Transport of Exogenous Fluorescent Phosphatidylserine Analogue to the Golgi Apparatus in Cultured Fibroblasts

Toshihide Kobayashi and Yasuaki Arakawa

Department of Hygiene and Preventive Medicine, School of Food and Nutritional Sciences, The University of Shizuoka, 395 Yada, Shizuoka 422, Japan

Abstract. We have examined intracellular transport and metabolism of the fluorescent analogue of phosphatidylserine, 1-palmitoyle-2-(N-[12-(7-nitrobenz-2-oxa-1,3-diazeole-4-yl)amino]dodecanoyl]-phosphatidylserine ([palmitoyl-C12-NBD]-PS) in cultured fibroblasts. When monolayer cultures were incubated with liposomes containing (palmitoyl-C12-NBD)-PS at 37°C, fluorescent PS was transported to the Golgi apparatus. NBD-containing analogues of phosphatidylcholine, phosphatidylethanolamine (PE), or phosphatidic acid did not accumulate in the Golgi apparatus under the same experimental conditions.

We suggest that the transport is not due to endocytosis, but is the result of incorporation and transbilayer movement of the (palmitoyl-C12-NBD)-PS at the plasma membrane followed by translocation of the lipid from plasma membrane to the Golgi apparatus via nonvesicular mechanisms. Uptake of fluorescent PS was inhibited by depletion of cellular ATP and was blocked by structural analogues of the lipid or by pretreatment of cells with glutaraldehyde or N-ethylmaleimide. After incorporation into the cell, fluorescent PS was metabolized to fluorescent PE. The intracellular distribution of fluorescence changed during the conversion. In addition to the Golgi apparatus, mitochondria also became labeled.

Our understanding of the mechanisms that regulate lipid traffic is still limited. Lipid molecules can move between intracellular membranes by several mechanisms: (a) transport of molecules from one organelle to another by vesicle budding and fusion; (b) transport of lipid monomers (either spontaneous or protein-mediated) through the cytosol; and (c) transport of molecules by lateral diffusion between organelles connected by membrane bridges (for reviews, see Sleight, 1987; Bishop and Bell, 1988; van Meer; 1989, Pagano, 1990). During biosynthetic membrane traffic, at least two mechanisms are operative for the transport of newly synthesized phospholipids. Transport of newly synthesized sphingolipids from the Golgi apparatus to the plasma membrane has been studied using a fluorescent precursor (Lipsky and Pagano, 1983, 1985a; van Meer et al., 1987; van't Hof and van Meer, 1990). Fluorescent sphingolipids were localized in the luminal leaflet of the Golgi membrane and the exoplasmic leaflet of the plasma membrane. The transport was inhibited during mitosis (Kobayashi and Pagano, 1989) or by inhibitors of protein secretion (Sleight and Pagano, 1983). Similar to PE transport, PC transport occurred under conditions where protein transport was inhibited (Kaplan and Simoni, 1985).

The movement of plasma membrane lipid along the endocytic pathway has been studied using several fluorescent lipid analogues. They are; 1-acyl-2-(N-[6-(7-nitrobenz-2-oxa-1,3-diozo-4-yl)amino]caproyl]-phosphatidylcholine (C6-NBD-PC) (Sleight and Pagano, 1984), N-(NBD-aminocaproyl)-sphingosylphosphorylcholine (C6-NBD-SM) (Koval and Pagano, 1989, 1990), and N-(NBD-aminocaproyl)-glucosylsphingosine (C6-NBD-GlcCer) (Kok et al., 1989). In these studies, fluorescent lipid analogues were inserted into the plasma membrane and their internalization was examined by fluorescence microscopy. Internalization of the fluorescent lipids is temperature and energy dependent. The data suggested that movement into the cell occurred by endocytosis. After internalization, fluorescent lipids were recycled back to the plasma membrane or were transported to the lysosomes for degradation. In the case of the SM analogue in CHO fibroblasts, the rate of transport to the lysosomes was 18–19-fold slower than the rate of recycling (Koval and Pagano, 1990).

These fluorescent lipids have a short chain fluorescent fatty acid and easily exchange between membranes. They are spontaneously inserted into the outer leaflet of the plasma membrane of the cultured cells during incubation. Once fluorescent PC, SM, and GlcCer are inserted into the outer leaflet of the plasma membrane, they do not flip-flop and are
restricted to the outer leaflet. The asymmetric distribution is not changed during recycling. On the other hand, when cells are incubated with 1-palmitoyl-2-(NBD-a-mocaproil)-phosphatidylserine ([palmitoyl-C12-NBD]-PS) (Martin and Pagano, 1987) or 1-palmitoyl-2-(NBD-a-mocaproil)-phosphatidylethanolamine ([palmitoyl-C6-NBD]-PE) (Sleight and Pagano, 1984; Martin and Pagano, 1987), transblayer movement of these lipids from the outer to the inner leaflet of the plasma membrane occurs. The process is ATP dependent and blocked by pretreatment of cells by N-ethylmaleimide (NEM) or glutaraldehyde (Martin and Pagano, 1987). After translocation to the inner leaflet of the plasma membrane, C6-NBD-PS and C6-NBD-PE are spontaneously distributed to intracellular membranes. Therefore, it is necessary to use nonexchangeable lipids in order to examine further transport of PS from the plasma membrane.

Several lines of evidence suggest that exogenous PS is transported to intracellular sites of PS synthesis and to the mitochondria. First, exogenously supplied PS is efficiently incorporated into cultured fibroblasts and suppresses endogenous PS formation (Nishijima et al., 1986; Hasegawa et al., 1989). Second, exogenous PS supports the growth of the mutant cells defective in PS synthesis (Voelker and Frazier, 1986). Third, exogenously supplied PS is metabolized to PE (Voelker and Frazier, 1986). In the present study, we have examined the interaction between liposomes containing a fluorescent analogue of PS, 1-palmitoyl-2-(NBD-a-mocodacanoyl)-phosphatidylserine ([palmitoyl-C12-NBD]-PS) and cultured fibroblasts. Spontaneous transfer of C12-NBD derivatives between membranes is 150-200 times slower than that of C6-NBD derivatives (Nichols and Pagano, 1982, 1983). We wanted to analyze whether this "relatively nonexchangeable" fluorescent lipid could be incorporated and targeted to specific organelles, and, if so, how the transport occurred. Fluorescent PS was incorporated into the cells and accumulated in the Golgi apparatus. Our results suggest that transblayer movement in the plasma membrane and vesicle-independent transport might be involved in PS transport to the Golgi apparatus. During incubation, some PS molecules were metabolized to PE. This conversion was accompanied by distribution of fluorescence to mitochondria.

**Glossary**

| Abbreviation | Description |
|--------------|-------------|
| B-PS         | bovine brain phosphatidylserine |
| B-SM         | bovine brain sphingomyelin |
| DOPE         | dioleoyl phosphatidylcholine |
| HCMF         | 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-buffered Puck's saline without calcium and magnesium (HCMF) |
| HMEM         | 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-buffered MEM, pH 7.4, without indicator |
| N'-Rh-PE     | N'-(lissamine rhodamine B sulfonyl)-dioleoyl phosphatidylethanolamine |
| (palmitoyl-C6-NBD)-PC | (1-palmitoyl-2-NBD-a-mocaproil)-phosphatidylcholine |
| (palmitoyl-C12-NBD)-PC | (1-palmitoyl-2-NBD-a-mocodacanoyl)-phosphatidylcholine |
| (palmitoyl-C12-NBD)-PA | (1-palmitoyl-2-NBD-a-mocodacanoyl)-phosphatidic acid |
| (palmitoyl-C12-NBD)-PE | (1-palmitoyl-2-NBD-a-mocodacanoyl)-phosphatidylethanolamine |
| (palmitoyl-C12-NBD)-PS | (1-palmitoyl-2-NBD-a-mocodacanoyl)-phosphatidylserine |
| P-PI         | pig liver phosphatidylinositol |

**Materials and Methods**

**Cell Culture**

CHO-K1 fibroblasts were the gift of Dr. R. Nozawa (The University of Shizuoka, Shizuoka, Japan). Cells were grown in MEM alpha medium (410-2000; Gibco Laboratories, Grand Island, NY) supplemented with 5% (vol/vol) FCS and 50 µg/ml gentamicin. Normal human skin (SF-TY) fibroblasts (Japanese Cancer Research Resource Bank, Tokyo) were grown in DMEM supplemented with MEM nonessential amino acids (320-1140; Gibco Laboratories), 10% (vol/vol) FCS, and 50 µg/ml gentamicin. Cells were grown at 37°C in an atmosphere of 5% CO2 and 5% O2, and maintained as monolayers on 150-mm diameter tissue culture dishes. 3-4-d-old cultures grown on 25-mm diameter (#1 thickness) glass coverslips were used in experiments.

Mitotic CHO cell populations were isolated from monolayer cultures grown in 850-cm2 plastic roller bottles (Falcon Labware, Oxnard, CA) as described previously (Kobayashi and Pagano, 1989). Briefly, each bottle was seeded with 8.8 x 106 cells in 100 ml of growth medium. After 48 h incubation at 37°C, the culture medium was replaced with fresh growth medium containing 0.4 µg/ml nocardazole (Zieve et al., 1980), and the cells were incubated for an additional 1.5 h at 37°C. Mitotic cell populations were then isolated by shaking (Klevecz, 1975).

**Lipids**

Dioloyl phosphatidylcholine (DOPC), dioloyl phosphatidylethanolamine (DOPE), (1-palmitoyl-2-NBD-a-mocodacanoyl)-phosphatidylcholine ([palmitoyl-C12-NBD]-PC), and N'-lissamine rhodamine B sulfonyl) dioleoyl phosphatidylethanolamine (N'-Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). Bovine brain sphingomyelin (B-SM) and pig liver phosphatidylinositol (P-PI) were from Serdary Research Laboratories Inc. (Ontario, Canada). Bovine brain phosphatidylserine ([B-PS]) was from Sigma Chemical Co. (St. Louis, MO). Fluorescent PS, PE and phosphatic acid (PA) were synthesized from (palmitoyl-C12-NBD)-PC as described (Martin and Pagano, 1987).

**Liposomes**

Liposomes containing (palmitoyl-C12-NBD)-PS and DOPE (20/80 mol %) were prepared by ethanol injection (Kremer et al., 1977). Briefly, a solution of 13.3 mM lipid in ethanol was injected in 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-buffered Puck's saline without calcium and magnesium (HCMF) (final concentration of lipid was 1 mM) and dialyzed overnight against 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-buffered, MEM, pH 7.4, without indicator (HMEM), or glucose-free HMEM as indicated. In some experiments the liposomes also contained 3 mol % N'-Rh-PE, a nonexchangeable lipid (Struck and Pagano, 1980). For experiments using fluorescent PA, PC, and PE, liposomes containing 20 mol % (palmitoyl-C12-NBD)-PA and 80 mol % DOPE, 40 mol % (palmitoyl-C12-NBD)-PC and 60 mol % DOPE, and 50 mol % (palmitoyl-C12-NBD)-PE and 50 mol % DOPE were prepared respectively by ethanol injection as described above. For the experiment in Table I, 5 mM phospholipid dispersions (DOPE, DOPE/B-PS [1:1], DOPE/DOPE [1:1], DOPE/P-PI [1:1], and DOPE/B-SM [1:1]) in HMEM were sonicated as described (Kobayashi et al., 1984) to prepare small unilamellar vesicles.

**Liposome-Cell Incubations**

Monolayer cultures grown on glass coverslips were washed three times with HMEM warmed at 37°C and incubated with liposomes containing fluorescent PS, PC, PE, or FA (200 µM total lipid) in HMEM for 30 min at 37°C. The cells were then washed three times with HMEM and photographed. In some experiments, the washed cells were further incubated at 37°C for 2 h. For the experiment in Fig. 5, cells were incubated with liposomes in the presence of 2 µg/ml Hoechst 33258.

For biochemical experiments, cells were scraped into 1 ml HMEM with a rubber policeman. The cell suspension was centrifuged at 500 g for 5 min at 4°C, and the resulting pellet was resuspended in phosphate saline (0.05 M Na2HPO4, 0.4 M NaCl) buffered to pH 4. Aliquots were removed for determination of DNA content (Labarca and Paigen, 1980) and for lipid extraction (Bligh and Dyer, 1959). DNA contents of human skin fibroblasts and CHO-K1 cells are 1.9 ± 0.2 and 2.0 ± 0.2 µg/10^6 cells (mean ± SD of three determinations), respectively. The amount of fluorescent NBD (λex = 475 nm; λem = 525 nm) and rhodamine (λex = 555 nm; λem = 585 nm) lipids...
present in the cell extracts were determined using a RF-5000 spectrophotofluorometer (Shimadzu Ltd., Kyoto, Japan). Lipid extracts were analyzed qualitatively by TLC on Silica Gel 60 thin-layer plates (E. Merck, Darmstadt, FRG) using chloroform/methanol/acetic acid/water (90:40:12:2) as developing solvent.

For mitotic cell experiments, cells were washed with HMEM and incubated with liposomes containing fluorescent PS (200 μM total lipid) in HMEM for 30 min at 37°C in the presence of 2 μg/ml Hoechst 33258. Then the incubation mixture was layered over a Percoll step gradient (Kobayashi and Pagano, 1989) and centrifuged at 2,500 g for 7 min at 7°C. The viable cells were collected and aliquots were removed for microscopy and for lipid extraction. Nocodazole (0.04 μg/ml) was present throughout the incubation.

**Incubation of Liposomes with Perforated Cells**

Perforated cells were prepared according to the method of Simons and Virta (1987) with slight modifications (Kobayashi and Pagano, 1988). Monolayer cultures were incubated with fluorescent PS as described above. Cells were then perforated and transferred to buffer B (25 mM HEPES-KOH [pH 7.0], 25 mM KCl, 2.5 mM Mg acetate, 0.25 M sucrose; Balch et al., 1984) containing an ATP-regenerating system (0.5 mM ATP, 4 mM creatine phosphate, [20 μg/ml] creatine phosphokinase [800 U/mg], Boehringer Mannheim Biochemicals, Indianapolis, IN; Davey et al., 1985), in the presence of 500 μM liposomes comprised of DOPC/N-Rh-PE (99/1 mol %). Incubations lasted for 10 min at 30°C. Cells were then washed with buffer B and photographed.

**Other Procedures**

Fluorescence microscopy was performed with an Olympus AH-2 microscope equipped with Planapo 40x and barrier filters which allowed no crossover of NBD and rhodamine fluorescence. Photomicrographs were taken using Kodak Tri-X film which was processed to ASA 1600 with Pando developer (Fuji film, Tokyo). Lipid concentrations were determined by phosphorous analysis (Rouser et al., 1966).

**Other Materials**

Chloroquine, cytochalasin B, nocodazole, and valinomycin were from Sigma Chemical Co. (St. Louis, MO). Monensin was from Calbiochem-Behring Corp. (La Jolla, CA). Tissue culture media were from Gibco Laboratories.

**Results**

**Exogenously Added (palmitoyl-C12-NBD)-PS Accumulates in the Golgi Apparatus**

When human skin fibroblasts were incubated with DOPC liposomes containing (palmitoyl-C12-NBD)-PS at 37°C, fluorescent PS was incorporated into the cell in a time-dependent manner (Fig. 1). The incorporation occurred without an appreciable lag. TLC analysis indicated that during incubation, a small percentage of fluorescent PS was metabolized to PE (see below). NBD-labeled fatty acid and lysocompounds were not detected during the incubation. At 2°C, the incorporation of fluorescent PS was 2.42 ± 0.76 pmol/μg DNA (mean of duplicate measurements ± deviations) after 30 min incubation. Fig. 2 a shows a fluorescent micrograph of human skin fibroblasts treated with fluorescent PS for 30 min at 37°C. The Golgi region became strongly fluorescent. Golgi labeling was also observed at 18°C. At 10°C, the Golgi region was weakly labeled, but at 2°C, it was not stained (data not shown). Incubation of CHO-K1 cells with (palmitoyl-C12-NBD)-PS gave diffuse staining of intracellular membranes.

The Golgi apparatus is easily identified in human skin fibroblasts by its characteristic extensive thread-like elements (Lipsky and Pagano, 1985). To find out whether the organelle in which (palmitoyl-C12-NBD)-PS accumulated was the Golgi complex or not, we examined colocalization of fluorescent PS with DOPC/N-Rh-PE (99:1) liposomes in perforated cells. When PC liposomes are incubated with perforated fibroblasts in the presence of ATP-regenerating system, liposomes fuse with the Golgi apparatus (Kobayashi and Pagano, 1988). In Fig. 3, cells incubated with (palmitoyl-C12-NBD)-PS were perforated and further incubated with DOPC/N-Rh-PE (99:1) liposomes in the presence of an ATP-regenerating system. Extensive overlap of NBD fluorescence and Rh fluorescence indicated that the Golgi apparatus was the site in which (palmitoyl-C12-NBD)-PS was found.

To examine the behavior of fluorescent PS when incorporated into cells together with other lipid components, human skin fibroblasts were incubated with DOPC liposomes containing N-Rh-PE, a liposome marker (Pagano et al., 1981; Struck et al., 1981), and (palmitoyl-C12-NBD)-PS (Fig. 4). Fluorescent PS again accumulated in the Golgi apparatus (Fig. 4 a), whereas N-Rh-PE stained intra and/or extracellular vesicles (Fig. 4 b). The molar ratio of NBD-lipid/N-Rh-PE in the cell after incubation with liposomes was 33.8, whereas the ratio in liposomes was 6.8. This means that five times more PS than N-Rh-PE was taken up by the cells. The same experiment was done using CHO-K1 cells. The molar ratio of NBD-lipid/N-Rh-PE in CHO-K1 cells was 33.1. These results indicate that (palmitoyl-C12-NBD)-PS was segregated from the liposomes before internalization.

**Uptake of (Palmitoyl-C12-NBD)-PS by Mitotic Cells**

A number of vesicular processes including endocytosis are
inhibited during mitosis (Fawcett, 1965; Berlin et al., 1978; Berlin and Oliver, 1980; Warren, 1985; Kobayashi and Pagano, 1989). To examine the mechanism of uptake of (palmitoyl-C12-NBD)-PS, we followed the process in mitotic CHO cells. When interphase CHO cells were incubated with liposomes comprised of DOPC/(palmitoyl-C12-NBD)-PS/N-Rh-PE (77:20:3) for 30 min at 37°C, NBD fluorescence accumulated in the Golgi apparatus (Fig. 5 a). On the other hand, rhodamine fluorescence was observed as dots throughout the cells (Fig. 5 c). In a mitotic cell, NBD fluorescence was dispersed into the cytoplasm, indicative of fragmentation of the Golgi apparatus which occurs at this stage of the cell cycle (Warren, 1985; Lipsky and Pagano, 1985b). No significant rhodamine fluorescence was observed in mitotic cells. When mitotic cells were isolated by mitotic arrest using nocodazole, the same results were obtained (Fig. 5, b, d, and f). In Fig. 5 d, cell surface rhodamine fluorescence was observed in two cells, suggesting the adhesion of liposomes to the cell surface. Incorporation of (palmitoyl-C12-NBD)-PS to interphase and mitotic CHO cells was 3.21 ± 0.11 and 5.40 ± 1.27 p mol/μg DNA (mean of duplicate measurements ± deviations), respectively. Quantitation showed that out of 200 mitotic cells, 97% displayed NBD fluorescence while 98% of the cells showed no internalization of N-Rh-PE.

Effects of Various Treatments on the Delivery of (Palmitoyl-C12-NBD)-PS to the Golgi Apparatus

In Table I, effects of PS analogues on the transport of (palmitoyl-C12-NBD)-PS to the Golgi apparatus were examined. Incubation of cells with DOPC liposomes did not affect Golgi labeling, while the inclusion of 50 mol % B-PS in DOPC liposomes inhibited labeling of the Golgi apparatus. Inclusion of 50 mol % DOPE, 50 mol % P-PI or 50 mol % B-SM in DOPC vesicles had no effect on labeling of the
Golgi apparatus. Glycerophosphoserine and glycerophosphoethanolamine inhibited Golgi labeling at high concentrations (10 mM) but not at low concentrations (1 mM). Glycerophosphocholine and L-serine did not inhibit the transport of (palmitoyl-C12-NBD)-PS to the Golgi apparatus.

Next, we examined the effects of various other treatments on the labeling of the Golgi apparatus by (palmitoyl-C12-NBD)-PS. ATP depletion, NEM pretreatment, or prefixation of cells with glutaraldehyde inhibited the incorporation of fluorescent PS (Table II, Fig. 2 b; NEM and glutaraldehyde pretreatment gave similar results as in Fig. 2 b). Nocodazole and monensin did not inhibit the internalization of fluorescent PS but the intracellular distribution of the fluorescence in drug-treated cells was different from that of control cells. Monensin caused vacuolization of the fluorescence, whereas nocodazole treatment led to fragmentation of the fluorescent structure (not shown). These drugs are known to induce vacuolization and fragmentation of the Golgi apparatus (Tar-takoff, 1983; Allan and Kreis, 1986).

(Palmitoyl-C12-NBD)-PS Is Metabolized to PE during Incubation

When cells incubated with (palmitoyl-C12-NBD)-PS for 30 min were washed and further incubated in HMEM at 37°C, conversion of PS to PE occurred (Fig. 6). After 2 h of chase period, 14% of PS was metabolized to PE. This value corresponds well to the rate of turnover of endogenous PS (t1/2 = 7.5 h [Voelker, 1985]). No other fluorescent metabolites were observed during incubation. During the chase period, Golgi fluorescence decreased and filamentous structures throughout the cell were stained. These filamentous structures colocalized with the mitochondria marker, rhodamine B (Fig. 7), suggesting that the fluorescent lipid(s) had moved from the Golgi apparatus to the mitochondria. These data are consistent with the previous finding that PS decarboxylase is located in the inner mitochondrial membrane (Dennis and Kennedy, 1972; Van Golde et al., 1974). Further chase (>3 h) gave diffuse fluorescence throughout the cell, suggesting the transport of fluorescent PE and/or PS to the other membranes.

Discussion

In this study, we have examined the transport and metabolism of (palmitoyl-C12-NBD)-PS in cultured fibroblasts. Using (palmitoyl-C6-NBD)-PS, Martin and Pagano (1987) showed that this fluorescent PS analogue was spontaneously incorporated into the outer leaflet of the plasma membrane of cultured fibroblasts at low temperature. They also showed that (palmitoyl-C6-NBD)-PS was transported from the outer to the inner leaflet of the plasma membrane in an ATP-dependent manner. Once transported to the inner leaflet of the plasma membrane, (palmitoyl-C6-NBD)-PS diffused through the cytosol to label intracellular membranes. Our results, obtained with the "relatively nonexchangeable" fluorescent PS analogue, (palmitoyl-C12-NBD)-PS, differ from those obtained with the C6 derivatives in several respects. First, incorporation of (palmitoyl-C12-NBD)-PS did not occur at low temperature. Second, ATP depletion and NEM or glutaraldehyde treatment of cells inhibited the incorporation of fluorescent PS. Third, fluorescent PS was accumulated in the Golgi apparatus in an ATP-dependent manner. Our data support the idea that fluorescent PS is transported to the Golgi apparatus by three consecutive processes. They are: (a) insertion of (palmitoyl-C12-NBD)-PS into the outer leaflet of the plasma membrane; (b) translocation of fluorescent PS from the outer to the inner leaflet of the plasma membrane; and (c) transport of fluorescent PS from the inner leaflet of the plasma membrane to the Golgi apparatus via a nonvesicular pathway.

We have also examined the transport of several other exogenous C12-NBD-phospholipid analogues. The distribution of (palmitoyl-C12-NBD)-PC in human skin fibroblasts was different from that of (acyl-C6-NBD)-PC in V79 cells (Sleight and Pagano, 1984), which labeled the Golgi region. Recently it was shown that different cell types show different patterns of internalization of (palmitoyl-C6-NBD)-PC (Sleight and Abanto, 1990). When human skin fibroblasts were incubated with (palmitoyl-C6-NBD)-PC, a large number of fluorescently labeled intracellular vesicles with no labeling in the region of the Golgi apparatus appeared (data not shown). At present, the reason for the different staining patterns of C6- and C12-NBD-PC is not clear. The diffuse intracellular fluorescence in Fig. 2 c suggests the accumulation of fluorescence in the ER. When cells are incubated with C6-NBD-
Figure 5. Uptake of fluorescent PS occurs in mitotic cells. Interphase (a, c, and e) and mitotic (b, d, and f) cells were incubated with DOPC/N-Rh-PE/(palmitoyl-C_12-NBD)-PS (77:3:20 mol %) liposomes in the presence of Hoechst 33258 as described under Materials and Methods. Microscope optics allowed direct visualization of NBD- (a and b), rhodamine (c and d), and Hoechst dye (e and f) fluorescence in the same specimen. Mitotic cell in interphase specimen was shown by arrows. Bar, 10 μm.
Table I. Effects of Structural Analogues of Phosphatidylserine on Labeling of the Golgi Apparatus

| Structural analogues | Concentration | Golgi labeling |
|----------------------|---------------|----------------|
| DOPC                | 500 μM        | +              |
| DOPC/B-PS (1:1)     | 500 μM        | -              |
| DOPC/DOPE (1:1)     | 500 μM        | +              |
| DOPC/PI (1:1)       | 500 μM        | +              |
| DOPC/B-SM (1:1)     | 500 μM        | +              |
| GPS                 | 1 mM          | +              |
| GPE                 | 1 mM          | 10 mM          |
| GPC                 | 10 mM         | +              |
| L-Serine            | 40 mM         | +              |

Human skin fibroblasts on glass coverslips were washed three times with HMEM and incubated with each reagent at the indicated concentrations for 30 min at 2°C. Cells were then washed and incubated with liposomes containing (palmitoyl-C12-NBD)-PS for 30 min at 37°C in the presence of each reagent. The cells were washed and examined fluorescence in the presence of each reagent. GPS, glycerophosphoserine; GPE, glycerophosphoethanolamine; GPC, glycerophosphocholine.

Table II. Effects of Various Treatments on Labeling of the Golgi Apparatus

| Treatment       | Concentration | Pretreatment conditions | Present throughout experiment | Golgi labeling |
|-----------------|---------------|-------------------------|-------------------------------|---------------|
|                 |               | Temperature °C | Time min |                     |               |
| Sodium azide +  | 5 mM          | 37            | 30      | +                  | -             |
| 2-Deoxyglucose* | 50 mM         | 37            | 30      | -                  | -             |
| NEM*            | 0.5 mM        | 2             | 30      | -                  | -             |
| Glutaraldehyde§ | 0.5 %         | 22            | 30      | -                  | -             |
| Valinomycin     | 5 ng/ml       | 37            | 30      | +                  | +             |
| Monensin        | 5 μM          | 37            | 4 h     | +                  | +             |
| Cytochalasin B  | 5 μg/ml       | 37            | 30      | +                  | +             |
| Colchicine      | 1 μM          | 37            | 30      | +                  | +             |
| Nocodazole      | 10 μg/ml      | 37            | 90      | +                  | +             |
| NH4Cl           | 10 mM         | 37            | 30      | +                  | +             |
| Chloroquine     | 100 μM        | 37            | 30      | +                  | +             |
| Trypsin         | 25 μg/ml      | 2             | 30      | -                  | +             |

Human skin fibroblasts on glass coverslips were washed three times with HMEM and incubated with each reagent at the indicated concentration, temperature, and time. The cells were then washed and incubated with liposomes containing (palmitoyl-C12-NBD)-PS for 30 min at 37°C. The cells were washed and examined fluorescence in the presence of each reagent. Where indicated, the reagents were also present throughout the experiment (i.e., during incubation with liposomes and during the microscopic observation).

* ATP depletion was performed by pretreating cells with 5 mM NaN3 and 50 mM 2-deoxyglucose in glucose-free HMEM for 30 min at 37°C and carrying out all subsequent incubations and washes in glucose-free HMEM containing these inhibitors.

† NEM treatment was followed by a 5-min treatment on ice with 0.5 mM DTT in HMEM and washing with HMEM before incubation with vesicles containing (palmitoyl-C12-NBD)-PS.

§ Fixative contained 0.5% glutaraldehyde, 5% sucrose (wt/vol), and 0.1 M Pipes, pH 7.0.

Insertion of Fluorescent PS into the Outer Leaflet of the Plasma Membrane

When human skin fibroblasts were incubated with DOPC liposomes containing N-Rh-PE and (palmitoyl-C12-NBD)-PS, fluorescent PS accumulated in the Golgi apparatus, whereas N-Rh-PE stained intra and/or extracellular vesicles. Higher NBD-lipid/N-Rh-PE molar ratio in cells, compared to that in the added liposomes, suggests that (palmitoyl-C12-NBD)-PS was segregated from liposomes before internalization. Our results suggest that (palmitoyl-C12-NBD)-PS was not endocytosed with other liposome components, but was transported to the Golgi apparatus across the plasma membrane.

(Palmitoyl-C12-NBD)-PS could be inserted into the outer leaflet of the plasma membrane either by monomer diffusion or by decylation of PS to lyso compound, which is readily inserted into the plasma membrane. Since lyso-PS was not detected throughout the incubation, a decylation pathway seems unlikely. ATP depletion, NEM and glutaraldehyde pretreatment, and structural analogues of PS inhibited the incorporation of (palmitoyl-C12-NBD)-PS into the cells. They are all inhibitors of transmembrane movement of PS across the plasma membranes (see below). These results suggest that the insertion of (palmitoyl-C12-NBD)-PS to the outer leaflet of the plasma membrane.
Figure 7. Colocalization of intracellular NBD fluorescence with mitochondria. Human skin fibroblasts were incubated with vesicles containing (palmitoyl-C12-NBD)-PS for 30 min at 37°C, washed, and incubated at 37°C in HMEM for an additional 30 min. Cells were then washed and incubated with 2 μg/ml rhodamine B for 3 min at room temperature. a, NBD fluorescence; b, Rh fluorescence. Fluorescence of the Golgi region is seen in the left hand corner of a. Bar, 10 μm.

leaflet of the plasma membrane may be coupled to the internalization of PS.

It should be noted that (acyl-C6-NBD)-PS inserted into the erythrocyte membrane can serve as a signal for triggering their in vivo recognition and clearance from the circulation (Tanaka and Schroit, 1983; Schroit et al., 1984, 1985). Similar results were obtained using PS-containing liposomes (Allen et al., 1988). Recently it has been shown that PS is recognized by the scavenger receptors on the surface of macrophages (Nishikawa et al., 1990). Specific recognition of PS on the surface of cultured fibroblasts has not been reported. However, receptor-mediated incorporation of PS may explain the inhibition of the incorporation of (palmitoyl-C12-NBD)-PS by exogenous natural PS, which does not exchange into the plasma membrane as rapidly as fluorescent PS analogues.

Translocation of Fluorescent PS from the Outer to the Inner Leaflet of the Plasma Membrane

Recently, several laboratories have demonstrated the presence of an aminophospholipid translocator at the plasma membrane of erythrocytes and nucleated cells (for review see Devaux, 1988). In these studies, exogenously supplied radioactive phospholipids or fluorescent, photoaffinity, or spin-labeled analogues have been used. Once PS and PE are inserted into the outer leaflet of the plasma membrane, they are rapidly translocated to the inner leaflet of the membrane bilayer by a process that is ATP dependent and protein mediated. The chain length of the fatty acid moiety of the phospholipids seems not to affect transbilayer movement. Rapid transbilayer movement is not observed for exogenous PC and SM.

Transbilayer movement of PS and PE in cultured fibroblasts has been examined using (palmitoyl-C6-NBD)-PS and (palmitoyl-C12-NBD)-PE (Sleight and Pagano, 1985; Martin and Pagano, 1987). Transmembrane movement was inhibited by depletion of cellular ATP levels and was blocked by treatment with structural analogues of lipid or by pretreatment of cells with glutaraldehyde or NEM. Flip-flop of (palmitoyl-C6-NBD)-PS in the plasma membrane of V79 fibroblasts was much faster than that of (palmitoyl-C12-NBD)-PE. Once (palmitoyl-C12-NBD)-PS is incorporated into the outer leaflet of the plasma membrane of fibroblasts, it is probably translocated to the inner leaflet by an aminophospholipid translocator.

Nonvesicular Transport of Fluorescent PS from the Inner Leaflet of the Plasma Membrane to the Golgi Apparatus

The transport of fluorescent PS from the plasma membrane to the Golgi apparatus could occur by vesicle-mediated membrane traffic or by movement across the cytosol. The following observations suggest that a vesicle-dependent mechanism is unlikely. First, fluorescent PS accumulated in the Golgi apparatus at 18°C, while transport of cell surface protein to the Golgi apparatus is blocked at this temperature (Jin et al., 1989). Second, although a number of vesicular processes including endocytosis are inhibited during mitosis (Warren, 1985), intracellular uptake of (palmitoyl-C12-NBD)-PS proceeded at this stage of the cell cycle. In mitotic cells, NBD fluorescence was dispersed into the cytoplasm. Since the ER as well as the Golgi apparatus are fragmented during mitosis (Featherstone et al., 1985), we cannot exclude the possibility that (palmitoyl-C12-NBD)-PS is not accumulated in the Golgi apparatus in mitotic cells. However, our results strongly suggest that the internalization of fluorescent PS is not mediated by endocytosis. It is more likely therefore that transport occurs through the cytosol. Spontaneous diffusion as seems to occur for (palmitoyl-C6-NBD)-PS is unlikely because of the low water solubility of (palmitoyl-C12-NBD)-PS (Nichols and Pagano, 1982). It is possible that the transport of (palmitoyl-C12-NBD)-PS from the inner leaflet of the plasma membrane to the Golgi apparatus is the function of a lipid transfer protein. A number of proteins that facilitate the exchange of lipids between membranes in vitro have been described, and their involvement in the rapid movement of lipids in living cells has been suggested (Yaffe and Kennedy, 1983; Kobayashi and Pagano, 1989). Recently, it has been shown that a phosphatidylcholine/phosphatidylinositol transfer protein in yeast is essential for Golgi function (Bankaitis et al., 1990).

We are grateful to Dr. M. Isemura for providing the fluorescent microscope. We thank Drs. R. E. Pagano, M. Nishijima, R. G. Parton, and K. Simons for critical reading of the manuscript.
Kobayashi was supported by grant-in-aid (No. 01771983) for scientific research from the Ministry of Education, Science and Culture of Japan.

Received for publication 1 October 1990 and in revised form 17 December 1990.

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