedure and satisfies most of the requirements. Although the conventional methods may yield more proteins per platelet cell, the convenience and the fidelity of the transfer procedure have more significance in most cases of platelet membrane protein preparation.

A previous observation by Kobayashi et al. implied [20] that DMPC was totally ineffective for the protein transfer under their experimental conditions. However, in this study, even DMPC was effective for the induction of the transfer of membrane proteins from human platelets to liposomes. The transfer of membrane proteins by coincubation of a cell with DMPC liposome has been shown for erythrocyte and other cells [13–18]. The result in this paper proves that the platelet is no exception. The apparent inconsistency between Kobayashi's result and ours may be explained by the difference in the platelet samples. Besides the difference in species, rabbit and human, we sometimes encountered only marginal transfer of the platelet membrane proteins when pure DMPC liposomes were used (data not shown). Apparently, the difficulty in the transfer was dependent on the batch of the platelet. However, the difficulty was abolished when more than 40 mol% of DDPC was added to the liposome preparation. Along with our recent reports [21,22] of the improvement of the protein transfer efficiency from human erythrocytes or from mouse B16 melanoma cells by incorporation of DDPC in the interacting liposome, this article showed that the effect of DDPC seems to be predominant and general to various kinds of cells.

For B16 melanoma, a significant selectivity was observed in the proteins transferred [22], while for human erythrocyte an induction period existed before the start of the actual protein transfer [17]. Nevertheless, those were not observed in the case of human platelet. The lack of these phenomena distinguishes the protein transfer of human platelet from that of other cells. It seems still hard to generalize this transfer phenomenon for any kind of cells or cell lines.

Two phenomena have been proposed as a possible explanation for the appearance of membrane proteins in the exterior of cell when the cell was exposed to liposome: vesiculation ('shedding') of cell membrane [13] and transfer of membrane proteins directly *onto* liposomal membrane [16]. Macroscopically, both of the two phenomena yield lipid vesicles bearing membrane proteins. Kobayashi and his co-workers claimed [20] that the interaction of platelet with a DLPC suspension resulted in the shedding of the platelet membrane. However, in the transfer from erythrocyte or murine lymphoma by using DMPC liposome, Huestis and Newton showed [15,18] that the majority of membrane proteins were on the DMPC liposome with the minor population of cell membrane fragments. These observations suggest the co-existence of the shedding and the direct protein transfer.

In order to obtain an insight into this point, we have examined the amount of LDH that was encapsulated in the liposomes after the interaction with platelets. Because LDH cannot permeate through lipid bilayer membrane, shed vesicles encapsulate platelet cytosolic LDH [20]. We found no detectable LDH (data not shown), and this result suggests that the extent of the shedding should be quite small, if any.

Although the apparent yields of platelet proteins are comparable between DMPC/DDPC liposome and DLPC [20], the DDPC containing liposome may have advantages over DLPC. Sunamoto and his co-workers have shown [23,34–36] that incorporation of DDPC in eggPC liposomes results in an improvement of reconstitution efficiency of glycophorin, an integral membrane protein, due to a stabilizing interaction between glycophorin and DDPC. Therefore, proteins in the DDPC-containing liposomal membrane are expected to be more stable than in membrane containing DLPC. The significant improvement of the transfer efficiency by the addition of DDPC in the interacting DMPC liposome described in this article can be attributed to the stabilization effect of DDPC to proteins in the accepting liposomal membrane. Also, membranes containing DLPC may be less stable because of DLPC's shorter acyl chains.

In any event, the detail of the protein transfer phenomenon is yet to be elucidated. We are currently

investigating the mechanism of the direct protein transfer, especially, the effect of the DDPC incorporation in the liposome on the mechanism.

Acknowledgments

The authors thank Dr. Manabu Oki, Kyoto Red Cross Blood Center, for the donation of the concentrated platelet. The authors are also grateful to Dr. Toshinori Sato, Department of Biomolecular Engineering, Tokyo Institute of Technology, and Dr. Yasuo Suda, Department of Chemistry, Osaka University, for helpful discussion. This research was supported by Grant-in-Aid for Scientific Research on Priority Areas from Ministry of Education, Science and Culture (No. 04204021).

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