GPI-Linked Proteins Do Not Transfer Spontaneously from Erythrocytes to Liposomes. New Aspects of Reorganization of the Cell Membrane†

Kenichi Suzuki* and Yukihisa Okumura

Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto, Japan

Received January 19, 2000; Revised Manuscript Received May 2, 2000

ABSTRACT: Exposure of cells to liposomes results in the release of integral membrane proteins. However, it is still controversial whether the release is due to spontaneous protein transfer from cells to liposomes or shed vesicles released from cells. We investigated this issue in an erythrocyte−liposome system by examining the location of acetylcholinesterase (AChE, an integral membrane protein marker), cholesterol (erythrocyte membrane lipid marker), hemoglobin (cytosolic protein marker), and a nonexchangeable lipid marker in liposomes in a sucrose density gradient at high resolution. The density distribution showed that AChE is not transferred to the liposomes but is located on small (about 50 nm) light (10−20 wt % sucrose) or large (about 200 nm) heavy shed vesicles (more than 30 wt % sucrose). AChE in the light shed-vesicle fraction markedly increased even after its level in the heavy fraction reached a plateau. AChE was also released from isolated heavy shed vesicles and accumulated in the small light shed-vesicle fraction in the presence of liposomes. After incubation of spherical erythrocytes (morphological index, 5.0) with liposomes, AChE hardly appeared in the heavy shed-vesicle fraction, and the majority (>99%) appeared in the light shed-vesicle fraction, indicating that AChE is released from both the erythrocytes and heavy shed vesicles to the light shed-vesicle fraction, which becomes rich in AChE. Our results demonstrated for the first time that GPI-linked proteins do not spontaneously transfer from erythrocytes to liposomes. Our study also suggests that in vivo GPI-linked membrane proteins do not spontaneously transfer between cell membranes but that some catalyst is needed.

Incubation of cells with liposomes results in the release of integral membrane proteins (*1*−*23*). When intact erythrocytes are incubated with 1,2-dimyristoylphosphatidylcholine (DMPC)[1] liposomes and the separated supernatant is subjected to sucrose density gradient centrifugation (see Figure 1 for a schematic presentation of the procedure), four major proteins including band 3 and acetylcholinesterase (AChE) are identified in the low-density fraction (10−20 wt % sucrose) (*2*, *3*), as well as in the shed-vesicle fraction (30−40 wt % sucrose). Because liposomes are located in the low-density fraction and the membrane proteins in the low-density fraction are incorporated in closed vesicles (*3*, *4*), the release of proteins has been regarded as "protein transfer" from cells to liposomes (*1*−*12*). On the other hand, when intact erythrocytes are incubated with DMPC liposomes and the separated supernatant is further centrifuged at a high speed (11000*g*), the lipid composition of the obtained pellet is similar to the remnant erythrocytes (Figure 1) (*13*−*20*). These experiments suggest that the release of integral membrane proteins is exclusively due to shed vesicles from erythrocytes (*13*−*20*).

However, the fact that the released membrane proteins are located in the same fraction with liposomes is not compelling evidence for the incorporation of membrane proteins in the low-density fraction into the liposomes. In this regard, the lipid composition in the supernatant after high-speed centrifugation has, to our knowledge, never been investigated. Thus, it is still unclear whether only shed vesicles are released or a spontaneous transfer of integral membrane proteins to liposomes occurs, although this is a basic question regarding the interaction between lipids and membrane proteins.

It has already been demonstrated that intermembrane protein transfer between cell membranes occurs in vivo (*24*−*28*). For example, glycosylphosphatidylinositol (GPI) anchored proteins expressed on the surface of transgenic mouse red blood cells were transferred in a functional form to endothelial cells in vivo (*24*). In some cases, transferred GPI-linked proteins show biological activity (*24*). This discovery has introduced a unique technique, called "protein painting", that exogenous GPI-linked protein can be incorporated on a cell membrane which cannot express the protein because of difficulty of gene transfer. However, the mechanisms of the intermembrane protein transfer between cell membranes, whether it occurs spontaneously or catalytically, have not been elucidated. Therefore, we investigated the mechanism of the protein transfer by employing a liposome−erythrocyte interaction system as a model system.

In the present study, we investigated the above issue by quantitatively determining the distribution of various markers

† This work was supported by Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Culture, and Sports (No. 07405044).

* To whom correspondence should be addressed at the Kusumi Membrane Organizer Project, ERATO, JST, Kumazaki Building, 5-11-33, Chiyoda Naka-ku, Nagoya 460-0012, Japan. Phone: +81-52-243-5222. Fax: +81-52-243-5211. E-mail: ksuzuki@kusumi.jst.go.jp.

[1] Abbreviations: AChE, acetylcholinesterase; DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; D_{14}DPC, 1,2-dimyristamido-1,2-deoxyphosphatidylcholine; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid; NBD-egg PE, egg [*N*-(4-nitrobenzo)-2-oxa-1,3-diazole]phosphatidylethanolamine.

10.1021/bi000113v CCC: $19.00
Published on Web 07/08/2000

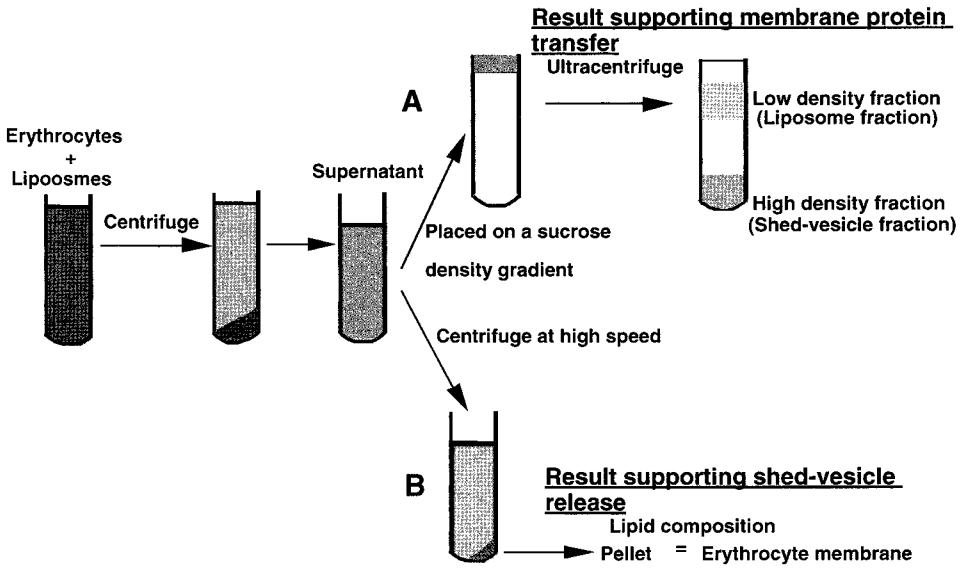

FIGURE 1: Schematic presentation of the present understanding of membrane protein transfer from erythrocytes to liposomes (A) and shed-vesicle release from erythrocytes (B).

from liposomes and erythrocytes using the sucrose density gradient centrifugation. For this purpose, several markers were used, including (a) AChE, a marker of an integral membrane protein, (b) hemoglobin, to trace a component originally located in the erythrocyte cytosol, (c) NBD-egg PE (nonexchangeable lipid marker), incorporated in liposomes, to determine the presence of liposomes, and (d) cholesterol, to evaluate the enrichment of AChE using other erythrocyte membrane components. Further, we used a shallower sucrose density gradient (5−20 wt %) than that used in previous studies for a more precise examination of the distribution of membrane components.

AChE (75 kDa), a glycosylphosphatidylinositol (GPI) anchored protein (*29*), was used as a marker of an integral membrane protein for two reasons. First, if we rule out the transfer of AChE to liposomes, it is self-evident that transmembranous proteins do not easily transfer to liposomes because the released amount of these proteins is much smaller (by about a factor of 10) than that of GPI-linked proteins (*21*). Second, although it has been demonstrated that GPI-linked proteins can transfer between cell membranes in vivo, the mechanism of such transfer, e.g., spontaneously or catalytically, is unknown as mentioned above.

The release of membrane proteins in the presence of liposomes commences following a critical change in erythrocyte morphology (*23*). The morphological change is induced by lipid transfer from liposomes to erythrocytes (*23*). The amount and rate of protein release depend on liposomal lipid composition (*23*). To determine the common mechanism, in this study we used phospholipids bearing various alkyl chain lengths, 1,2-dilauroylphosphatidylcholine (DLPC), DMPC, and 1,2-dipalmitoylphosphatidylcholine (DPPC). In addition, we used 1,2-dimyristamido-1,2-deoxyphosphatidylcholine (coded as D_{14}DPC) that has two amido groups instead of ester groups of DMPC (*30*). The order of transfer rate of phospholipids from liposomes to erythrocytes is DLPC > D_{14}DPC > DMPC ≫ DPPC. ">" means a difference of 10-fold, and "≫" means a difference of 100-fold (*23*, *31*).

In the present study, the new approach to examine the density of membrane components at high resolution under a variety of incubation conditions led us to conclude that GPI-linked membrane proteins do not transfer spontaneously from erythrocytes to liposomes. Instead, protein release was attributed to the process of protein condensation by the formation of high curvature of the lipid bilayer.

EXPERIMENTAL PROCEDURES

Materials. DLPC, DMPC, and DPPC were purchased from Sigma Chemical Co. (St. Louis, MO). D_{14}DPC and HEPES were obtained from Dojindo Laboratories (Kumamoto, Japan). Egg [*N*-(4-nitrobenzo)-2-oxa-1,3-diazole]phosphatidylethanolamine (NBD-egg PE) was from Avanti Polar Lipids, Inc. (Alabaster, AL). Acetylthiocholine iodide and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Wako Chemical Co. (Tokyo, Japan). Glutaraldehyde and sucrose were from Nacalai Tesque (Osaka, Japan). Throughout the study, 10 mM HEPES containing 150 mM NaCl (pH 7.4) was used as a buffer unless otherwise stated.

Preparation and Incubation of Liposomes and Erythrocytes. Liposomes (monodispersive 100 nm size) were prepared by the extrusion method as reported previously (*32*). As a nonexchangeable marker, NBD-egg PE (1 mol %) was added to DMPC (39 mol %)/D_{14}DPC (60 mol %) liposomes. The maximum content of D_{14}DPC was limited to 60 mol % because of the stability of liposomes. The human erythrocyte suspension was prepared and adjusted to a hematocrit (HCT, volume ratio of erythrocytes in the suspension) of 26.3%, as previously reported (*23*). The erythrocyte suspension was warmed for 10 min and exposed to an equal volume of a liposome suspension at 37.0 °C. Unless otherwise noted, equal volumes of erythrocyte suspension and liposome suspension were mixed and incubated in all experiments described below. The mixture was centrifuged at 2000*g* at 4 °C for 3 min to pellet erythrocytes.

Determination of Protein and Lipid Concentrations. The AChE activity was measured according to Ellman et al. (*33*). Briefly, to 3.0 mL of a HEPES buffer were added 10 μL of a sample suspension and 100 μL of 10 mM DTNB, and the mixture was incubated for 5 min at 37.0 °C. Then, the hydrolysis was started by the addition of 20 μL of 75 mM

acetylthiocholine iodide. Increase in absorbance at 412 nm was followed on a Hitachi U-3400 spectrophotometer (Tokyo). Phospholipid concentration was determined with phospholipid C-test Wako (Wako). Briefly, 100 μL of the sample solution was added to 100 μL of a solution including phospholipase D, choline oxidase, peroxidase, sodium 3,5-dimethoxy-*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-aniline (DAOS), 4-aminoantipyrine, and ascorbate oxidase. The mixture was incubated at 37 °C for 10 min. The absorbance of the produced blue dye at 600 nm was measured with a Bio-Rad microplate reader (Hercules, CA). The amount of cholesterol was measured with the cholesterol E test kit (Wako). Briefly, 100 μL of the sample solution was added to 100 μL of a solution containing cholesterol oxidase, peroxidase, DAOS, 4-aminoantipyrine, and ascorbate oxidase. This was followed by incubation and measurement of absorbance as well as determination of phospholipid concentration. The amount of hemoglobin was determined by the sodium lauroyl-hemoglobin method (*34*). Briefly, 100 μL of the sample solution was added to 100 μL of a hemoglobin kit solution (Wako) containing sodium lauroyl sulfate, and the absorbance of the product at 540 nm was measured with a microplate reader. When the experiments were performed in triplicate under five different conditions of incubation of liposomes with erythrocytes, the standard deviations of AChE activity, cholesterol concentration, phospholipid concentration, and hemoglobin concentration were less than 5% under all conditions. Although each of the experiments described in this study was only performed once, the results are reliable because of the high accuracy.

Fractionation of the Liposomal Supernatant by Sucrose Density Gradient Centrifugation. A discontinuous sucrose gradient was prepared by placing 9.7 mL of 27 wt % sucrose onto 0.5 mL of 40 wt % sucrose using a 10.9 mL polyallomer tube (Hitachi Koki ϕ15 mm × 96 mm) connected to a density gradient fractionator (Hitachi DGF-U). The supernatant (500 μL) separated from erythrocytes was immediately placed on the top of the gradient tube and centrifuged at 208000*g* for 2 h at 4.0 °C (Hitachi CP56GII with a P40ST swing bucket rotor). Fractions (0.37 mL each) were collected from the top of the tube for determination of AChE activity and the concentrations of phospholipid and cholesterol. The ratio of AChE activity in the low-density fraction to that in the heavy fraction did not change when a discontinuous or continuous sucrose gradient was employed. In experiments using DMPC (39)/D_{14}DPC (60)/NBD-egg PE (1) liposomes, continuous density gradient centrifugation was carried out with 5−20 wt % sucrose.

Incubation of Shed Vesicles and Liposomes. To examine the possible interaction of shed vesicles with liposomes, shed vesicles were isolated and incubated again with liposomes. After exposure of erythrocytes at 37 °C to DLPC liposomes (lipid concentration, 0.1 mM) for 5 min, DMPC (40)/D_{14}DPC (60) liposomes (0.1 mM) for 150 min, or DMPC liposomes (0.2 mM) for 530 min, they were pelleted by centrifugation. The supernatant was further centrifuged at 11000*g*, 4 °C, for 20 min to pellet shed vesicles. Sucrose density gradient centrifugation showed that the pellet only contained shed vesicles in the 30−40 wt % sucrose fraction. The phospholipid concentration of the shed-vesicle suspension was adjusted to 0.15 mM or 0.03 mM, which corresponds to 50% and 10% of the total phospholipid in the erythrocyte membrane (HCT, 26.3%), respectively. This concentration range of shed vesicles covers that of released shed vesicles by incubation of erythrocytes with liposomes. The shed-vesicle suspension was exposed to an equal volume of DLPC, DMPC (40)/D_{14}DPC (60), or DMPC liposome suspension (1.0 mM) at 37 °C. After fractionation of the mixture by density gradient centrifugation, we measured AChE activity and the amounts of cholesterol and hemoglobin.

Morphological Changes in Erythrocytes. Erythrocyte morphology was assessed as previously reported (*31*). Briefly, after fixation with 0.5 wt % glutaraldehyde (150 mM NaCl), erythrocytes were examined with an Olympus IMT-2 light microscope. Erythrocyte shape was classified into one of the following types: discocytes (normal shape), score = 0, and echinocytes (crenated erythrocytes), scores = +1 to +5 depending on the extent of morphological changes. The average score of erythrocyte shape was determined in 100 erythrocytes and was defined as the morphological index (MI). These experiments were performed in duplicate.

Incubation of Erythrocytes of MI 5.0 with Liposomes. To investigate the relationship between changes in erythrocyte morphology (crenation) and AChE release, erythrocytes and DLPC liposomes (lipid concentration, 1.0 mM) were incubated until the MI reached 5.0 (2 min) at 37 °C. Spherical erythrocytes with a smooth surface were washed and incubated with fresh DLPC liposomes (1.0 mM) for 2 min at 37 °C.

Measurement of Shed-Vesicle Size. The size of shed vesicles was measured by size exclusion chromatography using a Sepharose 4B column (Pharmacia) and by dynamic light scattering (Otsuka Electronic Co.).

RESULTS

Fractionation of Supernatants by Sucrose Density Gradient Centrifugation. To determine whether AChE is present on the liposomes after its release from erythrocytes, the obtained supernatant was subjected to sucrose density gradient centrifugation. When AChE density was simply compared with the phospholipid density under the conditions used in previous studies (sucrose, 10−40 wt %), the peaks were noticed to overlap with each other and AChE was apparently located on the liposomes (Figure 2). For a more precise evaluation of the location of AChE, a nonexchangeable marker lipid, 1 mol % NBD-egg PE was incorporated into DMPC (39)/D_{14}DPC (60) liposomes (lipid concentration, 1.0 mM) to differentiate between liposomes and other vesicles. The obtained supernatant was fractionated using a shallower density gradient (5−20 wt % sucrose) (Figure 3A−D). Shed vesicles appeared at more than 30 wt % sucrose as shown in Figure 2 and were pelleted under this centrifugation condition. Throughout the incubation, even after 300 min incubation more than 94% of NBD-egg PE was recovered in the low-density fraction (about 5−15 wt % sucrose) where the liposomes located, indicating that NBD-egg PE was not significantly transferred to erythrocytes or to shed vesicles but well kept in the liposomes.

As the incubation proceeded, AChE and cholesterol shifted to the higher density fraction, while NBD-egg PE slightly shifted to the lower density fraction (Figure 3). This shift was probably due to the loss of phospholipid by transfer to

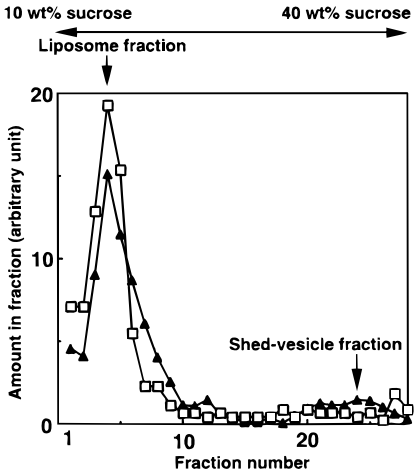

FIGURE 2: Density fractionation of the supernatant after incubation of erythrocytes (HCT, 26.3%) with DMPC (40)/D_{14}DPC (60) liposomes (lipid concentration, 1.0 mM) at 37 °C for 30 min. Continuous sucrose gradient (10 wt % sucrose at fraction 1 and 40 wt % at fraction 28) was used. Plots: phospholipid (□); AChE (▲).

erythrocytes. The phospholipid gradually separated to the lower density overlapped with NBD-egg PE and to the higher density fractions in which no NBD-egg PE was detected. After incubation for 300 min, which was longer than the time required for cessation of AChE release (see Figure 4B), only 14% of AChE was present over the fractions numbered 1−9 (liposome fraction), while more than 95% of NBD-egg PE was found in this range. More clearly, after 400 min incubation with DMPC liposomes (1.0 mM) (Figure 3E), more than 94% of NBD-eggPE was detected in fractions numbered 1−7, where only 5% of AChE was located. This discrepancy in the distribution between AChE and NBD-egg PE clearly indicates that AChE is not located on the liposomes and, in terms of density, there are two types of shed vesicles. We defined these vesicles as light shed vesicles (sucrose, 10−20 wt %) and heavy shed vesicles (sucrose, ∼30 wt %).

Figure 3 shows a synchronous change in the distribution of AChE and cholesterol. Preliminary experiments in our laboratory showed that cholesterol did not significantly change the density of liposomes (data not shown), while incorporation of membrane proteins increased the density (*5*). These results indicate that cholesterol and AChE are simultaneously released in the same shed vesicles, and therefore we can evaluate the accumulation of AChE relative to cholesterol in the light shed vesicles as shown below (see Figure 8).

AChE Release Observed at Light and Heavy Fractions. To further characterize AChE release, we monitored the serial changes in AChE activity in light and heavy shed-vesicle fractions using a variety of liposomal lipids (Figure 4). With DLPC liposomes (1.0 mM), 90% of AChE activity was detected in the light shed-vesicle fraction at 5 min (Figure 4A). On the other hand, with DMPC liposomes (1.0 mM), approximately equal amounts of released AChE were observed in both fractions (Figure 4C). Although with a high concentration of DMPC (40)/D_{14}DPC (60) liposomes (1.0 mM), 80% of AChE was located in the light shed-vesicle fraction (Figure 4B), low concentration (0.1 mM) induced only the heavy shed vesicles (Figure 4D). These results

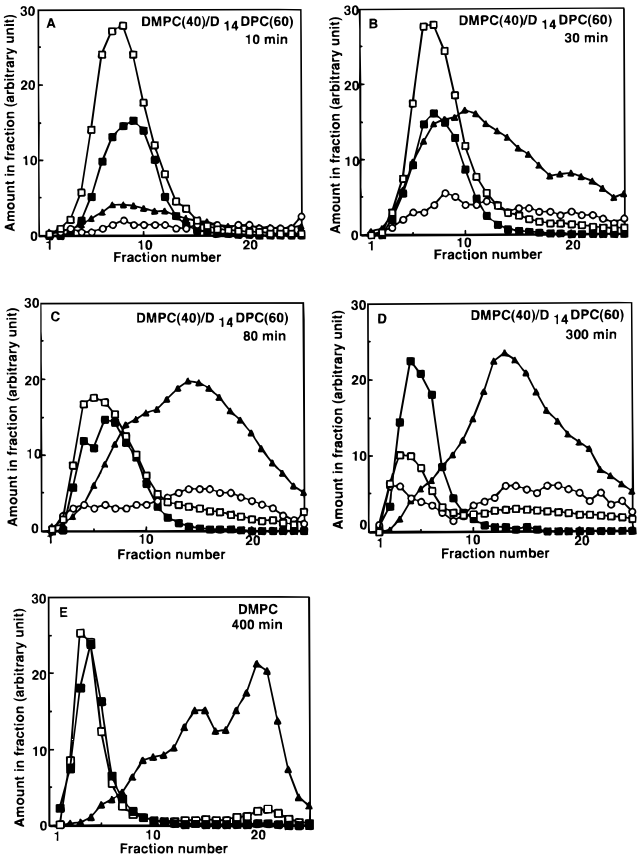

FIGURE 3: Density fractionation of the supernatant after incubation of erythrocytes (HCT, 26.3%) with a DMPC (39)/D_{14}DPC (60)/NBD-egg PE (1) liposome (lipid concentration, 1 mM) suspension at 37 °C for (A) 10 min, (B) 30 min, (C) 80 min, or (D) 300 min. (E) Erythrocytes and DMPC (99)/NBD-egg PE (1) liposomes (lipid concentration, 1 mM) were incubated for 400 min at 37 °C. Continuous sucrose gradient (5 wt % sucrose at fraction 1 and 20 wt % at fraction 25) was used. Plots: NBD-egg PE (■); phospholipid (□); AChE (▲); cholesterol (○). Incubation time is shown in each section. Under the conditions employed, in the case of DMPC (39)/D_{14}DPC (60)/NBD-egg PE (1) liposomes, AChE release started at 6 min incubation time and leveled off after 30 min (see Figure 4B).

indicate that AChE activity in the light shed-vesicle fraction relative to that in the heavy shed-vesicle fraction largely depends on the lipid composition and concentration.

On the other hand, for liposomes that induced AChE release to the light shed-vesicle fraction (Figure 4A−C), AChE release commenced simultaneously in both the light and heavy shed-vesicle fractions as previously reported (*23*). However, for the liposomes that induced AChE release to the light shed-vesicle fraction (Figure 4A−C), AChE continued to be released to the light shed-vesicle fraction even after cessation of its release to the heavy fraction. Termination of the release of the heavy shed vesicles was also confirmed by the saturation of phospholipid and hemoglobin in the fraction (data not shown). Figure 4 also shows that AChE release to both fractions commenced at an MI of 2.8, as reported previously (*23*), and that the release of the heavy shed vesicles terminated at an MI of 5.0. These results suggest that initiation of the release to both fractions and termination of the release to the heavy fraction are determined by the shape of erythrocytes, while termination of the release to the light fraction is determined by other factors.

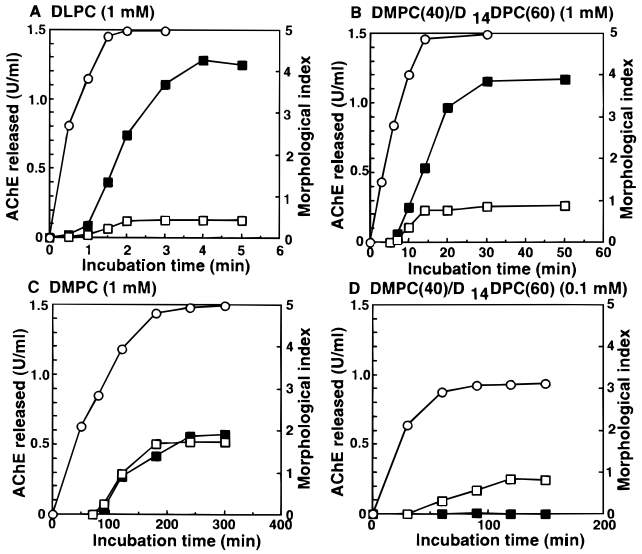

FIGURE 4: AChE activity (units/mL) in the light shed-vesicle fraction (~25 wt % sucrose, ■), heavy shed-vesicle fraction (30−40 wt % sucrose, □), and MI of erythrocytes (○) upon incubation of erythrocytes (HCT, 26.3%) and various liposomes at 37 °C: (A) DLPC (lipid concentration, 1 mM), (B) DMPC (40)/D_{14}DPC (60) (1 mM), (C) DMPC (1 mM), and (D) DMPC (40)/D_{14}DPC (60) (0.1 mM).

As shown in Figure 4, AChE activity in the light shed-vesicle fraction relative to that in the heavy shed-vesicle fraction depended on the lipid composition and concentration. In the next series of experiments, we explored this issue in more detail. AChE activity in the supernatant from erythrocytes (Figure 5A) and in the light shed-vesicle fraction (Figure 5B) after AChE release reached a plateau (the time at which the release reached a plateau is indicated in Figure 5C), monotonically increased with liposomal lipid concentration. For DPPC liposomes, no AChE activity was observed at all in the light shed-vesicle fraction even at high concentrations. Figure 6 shows that AChE activity in the light shed-vesicle fraction correlated closely with whole AChE activity in the supernatant regardless of the liposomal lipid composition. Irrespective of the liposomal lipid composition, released AChE was almost exclusively located on the heavy shed-vesicle fraction when less than 50% of AChE in erythrocytes was released in the supernatant. As AChE release in the supernatant increased (>50%), more AChE appeared in the light shed-vesicle fraction. When 95% of AChE in erythrocytes was released in the supernatant, most AChE was found in the light shed-vesicle fraction.

Further Release of AChE from the Heavy Shed-Vesicle Fraction to the Light Fraction. To study how AChE is released to the heavy shed-vesicle fraction, we also examined AChE activity and the amount of phospholipid in the fraction under conditions similar to those described in Figure 5. AChE activity in the heavy shed-vesicle fraction reached a peak level with a rather narrow range of liposomal lipid concentration (Figure 7A) although the amount of phospholipid did not significantly change at liposomal concentration higher than 0.2 mM (Figure 7B). At high liposomal concentration, the amount of hemoglobin in the heavy shed-vesicle fraction also did not change significantly [about 120 μg/mL in case of DLPC liposomes and ~200 μg/mL in the case of DMPC (40)/D_{14}DPC (60) and DMPC liposomes]. These results indicate that the amount of heavy shed vesicles did not

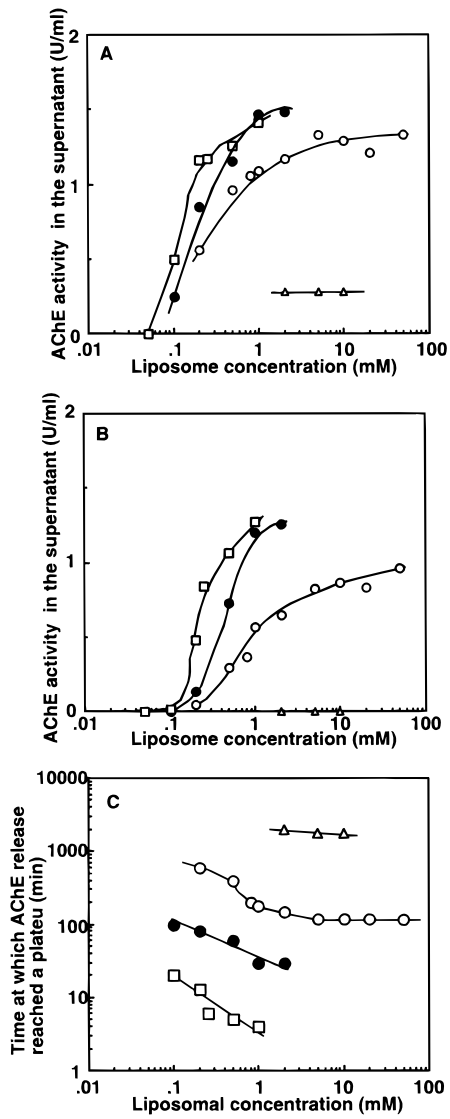

FIGURE 5: AChE activity (units/mL) after termination of release (A) in the supernatant and (B) in the light shed-vesicle fraction. The time at which AChE release reached a plateau is indicated in (C). Erythrocytes (HCT, 26%) and liposomes were incubated at 37 °C: (□) DLPC; (●) DMPC (40)/D_{14}DPC (60); (○) DMPC; (△) DPPC liposomes.

change in the liposomal lipid concentration range. To estimate the released AChE activity per known amount of heavy shed vesicles, we determined AChE activity per known amount of phospholipid in the heavy shed-vesicle fraction (Figure 7C). Increased concentrations of liposomal lipid were associated with reduced AChE/phospholipid in the heavy shed-vesicle fraction. These results suggest that shed vesicles including rich AChE are further released from the heavy shed-vesicle fraction to the light density fraction, which result in the reduction of AChE density in the heavy shed vesicles.

To inspect the above possibility, the isolated heavy shed vesicles were incubated with DLPC, DMPC (40)/D_{14}DPC (60), or DMPC liposomes (1.0 mM). This resulted in the appearance of the light shed-vesicle fraction containing AChE and cholesterol (Figure 8). Regardless of the amount of the incubated heavy shed vesicles, the ratio of AChE to cholesterol in the light shed-vesicle fraction was more than 2 in the cases of DLPC and DMPC (40)/D_{14}DPC (60) liposomes. Since cholesterol and AChE are simultaneously

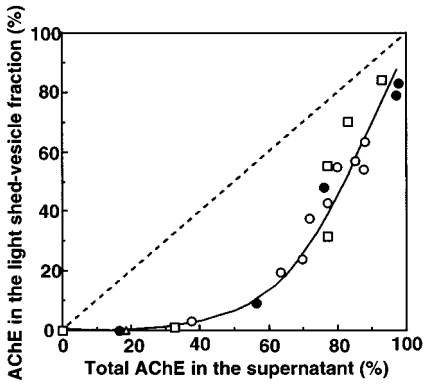

FIGURE 6: Relationship between the ratio of AChE in the light shed-vesicle fraction to that in erythrocytes (%) and the ratio of AChE in the supernatant to that in erythrocytes (%) at the end of AChE release. Erythrocytes (HCT, 26.3%) and liposomes were incubated at 37 °C: (□) DLPC; (●) DMPC (40)/D_{14}DPC (60); (○) DMPC; (△) DPPC liposomes. At the dotted line, the entire AChE released into the supernatant is located on the small light shed vesicles.

released on the same vesicle as shown in Figure 3, this result indicates that AChE was enriched in the process of release of the light shed vesicles from the heavy shed vesicles. On the other hand, after long incubation cholesterol spontaneously transfers between membranes (*35*). In this case, spontaneous cholesterol transfer to liposomes should be taken into account in addition to cholesterol release by small shed-vesicle release. This explains the equal release of AChE and cholesterol in the presence of DMPC liposomes (Figure 8C).

On the other hand, hemoglobin was hardly found in the light shed-vesicle fraction (Figure 8), indicating the small size of light shed vesicles. This result also agrees with the finding that the amount of hemoglobin in the heavy shed-vesicle fraction did not change significantly at higher liposomal concentration when shed vesicles including rich AChE were further released from heavy shed vesicles to the light density fraction under the conditions of Figure 7. Using size exclusion chromatography and dynamic light scattering, the size of the vesicles was estimated to be about 50 nm, which was markedly smaller than that of the heavy shed vesicles (200 nm). When only shed vesicles were incubated in control experiments in the buffer at 37 °C, AChE and cholesterol were not released to the light fraction. These results indicate that AChE was further released from the heavy shed vesicles to accumulate on the small light vesicles.

The transformation of shed vesicles to smaller vesicles was likely due to an additional lipid transfer from liposomes to shed vesicles, similar to the lipid transfer from liposomes to erythrocytes (*23*) as explained below. The rate of phospholipid transfer between membranes depends on the alkyl chain length of the phospholipids (*31*). Phospholipids bearing shorter alkyl chains transfer between membranes faster than those bearing longer chains (*31*). Because the alkyl chain length of the phospholipids in the shed vesicles (>16) is longer than that of the liposomes (12 and 14 in Figure 8), the rate of phospholipid transfer from the liposomes to the shed vesicles is much faster than that from the shed vesicles to the liposomes. Therefore, the transferred phospholipids from liposomes accumulate in the shed vesicles. Because the phospholipids of the shed vesicles distribute asymmetrically in the outer leaflet and the inner leaflet as well as those of erythrocyte membranes (*16*), and the flip-flop rate of the phosphatidylcholines is very slow (*36*), the transferred phosphatidylcholines from the liposomes to the shed vesicles should accumulate in the outer leaflet of the shed vesicles. Because membranes are crenated when the volume of the outer leaflet is larger than the inner leaflet (bilayer couple balance) (*37*), shed vesicles would be crenated as observed in erythrocyte membranes (*23*, *31*) and new smaller vesicles would be formed. This is supported by previous results demonstrating that excessive incorporation of exogenous phosphatidylcholine, which has a short alkyl chain, into liposomes composed of a longer alkyl chain can cause reorganization of the liposomes into multiple smaller vesicles (*38*). Furthermore, the fact that the rate of AChE release from the heavy shed-vesicle fraction to the light fraction (Figure 8) is in parallel with the rate of transfer of the phosphatidylcholines between membranes endorses this notion.

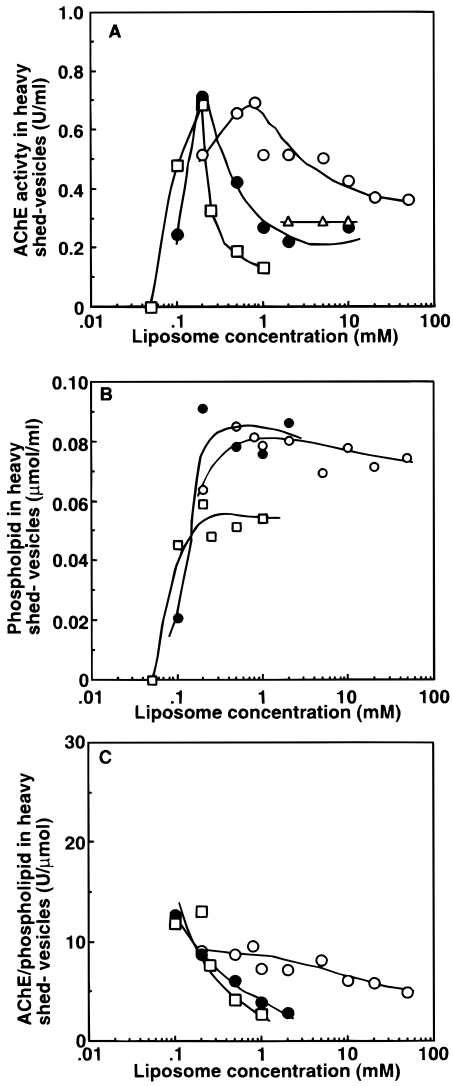

FIGURE 7: (A) AChE activity (units/mL), (B) phospholipid amount (μmol/mL), and (C) AChE/phospholipid (units/μmol) in the heavy shed-vesicle fraction after termination of AChE release. The experimental conditions were similar to those described in Figure 5. Plots: (□) DLPC; (●) DMPC (40)/D_{14}DPC (60); (○) DMPC; (△) DPPC liposomes.

Incubation of Erythrocytes of MI 5.0 with Liposomes. Because AChE activity in the heavy shed vesicles was

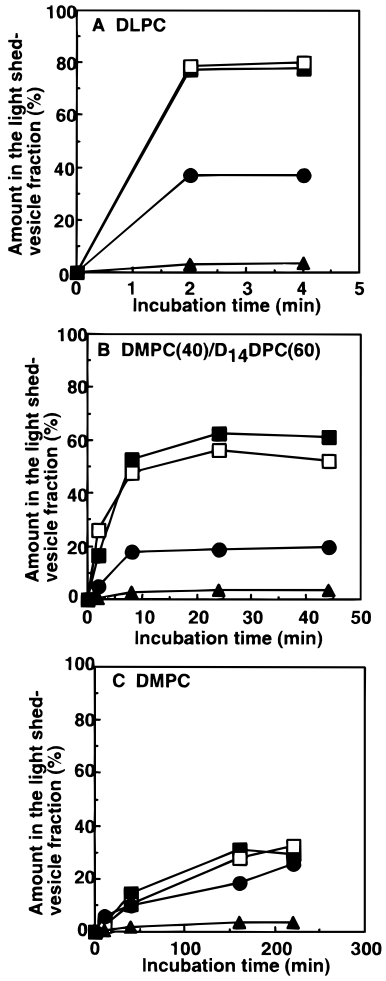

FIGURE 8: Serial changes in the release of AChE (■, phospholipid concentration of shed vesicles = 0.15 mM; □, phospholipid concentration of shed vesicles = 0.03 mM), cholesterol (●, phospholipid concentration of shed vesicles = 0.15 mM), and hemoglobin (▲, phospholipid concentration of shed vesicles = 0.15 mM) from the heavy shed-vesicle fraction to the light fraction in the presence of various liposomes. The liposome suspension (lipid concentration, 1 mM) was incubated with the shed-vesicle suspension at 37 °C: (A) DLPC, (B) DMPC (40)/D_{14}DPC (60), and (C) DMPC liposomes. The ordinate represents the amount in the light shed-vesicle fraction relative to that in the original heavy shed vesicle.

unchanged at an MI of 5.0, the continuous increase of AChE activity in the light shed-vesicle fraction is unlikely to be due to the release from the heavy to light shed vesicles. To investigate whether the light shed vesicles are directly released from erythrocytes, the latter, at MI 5.0, were incubated with DLPC liposomes (1.0 mM). Most of the released AChE (>99%) was located in the light shed-vesicle fraction but hardly released to the heavy fraction. The ratio of AChE to cholesterol in the light shed-vesicle fraction was similar to that shown in Figure 8A. The same finding was also observed in other liposomes. These results indicate that the light shed vesicles are also directly released from erythrocytes and the light shed vesicles are rich in AChE.

DISCUSSION

In this study, the sucrose density gradient centrifugation (Figure 3) was carefully carried out to inspect whether GPI-linked proteins are transferred from erythrocytes to liposomes. There are reasons to investigate this issue by the sucrose density gradient centrifugation. First, previous studies were performed using the sucrose density gradient centrifugation (*2*−*7*). Second, it is often difficult to completely separate liposomes from shed vesicles by gel chromatography due to the low resolution (*2*). Our results showed that AChE did not transfer from erythrocytes to liposomes but was rather released into the small light shed vesicles, which are rich in AChE. Considered together, the major finding of our study can be schematically presented in Figure 9, which illustrates AChE release and the formation of heavy and light shed vesicles.

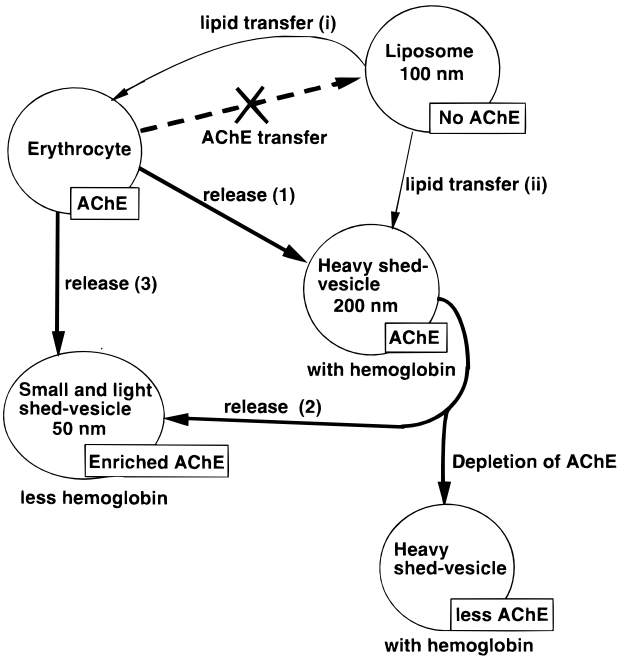

FIGURE 9: Outline of AChE release in the heavy and light shed vesicles from erythrocytes in the presence of liposomes.

When intact erythrocytes are exposed to liposomes, which contain easily exchangeable lipid, liposomal lipid transfers from the liposomes to erythrocytes [lipid transfer (i)], which results in crenation of the erythrocyte membrane (*23*, *31*). This occurs during the induction period before AChE release from erythrocytes as reported earlier (*23*). Following critical morphological changes (*23*), erythrocytes release shed vesicles to the heavy fraction (release 1, Figure 9). With less than 50% of AChE released from erythrocytes, AChE is only released to the heavy fraction. When more than 50% of AChE is released from erythrocytes, further lipid transfer continues from the liposomes to the heavy shed vesicles [lipid transfer (ii)], leading to the release of light shed vesicles rich in AChE (release 2). The release of shed vesicles from erythrocytes to the light fraction continues (release 3, Figure 9) even after cessation of the release of heavy shed vesicles.

Because shed vesicles contain hemoglobin, the density of the heavy shed vesicles is sufficiently high to be separated by centrifugation. However, the light shed vesicles are similar to the liposomes with respect to size and density. Therefore, it has been very difficult to separate these structures from the original liposomes even by density gradient centrifugation (10−40%). In fact, unless erythrocytes were incubated with liposomes for prolonged periods and unless a shallow-density gradient (5−20%) of sucrose was used, the discrepancy in

the density between the cargo of membrane proteins and liposomes was not clear. This is the major reason for the wrongly held notion that membrane proteins transfer directly from erythrocytes to liposomes during their incubation. As shown in Figure 9, it is now clear that a direct transfer does not occur.

The present study also suggests that in vivo GPI-linked proteins do not spontaneously transfer between cell membranes, but some catalyst is needed. Since transfer of GPI-linked proteins between cell membranes has been applied to the protein painting technique (*24*−*28*) as mentioned in the introduction, this study should provide important information in this field. However, it should be mentioned that the present results do not completely exclude the possible transfer of GPI-linked proteins. Because lipid concentration inside the cell membrane is a little bit higher (∼10−100 mM) than that used in this study (∼50 mM), and because it is reported that the rate of lipid transfer between liposomes depends on the concentrations of both donor and acceptor membranes at high lipid concentration (*39*, *40*), GPI-linked proteins might still spontaneously transfer to the inside of cells in a similar mechanism. Further studies are necessary to examine this issue.

We previously showed that the release of both a GPI-anchored protein, AChE, and a transmembrane protein, band 3, commenced simultaneously after the same induction period and was completed concurrently. The amount of released band 3 from erythrocytes was much smaller (by about a factor of 10) than that of AChE (*21*). Therefore, band 3 probably does not transfer spontaneously from erythrocytes to liposomes. However, it is naive to extend these results to conclude that neither GPI-anchored proteins nor transmembrane proteins in other cells transfer spontaneously from cells to liposomes because it is generally known that GPI-anchored proteins locate in "lipid rafts" in which sphingolipids and cholesterol are enriched; meanwhile, transmembrane proteins distribute differently from GPI-anchored proteins (*41*, *42*). Further studies are needed to elucidate this issue.

Along with the negation of spontaneous GPI-linked protein transfer from erythrocytes to liposomes, an important finding of this study was that the small shed vesicles were richer in AChE than the large (heavy) shed vesicles (Figure 8). Because the large (heavy) shed vesicles are richer in AChE than erythrocytes (*13*), our finding indicates that AChE is condensed in the membranes after every shedding process. To explain why the large (heavy) shed vesicles from erythrocytes are rich in GPI-anchored proteins such as AChE, the following experiments were carried out (*43*). Fluorescence imaging micropipet experiments showed that GPI-linked protein was excluded from entry into the crenated membrane region of erythrocyte and that such proteins were abundant on the cap of the membrane crenated area. At entry, integral membrane proteins are trapped by cytoskeletons and subsequently condensed (*43*). On the basis of these changes, it has been postulated that enrichment of the GPI-linked protein is due to the volume exclusion effect by integral membrane proteins (*43*). However, since the heavy shed vesicles do not contain cytoskeletons as previously reported (*13*, *18*), the volume exclusion effect is inconceivable, based on AChE enrichment in the release of the small light shed vesicles from the heavy (large) shed vesicles. Because theory predicts that membrane proteins are preferentially located in large curvature bilayers (*44*), the curvature is likely to be important for the enrichment in this case. Because the size of the shed vesicles from Golgi and endoplasmic reticulum (ER) membranes is small (about 60 nm) (*45*), it is interesting to consider that the difference of curvature is also utilized for efficient assembling of proteins in vivo.

In summary, our study demonstrated that the GPI-linked membrane protein does not transfer spontaneously from erythrocytes to liposomes but rather to two types of shed vesicles, which differ in size and density (light shed and heavy shed vesicles). The small (light) shed vesicles are released from both erythrocytes and the large (heavy) shed vesicles. The driving force that allows AChE to be released to the small light shed-vesicle fraction is the incorporation of phospholipid from liposomes into erythrocytes or into the heavy shed vesicles. AChE accumulated upon the release of the small (50 nm) shed vesicles, probably due to the formation of the high curvature bilayer.

ACKNOWLEDGMENT

We thank Drs. Nobuhiro Morone (ERATO, JST), Masamitsu Goto (Kyoto University), and Ken Ritchie (ERATO, JST) for helpful discussions and critical reading of the manuscript.

REFERENCES

1. Dunnick, J. K., Rooke, J. D., Aragon, S., and Kriss, J. P. (1976) *Cancer Res. 36*, 2385−2389.
2. Bouma, S. R., Drislane, F. W., and Huestis, W. H. (1977) *J. Biol. Chem. 252*, 6759−6763.
3. Newton, A. C., Cook, S. L., and Huestis, W. H. (1983) *Biochemistry 22*, 6110−6117.
4. Huestis, W. H., and Newton, A. C. (1986) *J. Biol. Chem. 261*, 16274−16278.
5. Newton, A. C., and Huestis, W. H. (1988) *Biochemistry 27*, 4645−4655.
6. Newton, A. C., and Huestis, W. H. (1988) *Biochemistry 27*, 4655−4659.
7. Waters, S. I., Sen, R., Brunauer, L. S., and Huestis, W. H. (1996) *Biochemistry 35*, 4002−4008.
8. Shibata, R., Noguchi, T., Sato, T., Akiyoshi, K., Sunamoto, J., Shiku, H., and Nakayama, E. (1991) *Int. J. Cancer 48*, 434−442.
9. Nakamura, M., Tsujii, K., Katsuragi, Y., Kurihara, K., and Sunamoto, J. (1994) *Biochem. Biophys. Res. Commun.*, 415−422.
10. Dalencon, F., Rosilio, V., Puisieux, F., Baszkin, A., and Wautier, J. L. (1996) *Biochim. Biophys. Acta 1302*, 241−248.
11. Kogure, K., Nakamura, C., Okuda, O., Hayashi, K., and Ueno, M. (1997) *Biochim. Biophys. Acta 1329*, 174−182.
12. Kogure, K., Okuda, O., Nakamura, C., Hayashi, K., and Ueno, M. (1999) *Chem. Pharm. Bull. 47*, 1117−1120.
13. Ott, P., Hope, M. J., Verkleij, A. J., Roelofsen, B., Bradbeck, U., and van Deenen, L. L. N. (1981) *Biochim. Biophys. Acta 641*, 79−87.
14. Buetikofer, P., and Ott, P. (1985) *Biochim. Biophys. Acta 821*, 91−96.
15. Frenkel, E. J., Kuypers, F. A., Op den Kamp, J. A. F., Roelofsen, B., and Ott, P. (1986) *Biochim. Biophys. Acta 855*, 293−301.
16. de Jong, K., and Ott, P. (1993) *FEBS Lett. 334*, 183−188.
17. Belenznay, Z., Zachowski, A., Devaux, P. F., Navazo, M. P., and Ott, P. (1993) *Biochemistry 32*, 3146−3152.
18. Takahashi, K., Kobayashi, T., Yamada, A., Tanaka, Y., Inoue, K., and Nojima, S. (1983) *J. Biochem. 93*, 1691−1699.
19. Yamaguchi, T., Yamada, S., and Kimoto, E. (1994) *J. Biochem. 115*, 659−663.
20. Greidzial, M., Ehrke, R., Baust, B., Torchilin, V. P., and Lasch, J. (1990) *Biomed. Biochim. Acta 49*, 189−200.

21. Suzuki, K., Okumura, Y., Sato, T., and Sunamoto, J. (1995) *Proc. Jpn. Acad., Ser. B 71*, 93−97.
22. Suzuki, K., Okumura, Y., Sato, T., Yasuda, T., Oki, A., Oki, M., and Sunamoto, J. (1996) *Transfusion 11−12*, 966−968.
23. Suzuki, K., Okumura, Y., and Sunamoto, J. (1999) *J. Biochem. 125*, 876−882.
24. Kooyman, D. L., Byrne, G. W., McClellan, S., Nielsen, D., Tone, M., Waldmann, H., Coffman, T. M., McCurry, K. R., Platt, J. L., and Logan, J. S. (1995) *Science 269*, 89−92.
25. Medof, M. E., Nagarajan, S., and Tykocinski, M. L. (1996) *FASEB J. 10*, 574−586.
26. Anderson, S. M., Yu, G., Giattina, M., and Miller, J. L. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 5894−5898.
27. Sloand, E. M., Maciejewski, J. P., Dunn, D., Moss J., Brewer, B., Kirby, M., and Young, N. S. (1998) *Blood 92*, 4439−4445.
28. Brunschwig, E. B., Fayen, J. D., Medof, M. E., and Tykocinski, M. L. (1999) *J. Immunother. 22*, 390−400.
29. Roberts, L., Santikarn, S., Reinhold, V. N., and Rosenberry, T. L. (1988) *J. Biol. Chem. 263*, 18776−18784.
30. Sunamoto, J., Goto, M., Iwamoto, K., Kondo, H., and Sato, T. (1990) *Biochim. Biophys. Acta 1024*, 209−219.
31. Ferrell, J. E., Jr., Lee, K.-J., and Huestis, W. H. (1985) *Biochemistry 24*, 2849−2857.
32. Olson, F., Hunt, C. A., Szoka, F. C., Vail, W. J., and Papahadjopuolos, D. (1979) *Biochim. Biophys. Acta 557*, 9−23.
33. Ellman, J. L., Coutney, K. D., Andres, V., Jr., and Fetherstone, R. M. (1961) *Biochem. Pharmacol. 7*, 88−95.
34. Oshiro, I., Tsutsui, M., Fujii, M., Ueyama, M., Takenaka, T., and Maeda, J. (1981) *Rinsho Byori 29*, 203−209.
35. Kavecansky, J., Joiner, C. H., and Schroeder, F. (1994) *Biochemistry 33*, 2880−2890.
36. Wimley, W. C., and Thompson, T. E. (1991) *Biochemistry 30*, 1702−1709.
37. Sheetz, M. P., and Singer, S. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4457−4461.
38. Gabriel, N. E., and Roberts, M. F. (1984) *Biochemistry 23*, 4011−4015.
39. Jones, J. D., and Thompson, T. E. (1990) *Biochemistry 29*, 1595−1600.
40. Jones, J. D., and Thompson, T. E. (1989) *Biochemistry 28*, 129−134.
41. Verkade, P., Harder, T., Lafont, F., and Simons, K. (2000) *J. Cell Biol. 148*, 727−739.
42. Simons, K., and Ikonen, E. (1997) *Nature 387*, 569−572.
43. Discher, D. E., Mohandas, N., and Evans, E. A (1994) *Science 266*, 1032−1035.
44. Kim, K. S., Neu, J., and Oster, G. (1998) *Biophys. J. 75*, 2274−2291.
45. Jesch, S. A., and Linstedt, A. D. (1998) *Mol. Biol. Cell 9*, 623−635.

BI000113V