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Rapid Report

Evidence for the activity of a phospholipid exchange protein in vivo

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Liposomes containing a self quenching concentration of a fluorescent phosphatidylethanolamine analog, were microinjected into Chinese hamster ovary cells. Immediately after microinjection, little intracellular fluorescence was observed. 10 min post-injection, labeling of the nuclear envelope and mitochondria became evident. Combined with control studies, our results suggest that phospholipid exchange protein(s) facilitates phosphatidylethanolamine movement in vivo.

Phospholipid exchange proteins (PLEPs) facilitate the transfer of lipid monomers between both artificial and natural membranes in vitro (see Refs. 1 and 2 for reviews). These cytoplasmic proteins have been purified from plants [3], animals [1] and prokaryotic organisms [4]. Helmkamp has suggested that PLEPs are likely to be present in every eukaryotic organism [5]. Their wide distribution in nature and activity in vitro have led most investigators to postulate that PLEPs play a critical role in the establishment and maintenance of organelle phospholipid composition. Although a great deal is known about the activity of PLEPs in vitro, their function in vivo remains obscure. Dowhan and coworkers have recently demonstrated that a phosphatidylinositol/phosphatidylcholine exchange protein in yeast is coded for by the *sec*14 gene [6]. This protein is essential for cell viability [7] and is known to play a role in stimulating Golgi secretory functions [8]. Mutations in the CDP-choline pathway for phosphatidylcholine biosynthesis result in phenotypic reversion of *sec*14 mutants [9]. Based on this, it has been proposed that the yeast PLEP functions in maintaining phospholipid equilibration in vivo [9]. However, it has not been demonstrated that phenotypic changes in *sec*14 mutants are caused by either alterations in lipid transport rates or lipid distribution in vivo.

The best evidence for a role of PLEPs in the transport of lipids in vivo comes from following the movement of radiolabeled lipids [10–12]. When cultured cells are fed radiolabeled lipid precursors such as choline and ethanolamine, the corresponding radiolabeled phospholipids rapidly appear in a variety of organelles. It has been suggested that the rapid appearance of the radiolabeled phospholipids in multiple organelles is inconsistent with vesicle-mediated transport, and that a more likely explanation of the data is that PLEPs transport newly synthesized phospholipids. Yaffe and Kennedy have suggested that this interpretation of the data may be incorrect since in vitro assays of PLEP activity toward specific phospholipids sometimes disagree with the observed transport of newly synthesized phospholipids in vivo [13]. To obtain an understanding of the biological significance of PLEPs, an in vivo assay for PLEP activity must first be developed. In this report we describe the use of a fluorescent phospholipid analog to demonstrate lipid transport consistent with PLEP activity in vivo.

We and others have used fluorescently labeled lipids to follow the transport of lipids in vivo [14–16]. Phospholipids having a fluorophore attached to one of their

Abbreviations: C_6-NBD-PC, 1-acyl-2-(6-[7-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidylcholine; CHO, Chinese hamster ovary; DOPC, dioleoylphosphatidylcholine; N-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine; nsPLEP, nonspecific phospholipid exchange protein; PLEPs, phospholipid exchange proteins; Rh-dextran, Rhodamine-labeled dextran.

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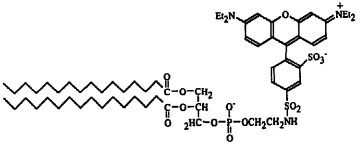

Fig. 1. Structure of *N*-(lissamine rhodamine B sulfonyl)-dipalmitoylphosphatidylethanolamine (N-Rh-PE). This compound was obtained from Avanti Polar Lipids, Pelham, AL and purified by preparative TLC before use.

fatty acid chains are excellent substrates for PLEP-catalyzed transport in vitro [17,18]. Typically, these fluorescent analogs transfer spontaneously between membranes at a rate that is dependent on the temperature, lipid composition of the donor and acceptor membranes, and the composition of the incubation medium [19]. The presence of a PLEP greatly increases the rate of acyl chain-labeled fluorescent phospholipid transfer [17]. For example, 1-palmitoyl, 2-C_6-NBD-PC spontaneously equilibrates between dioleoylphosphatidylcholine (DOPC) vesicles at a rate of 94.4%/min [17]. In the presence of 14 μg/ml bovine liver nonspecific phospholipid exchange protein (nsPLEP), the rate increases to 198%/min [17]. Because acyl chain-labeled fluorescent phospholipids have relatively high rates of spontaneous transfer between membranes, they are of limited use in investigating PLEP activity in vivo.

The structure of N-Rh-PE, a head group-labeled fluorescent analog of phosphatidylethanolamine, is presented in Fig. 1. Unlike the acyl chain-labeled phospholipids, this head group-labeled analog has a very slow spontaneous rate of exchange between membranes [17,20,21]. When cultured cells are incubated in the presence of liposomes containing acyl chain-labeled fluorescent lipid and N-Rh-PE, the acyl chain-labeled lipid rapidly inserts into the plasma membrane while the N-Rh-PE remains associated with the liposomes [15,16,20–22]. Over a period of several hours, the spontaneous movement of N-Rh-PE from liposomes into the plasma membrane does not occur to an extent that can be detected by fluorescence microscopy. Although the spontaneous transfer of N-Rh-PE is very slow, we have found that it is a substrate for bovine liver nonspecific phospholipid exchange protein (nsPLEP) (Fig. 2).

We hypothesized that if PLEPs functioned to transfer phospholipid monomers in vivo, N-Rh-PE would move into organelle membranes after microinjection of liposomes containing N-Rh-PE into cells. To allow for the visualization of this transfer, small unilamellar liposomes were prepared containing 25 mol% N-Rh-PE and 75 mol% DOPC. We determined that at this concentration, greater than 98% self quenching of N-Rh-PE occurs. Therefore, if lipid monomers of N-Rh-PE moved from microinjected liposomes to organelles, they would leave a highly quenching environment and become brightly fluorescent in the accepting organelle membrane.

Immediately after microinjection of the liposomes into Chinese hamster ovary (CHO) cells, the cells appeared dimly fluorescent throughout the cytosol. At this time the fluorescent lipid in the cells was impossible to photograph due to the low fluorescence intensity. As seen in Figs. 3A and B, after 10 min at room temperature, some intracellular organelles became fluorescently labeled. Most of the fluorescence appeared in mitochondria and the nuclear envelope. Although the result shown in Fig. 3 is consistent with PLEP-mediated transfer of N-Rh-PE monomers from liposomes to organellar membranes, other mechanisms would produce similar results. Possible alternative mechanisms are (i) fusion of liposomes with organelles, (ii) adsorption of liposomes with organelles and (iii) metabolism of N-Rh-PE to fluorescent compounds able to transfer spontaneously to organelles.

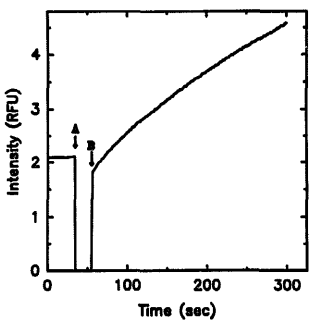

Fig. 2 PLEP-mediated transfer of N-Rh-PE between liposomes. Donor liposomes containing 25 mol% N-Rh-PE and 75 mol% dioleoylphosphatidylcholine (DOPC), and acceptor liposomes composed entirely of DOPC were prepared by ethanol injection [23]. The fluorescence of a solution containing 25 μM donors and 250 μM acceptors was determined (time 0). At arrow A, a shutter was closed to block light from the fluorescence detector and 75 μg of nsPLEP [24,25] was added. After mixing the sample, the shutter was opened and the change in rhodamine fluorescence recorded (arrow B). The increase in fluorescence intensity represents the movement of N-Rh-PE from the highly quenching environment of the donors to the nonquenching environment of the acceptors. When an identical experiment was performed substituting bovine serum albumin for the nsPLEP, no increase in fluorescence intensity was observed (not shown). Fluorescence measurements were obtained using a Specs 1681 0.22 spectrometer (Ex = 560 nm, Em = 590 nm, 1 nm slit widths).

To eliminate the first two possibilities, we microinjected liposomes containing rhodamine-labeled dextran (Rh-dextran) at a concentration of 35 μM. At this concentration we determined that the Rh-dextran was 95% self quenched. The large Rh-dextran molecules cannot pass through the liposomal membrane. Immediately after injection of liposomes, the cells were uniformly labeled throughout their cytoplasm. Although the fluorescence in these cells was dim, it was brighter than that observed after microinjection of N-Rh-PE

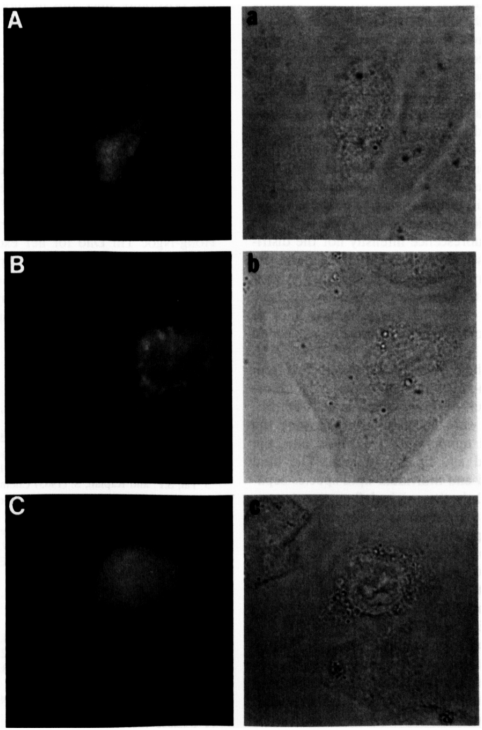

Fig. 3. Fluorescence (upper case) and phase contrast (lower case) photomicrographs of CHO cells. The photomicrographs labeled A, a, B and b were taken 10 min after microinjection of small unilamellar liposomes composed of 25 mol% N-Rh-PE and 75 mol% DOPC. Liposomes were prepared by ethanol injection as described by Kremer et al. [23] except that injection buffer was used as an aqueous solvent. The injection buffer consisted of 140 mM KCl, 10 mM Hepes, 10 mM glucose, 5 mM NaCl, 2 mM $MgCl_2$, 1.1 mM EGTA, (pH 7.0), 316 mOsm). Immediately before microinjection, the liposome preparation was centrifuged at 100 000 $\times$ g_{av} in a Beckman Instruments Airfuge for 5 min to remove any aggregates. Photomicrographs labeled C and c are of a CHO cell microinjected with small unilamellar liposomes containing entrapped 35 μM Rh-dextran. The Rh-dextran was purchased from Sigma Chemicals, St. Louis, MO and dialyzed for several days before entrapment in lipid vesicles to remove uncoupled rhodamine. The liposome preparation was fractionated by gel filtration chromatography (Sepharose 6B-100) to remove unencapsulated Rh-dextran before centrifugation.

labeled liposomes and could be photographed (Fig. 3c). Our ability to visualize liposomes loaded with Rh-dextran is probably due to the lower self quenching of the Rh-dextran compared to N-Rh-PE labeled vesicles. Several pieces of evidence suggested that liposomes loaded with Rh-dextran did not become leaky after microinjection into the CHO cells. (i) Liposomes injected outside the cells initially appeared as a bolus and eventually diffused throughout the bathing medium. The initial bolus of liposomes outside the cells appeared to have the same fluorescence intensity as liposomes injected into the cells. (ii) After adding Triton X-100 to the medium bathing cells microinjected with liposomes encapsulating self quenched Rh-dextran, a dramatic increase in rhodamine fluorescence was observed. This fluorescence quickly decreased as free Rh-dextran diffused into the bathing medium. (iii) Microinjection of 50 fold less free Rh-dextran (as compared to the amount encapsulated in liposomes) resulted in brilliant labeling of the cells.

After microinjection of liposomes containing Rh-dextran, neither the fluorescence intensity nor the distribution of the fluorescence in the cells changed over 30 min at room temperature. This suggests that the vesicles neither fused to organelles, which would have brightly labeled the organelles due to dequenching of the fluorophore, nor became specifically associated with organelles.

To determine if the movement of fluorescence from microinjected N-Rh-PE containing liposomes to organelles was the result of the metabolism of N-Rh-PE, 896 individual cells were injected. After allowing the cells to sit at room temperature for 20–30 min, the cells were harvested and lipids extracted. The lipid extracts were pooled and individual lipid species separated by HPTLC. Only the original fluorescent compound was found in the pooled lipid extract, N-Rh-PE. No fluorescent compounds were present in the aqueous phase of the lipid extract. Therefore, the transfer of fluorescence from microinjected N-Rh-PE containing liposomes cannot be explained by the production of new fluorescent compounds.

Why is N-Rh-PE preferentially transferred to the nuclear envelope and mitochondria, as shown in Fig. 3? Although the answer to this question remains obscure, this finding is not unprecedented. When liposomes, containing fluorescently labeled lipids that are able to transfer spontaneously between membranes (i.e., acyl chain-labeled lipids) are microinjected into cultured cells, they accumulate preferentially in mitochondria and the nuclear envelope [15]. Since these lipids move spontaneously one may have expected them to distribute evenly into all membranes. However, as mentioned above, the acyl chain-labeled phospholipid analogs are excellent substrates for PLEPs in vitro. Therefore, it is possible that cellular PLEPs direct and concentrate both N-Rh-PE and the acyl chain-labeled phospholipids to the mitochondria and nuclear envelope.

We have demonstrated that microinjected N-Rh-PE transfers from microinjected liposomes to cellular organelles in a manner consistent with PLEP activity. We believe that similar results can be obtained after fusing labeled liposomes to cells. This is a particularly attractive possibility since this method should allow a large number of cells to become labeled. Because cells labeled with fluorescent lipid analogs are significantly more sensitive to high intensity light cytotoxicity as compared to unlabeled cells (unpublished observation), the use of fluorescent lipid analogs may provide a means for the selection of PLEP-defective mutants in higher eukaryotes. Thus, the use of fluorescent lipid analogs may finally allow the function and biological significance of PLEPs to be determined.We thank Dr. J. Wylie Nichols, Emory University, for providing a partially purified preparation of nsPLEP that permitted us to perform pilot experiments. We also thank Hong-Ngoc Thi Dao for her excellent technical assistance in isolating the nsPLEP. Special thanks are given to Dr. Raymund Pun, Department of Physiology and Biophysics, for use of his microinjection equipment and for discussions. This work was supported by NIH Grant GM39035 to R.G.S.

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