Importance of Phosphatidylethanolamine for the Interaction of Apocytochrome *c* with Model Membranes Containing Phosphatidylserine

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ABSTRACT: The effect of phosphatidylethanolamine (PE) on the binding of apocytochrome *c* to model membranes was examined. When 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) of the standard vesicles composed of 80% of this lipid and 20% of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) was gradually replaced with upward of 50% of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), the binding increased appreciably. Ca^{2+}, causing the phase separation of PS, also brought about increased binding of apocytochrome *c* in the PC/PS system, underlining the importance of PS properties in membranes for the protein binding. The resonance energy transfer between Trp-59 in apocytochrome *c* and pyrene-PS incorporated into bilayers showed that the replacement of PC with PE increased the extent of apocytochrome *c* penetration into membranes by a PE concentration-dependent manner. However, in the absence of PS, PE had no apparent effect on these functions of apocytochrome *c*, suggesting that PE-induced change(s) of acidic membrane properties is important to the association of apocytochrome *c* with vesicles. From the observations that the excimer to monomer fluorescence ratio of pyrene-PS increased and the fluorescence of NBD-PS was quenched with increasing concentration of PE, it was deduced that PE caused PS-enriched domains in PC/PE/PS membranes. The colocalization of pyrene-PS with BODIPY-PS by PE further supported the possibility. We suggest that PE-induced formation of PS-enriched domains acts as binding sites for apocytochrome *c* in membranes.

The majority of mitochondrial proteins are synthesized in the cytoplasm and translocated across the membranes to their compartments after translation is completed. A large number of proteineous components, participating in the translocation, are required, depending on the final destination of the mitochondrial proteins. An exception to the complicated mechanism is the translocation of apocytochrome *c*, the heme-free precursor of cytochrome *c*, into mitochondria. The import does not require a cleavable amino-terminal signal peptide, the general protein translocation machinery, and protease-sensitive components of the outer membrane. Neither membrane potential nor ATP is involved in the translocation (*1*, *2*). Instead, apocytochrome *c* can associate specifically with mitochondrial outer membrane and insert spontaneously into the membrane. It is recognized and bound to cytochrome *c* heme lyase (CCHL)[1] in a complex, making the translocation unidirectional (*3*, *4*). Translocation into the intermembrane space is believed to be driven by the refolding of the polypeptide as a result of the CCHL-catalyzed covalent attachment of heme (*5*).

Because of the relatively simple mechanism for the translocation of apocytochrome *c*, extensive studies have been done with model membrane systems. An interesting observation was that apocytochrome *c* binds to negatively charged phospholipids such as PS, PI, PG, and cardiolipin with high affinity (*6*−*8*). It has been shown that these acidic lipids promote the binding, insertion, and concomitant translocation event of apocytochrome *c* into membranes. However, these studies have used a single acidic lipid or binary mixture containing negatively charged phospholipid and PC as model membranes. Therefore, it is not available how it can be related between the regulation of membrane dynamics of acidic phospholipids by other lipid components and the translocation. Moreover, very little is known what the role of acidic phospholipids is in the import of apocytochrome *c* into membranes.

PC is the most abundant glycerophospholipid in all mammalian membranes. PE is the second most abundant phospholipid of cell membranes and is especially enriched in the mitochondria. PE accounts for >20% of outer membrane lipid composition of mitochondria in most of the cell types. PS is a minor lipid component of cells, with the exception of brain tissue, where it can amount to 15% of the total lipid (*9*). It is known that mitochondrial outer membranes have a high PE content (*10*) and that PE and PS

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[1] Abbreviations: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; NBD-PS, 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzodiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phophoserine; pyrene-PS, 1-palmitoyl-2-(pyrenedecanoyl)-*sn*-glycero-3-phosphoserine; BODIPY-PS, 2-(4,4-difluoro-5-methyl-4-boro-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CCHL, cytochrome *c* heme lyase; GdnHCl, guanidine hydrochloride; DTT, dithiothreitol; LUVs, large unilamellar vesicles.

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are distributed primarily on the cytoplasmic side of the membranes (*11*). Recently, we have shown that the mixing properties of PS in the PC matrix are not random but that phase separation of domain formation occurs in the liquid-crystalline phase of membranes (*12*). In addition, PS has been shown to have a high affinity to apocytochrome *c* (*6*−*8*). Therefore, we used PS as a model anionic phospholipid to study the effect of PE on the interaction of apocytochrome *c* to membranes in the PC/PS system.

In this study, we propose that PE, a major phospholipid of mitochondrial outer membrane, induces the lateral segregation of PS, an acidic phospholipid, and that the induced PS domain promotes the binding and insertion of apocytochrome *c* into the lipid bilayer.

MATERIALS AND METHODS

Materials. Horse heart cytochrome *c* (type VI) was obtained from Sigma (St. Louis, MO). All phospholipids and NBD-PS were purchased from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. Pyrene-PS and BODIPY-PS were synthesized from pyrene-PC as described (*13*). Chloroform solutions of lipids were stored in sealed ampules under argon at −20 °C. Other chemicals were of the highest grade commercially available.

Apocytochrome c Preparation. Apocytochrome *c* was prepared from purified cytochrome *c* as previously described (*14*) and was subjected to a renaturation procedure as described (*15*). The apocytochrome *c* showed one single band on SDS−polyacrylamide gel electrophoresis. The protein was stored at −70 °C in 25 mM Mes (pH 6.5) buffer containing 50 mM NaCl and 1 mM DTT until used. In all experiments, we used this buffer condition unless described otherwise.

Liposome Preparation. Most liposomes were prepared using the extrusion method with 100 nm pore size polycarbonate membrane (*16*). Vesicles of mixed lipid composition of POPC/POPS (80:20, by molar ratio) were used as a standard liposome throughout these investigations. The mole concentration of POPS was fixed at 20%, but the initial 80% of POPC in the standard vesicles was varied by replacing it with upward of 50% of POPE. To prepare vesicles containing extrinsic fluorophores such as pyrene-, BODIPY-, or NBD-labeled phospholipids, 1 mol % of pyrene-PS, 1 mol % of BODIPY-PS, or 10 mol % of NBD-PS was incorporated into liposomes instead of normal PS. The concentrations of nonfluorescent phospholipids were determined by a phosphorus assay (*17*). The concentrations of fluorescent probes were determined spectrophotometrically at 342 nm using 42 000 cm^{-1} for pyrene-PS and 80 000 cm^{-1} for BODIPY-PS and that of NBD-PS at 465 nm using 22 000 cm^{-1} as the molar extinction coefficient, respectively.

Fluorescence Measurements. Fluorescence was measured with a Shimadzu RF-5301 PC spectrofluorometer equipped with a thermostated cuvette compartment maintained at 35 °C. Emission spectra of the Trp residue in apocytochrome *c* were recorded in the range of 320−370 nm with the excitation wavelength of 295 nm. For the measurement of the excimer (E) and the monomer (M) of pyrene-containing liposomes, the excitation wavelength was 342 nm and the emission wavelength was 360−500 nm, respectively. The E/M ratio of the pyrene-labeled PS was calculated by measuring fluorescence intensity at 375 nm (for monomer) and 480 nm (for excimer). In all fluorescence experiments, each measurement under the experimental condition was corrected for inner filter effect due to light scattering and absorption, as described elsewhere (*18*).

Apocytochrome c Binding into Model Membranes. The binding of apocytochrome *c* to lipid bilayers was characterized by measuring an increase of single Trp fluorescence in apocytochrome *c* and a blue shift of maximal emission intensity as demonstrated previously (*19*).

The amount of apocytochrome *c* bound to membranes was measured using the precipitation method using biotinylated PC and immobilized avidin (*16*). Briefly, 14 μg of apocytochrome *c* in 150 μL of buffer was incubated with liposomes to an L/P ratio of 100 and further incubated for 10 min at 35 °C. After addition of immobilized avidin (10 μL of a 7.5 mg/mL suspension) and incubation for 10 min, the liposomes were precipitated by microcentrifugation for 5 min at 14 000 rpm. The supernatant was assayed for the protein concentration. The membrane-bound apocytochrome *c* was separated from the immobilized avidin−liposome complex, and its concentration was also measured. As a control experiment, we measured the concentration of phospholipid left in the supernatant by phosphrous assay (*17*) after precipitating vesicles in the absence of apocytochrome *c*. Less than 3 mol % phospholipid of total added phospholipids remained in the supernatant. This indicates that nearly all liposomes can be precipitated under the experimental condition.

The effect of phase separation of PS induced by $CaCl_2$ on the binding was also investigated using Trp fluorescence and the precipitation method. In all experiments concerning the interaction of apoprotein with model membranes, the stock solution of apocytochrome *c* dissolved in 3 M GdnHCl was used and rapidly diluted 50-fold in the presence of vesicle solution.

Protein concentrations were determined using bicinchoninic acid according to the manufacturer's instruction (Sigma).

Penetration of Apocytochrome c into Model Membranes. The penetration of apocytochrome *c* into membranes was measured by the energy transfer between Trp residues of apocytochrome *c* and pyrene-PS (the pyrene group is located at the end of *sn*-2 position of PS) incorporated into lipid bilayers as described (*20*). The reaction samples were incubated for 10 min at 35 °C, and then the fluorescence of the Trp residue at 340 nm or the pyrene intensity at 375 nm (342 nm of excitation wavelength) was measured to see the extent of the energy transfer between the fluorescence of Trp residues and pyrene probes.

Phase Properties of Lipids. When the excimer (E) to monomer (M) fluorescence ratio (E/M) of pyrene-PS was determined, the excitation wavelength was 342 nm and the emission wavelength was in the range of 360−500 nm. The fluorescence intensities at 375 nm (for monomer) and 480 nm (for excimer) were selected to calculate the E/M ratio. The emission fluorescence of NBD-PS was measured at 534 nm with an excitation wavelength of 465 nm. To determine the colocalization of fluorescent probes, the excimer fluorescence intensities of pyrene-PS were measured in the presence and absence of BODIPY-PS. To prevent the excimer fluorescence quenching effect of oxygen, the buffer solution was saturated with argon gas for more than 2 h before use. In all experiments measuring phase properties

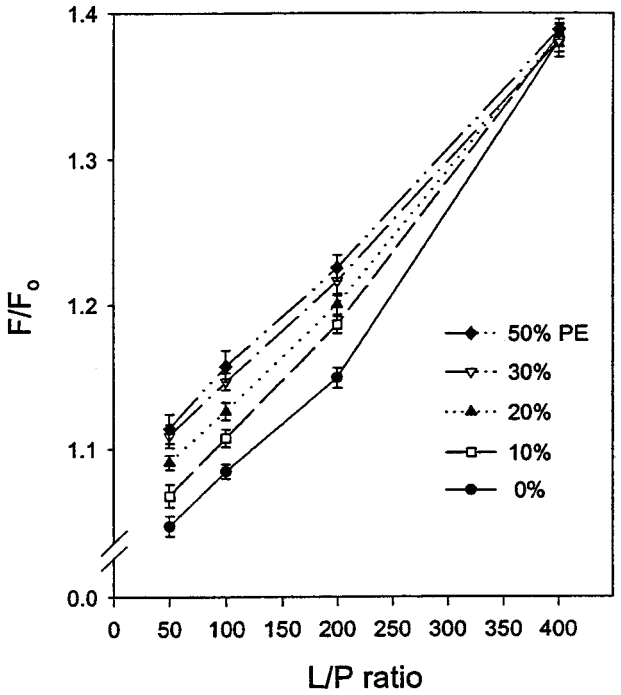

FIGURE 1: Titration of Trp fluorescence of apocytochrome *c* (3 μM, 35 °C) by model membranes. Maximal emission fluorescence of Trp was monitored by adding liposomes with different content of PE in the PC/PE/PS system. F_0 and F represent the emission intensities in the absence (F_0) or presence (F) of liposomes, respectively. Data points represent mean ± SE of five independent experiments.

of lipids, 5 μM phospholipid in 25 mM Mes (pH 6.5) buffer containing 50 mM NaCl and 1 mM NaEDTA was used, and the fluorescence intensities were monitored at 35 °C.

RESULTS

Effect of PE on the Binding of Apocytochrome c to PC/PE/PS Membranes. To examine the effect of PE on the interaction of apocytochrome *c* to model membranes, we first monitored the fluorescence change of the Trp residue in apocytochrome *c* with increasing concentration of phospholipid vesicles containing different mole fractions of PE in the PC/PE/PS system. Apocytochrome *c* has only one Trp residue (59th amino acid), and this has been used as a chromophore for the spectroscopic studies. Figure 1 shows a titration of Trp fluorescence of apocytochrome *c* by vesicles. The Trp fluorescence increased with increasing concentration of phospholipid (as shown by an increase of F/F_0) and converged to the same value at around 400 of lipid/protein (L/P) ratio regardless of the presence of PE in vesicles containing a fixed amount of PS. However, PE-containing liposomes showed higher intensities of Trp fluorescence at a range of $\leq$200 of L/P ratio than the value of without PE with a PE concentration-dependent manner.

To confirm this result, we quantified the amount of the protein bound to liposomes that initially contained 80% of PC and 20% of PS by replacing PC with upward of 50% of PE at a fixed L/P ratio of 100. As shown in Figure 2A, the binding was increased as a function of PE content, and at >20% of PE the amount of apocytochrome *c* bound to membranes was enhanced by about 50% when compared with the binding with standard vesicles as revealed by the precipitation method. However, in the absence of PS, only about 10% of apocytochrome *c* was bound to 100% PC membranes, and more interestingly, replacement of PC with PE had very little effect on the increase of apocytochrome *c* binding to vesicles.

To obtain more insight into the relation between membrane property and apocytochrome *c* binding, we measured the amount of apocytochrome *c* bound to standard vesicles in the presence of $CaCl_2$. As shown in Figure 2B, the protein binding was enhanced by increasing Ca^{2+} concentration. However, in the absence of PS, Ca^{2+} had no apparent effect (<2%) on the protein binding to the PC/PE (1:1) system.

We also measured the possible structural changes of apocytochrome *c* without liposomes by stepwise increasing the concentration of $CaCl_2$. When we used a spectropolarimeter for circular dichroism and a spectrofluorometer for Trp fluorescence, any Ca^{2+}-induced conformational change of the protein was not observed. Also we could not detect Ca^{2+}-induced fusion of standard vesicles and liposomes containing PE when assayed by the lipid mixing as described (*7*) (results not shown).

Incorporation of Apocytochrome c into Lipid Bilayers. The effect of PE on the insertion of apocytochrome *c* into lipid bilayers was tested with energy transfer between the Trp and pyrene group of pyrene-PS with the same method described (*20*). The emission of Trp fluorescence and the excitation of pyrene show strong spectral overlap. Because the pyrene group is attached to the end of the decanoyl chain at the *sn*-2 position of PS, it is possible to use the energy transfer between these two fluorophores to determine the extent of apocytochrome *c* insertion into lipid bilayers. The extent of energy transfer increased with increasing concentration of PE in the membranes, as shown by the decreasing ratio of F/F_0, which represents the fluorescence intensity ratio for the sample with (F) and without (F_0) pyrene-PS at 340 nm of emission wavelength (Figure 3). However, in the absence of PS, PE had no apparent effect on the F/F_0 ratio.

Formation of the PS-Enriched Domain Induced by PE. From the results described above, we expected that increases of apocytochrome *c* binding and insertion into membranes are caused by PE-induced changes of membrane property in the PC/PE/PS system, but those effects are remarkably diminished in the absence of PS. To test this notion, we used the fluorescence of pyrene-PS incorporated into membranes. Excited-state pyrene molecules give two characteristic emission fluorescence spectra for the monomer (M) and excimer (E), respectively. The E/M ratio is strictly dependent on the rate of collision of the pyrene molecules. Consequently, in the lipid bilayer, pyrene-labeled lipids reflect the lateral diffusion rate of the probes (*22*) or their local concentration (*23*) or both. To determine the effect of PE on lipid dynamics of PS, 1 mol % of pyrene-PS was incorporated into a standard vesicle, and the E/M ratio was examined by replacing PC with PE. When the concentration of PE increased to 50%, the E/M ratio was enhanced by about 31% compared to the value for a standard liposomes (Figure 4).

To corroborate the possibility that PE molecules cause the lateral segregation of pyrene-PS, we utilized the self-quenching of the fluorescence of NBD-labeled phospholipid (*24*) providing the information on phospholipid clustering in lipid bilayers. Figure 5 shows that the quenching efficiency was enhanced as PE concentration increased. At 50% of PE, the fluorescence of NBD-PS decreased by about 22% as

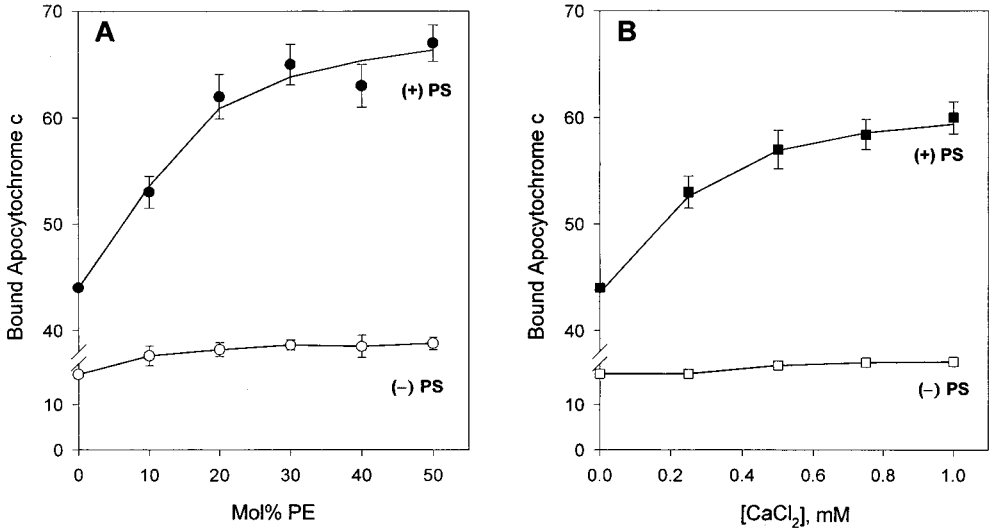

FIGURE 2: Binding of apocytochrome *c* to large unilamellar vesicles composed of mixtures of PC/PS, PC/PE, or PC/PE/PS. Binding was determined by replacing PC in standard vesicles with PE (A, ●) or by increasing the concentration of $CaCl_2$ outside the standard vesicles (B, ■). Open circles and open squares represent the association of apoprotein to membranes without PS by increasing the concentration of PE (A) or by increasing the concentration of $CaCl_2$ at 50% of PE (B), respectively. For other details, see Materials and Methods. Data points represent mean ± SE of three independent experiments.

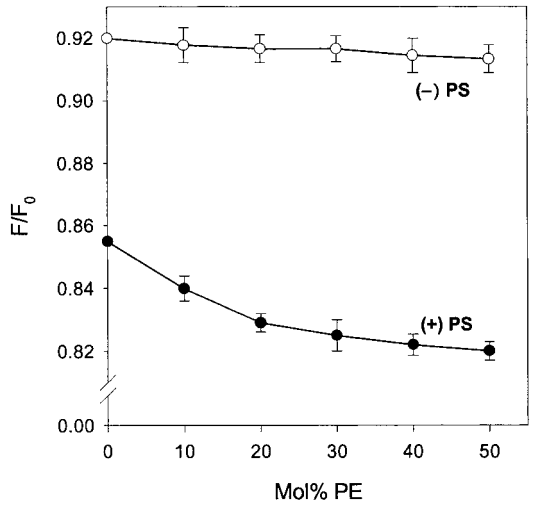

FIGURE 3: PE-dependent insertion of apocytochrome *c* into membranes. The energy transfer between Trp in the protein and pyrene-PS was examined by replacing PC with PE in the presence (●) or absence (○) of PS. Data points represent mean ± SE of three independent experiments.

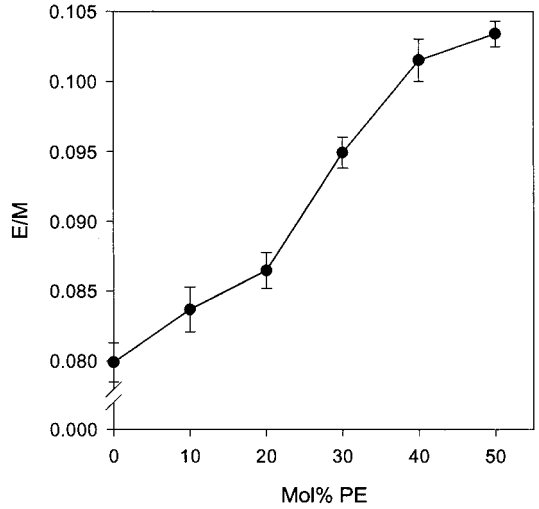

FIGURE 4: Effect of PE concentration in membranes on the E/M ratio of pyrene-PS. The monomer (M) and excimer (E) fluorescence of pyrene-PS (1%) incorporated into liposomes was examined by replacing PC with PE. Data points represent mean ± SE of three independent experiments.

compared to the value without PE. This result provides evidence that PE molecules induce the lateral separation of PS in membranes.

To ascertain that phase separation of PS in PC/PE/PS mixtures is responsible for the increase in the E/M ratio of pyrene-PS and the fluorescence quenching of NBD-PS, we utilized resonance energy transfer between pyrene-PS and BODIPY-PS. As the dipyrromethene boron difluoride group of BODIPY shows a maximum absorption spectrum at around 500 nm (*25*), it was possible to use the energy transfer between two fluorophores to estimate their colocalization as demonstrated before (*12*). We determined the quenching efficiency (F/F_0) as a function of PE concentration, where F and F_0 are the intensities of excimer emission of pyrene-PS measured in the presence (F) and absence (F_o) of the quencher, BODIPY-PS. When the concentration of PE was increased, the quenching was gradually enhanced (Figure 6).

DISCUSSION

It has been demonstrated that negatively charged phospholipids play an important role in the translocation process of apocytochrome *c* into mitochondrial outer membrane (*7*, *8*). Especially, with the model membrane system, it was observed that apocytochrome *c* binds with high affinity (for example, $K_d = 0.017$ μM for dioleoylphosphatidylserine) to acidic phospholipids (*26*). It has also been known that a signal peptide and other proteineous components are not necessary for the import of apocytochrome *c* into mitochondria. The anionic lipids might be important to provide a proper environment for the translocation of apocytochrome *c*. This suggestion is supported by the observation that only with higher concentrations of acidic phospholipids (e.g., 30−50% of PS) than that of mitochondrial outer membrane in vivo, apocytochrome *c* can translocate the lipid bilayers (*7*). But it has remained as a puzzle why model membrane

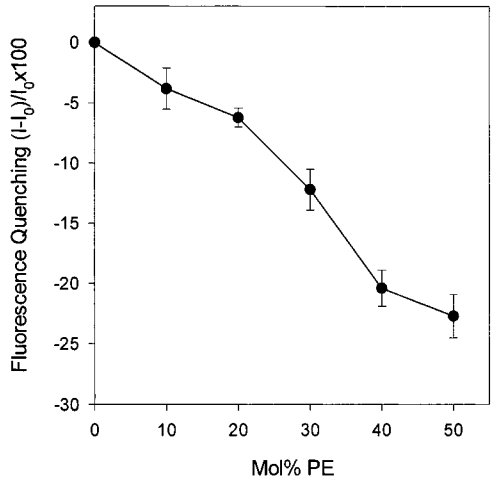

FIGURE 5: Quenching of NBD-PS fluorescence by PE. The initial composition of vesicles (PC:PS:NBD-PS = 80:10:10) was gradually changed by replacing PC with the indicated amount of PE. I and I_0 are the fluorescence intensities at 534 nm in the absence (I_0) or in the presence (I) of PE. Data points represent mean ± SE of three independent experiments.

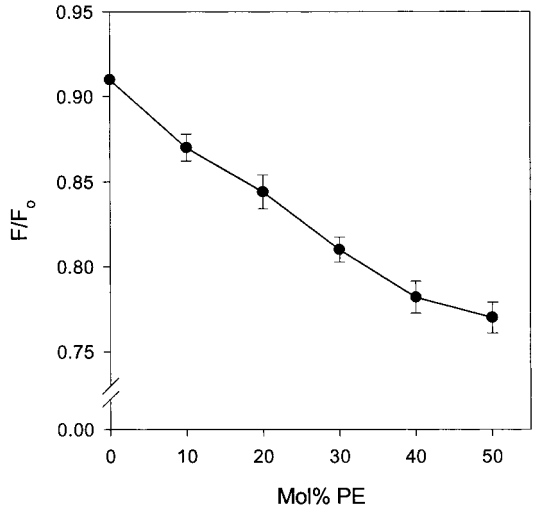

FIGURE 6: Quenching of excimer fluorescence of pyrene-PS by BODIPY-PS with increasing concentration of PE. F/F_0 is the ratio of the fluorescence intensities at 480 nm for LUVs containing only 1 mol % pyrene-PS (F_0) or 1 mol % pyrene-PS and 1 mol % BODIPY-PS (F). Data points represent mean ± SE of three independent experiments.

systems require higher amounts of PS (and acidic phospholipids) for the efficient translocation of the apoprotein compared with that of mitochondrial outer membrane.

PE-containing liposomes showed higher intensities of Trp fluorescence than the value of without PE (Figure 1), suggesting that the binding extent of apocytochrome *c* to membranes is related to the PE content in acidic liposomes. The fluorescence change of the Trp residue in apocytochrome *c* seems to be small upon interaction with model membranes containing various content of PE. However, the differences in F/F_0 ratio shown in Figure 1 are in agreement with the previous data presented by Berkhout et al. (*19*). They found that only about 0.1 of $\Delta F/F_0$ resulted from the titration of Trp fluorescence with pure PS and PS/PC (7:3, mol/mol) or with PS/PC (7:3, mol/mol) and PS/PC (1:1). But a large difference was observed at dissociation constants (K_d): 0.04, 0.19, and 0.25 μM for 100% PS, PS/PC (7:3), and PS/PC (1:1), respectively. Therefore, we consider that small changes of Trp fluorescence are valuable and sufficient to estimate the PE effect on the interaction of apocytochrome *c* to membranes. Furthermore, to obtain more insight into the association of apocytochrome *c* with liposomes, we performed the direct binding assay of apocytochrome *c* to membranes (Figure 2). Significant differences of the membrane-bound amount of apocytochrome *c* were observed upon increasing the mole percent of PE in vesicles. These results indicate that PS, an acidic phospholipid, results in the efficient binding of apocytochrome *c* to lipid bilayers, which agrees well with the previous observations (*26*), and PE stimulates the binding effectively only in the presence of PS.

Binding of apocytochrome *c* to membranes was enhanced by increasing Ca^{2+} concentration in the presence of PS (Figure 2B). It has been established that Ca^{2+} causes the lateral formation of the PS domain in membranes (*21*). Ca^{2+} could induce domain formation of PS and cause charge screening. Charge screening reduces the electrostatic interaction between apocytochrome *c* and PS. A possible hypothesis is that simultaneous interaction between all three components (apocytochrome *c*, phospholipids, and Ca^{2+}) can take place at interfacial binding/insertion sites. In this situation, apocytochrome *c*, a basic protein that has nine positively charged amino acids at neutral pH, can bind to the limited number of PS molecules in the spontaneously formed microdomain of PC/PS membranes without Ca^{2+} (*12*). After binding to membranes, apocytochrome *c* should collect a stoichiometric number of negatively charged phospholipid molecules around itself to maintain the efficient membrane-bound state. It was suggested that one apocytochrome *c* molecule binds to the 8−11 of PS molecules (*7*). We propose that Ca^{2+} facilitates this process by causing a lateral redistribution of PS in the PC matrix. Currently, this model is just one of the various possibilities to explain the role of Ca^{2+} in stimulating the binding of apocytochrome *c* to vesicles.

We demonstrate that the replacement of PC with PE enhances the binding and insertion of apocytochrome *c* to model membranes originally consisting of PC/PS (Figures 2 and 3). From the observations that the excimer to monomer fluorescence ratio of pyrene-PS increased and the fluorescence of NBD-PS was quenched with increasing concentration of PE (Figures 4 and 5), it can be suggested that PE causes PS-enriched domains (lateral segregation or exclusion of PS from other phospholipids) in PC/PE/PS membranes. The colocalization of pyrene-PS with BODIPY-PS by PE further supports the possibility (Figure 6). The PE-induced phase separation of PS might serve as binding sites for apocytochrome *c* and promote insertion and concomitant import event in the end. Therefore, it can be deduced that, despite the lower concentration of PS in mitochondrial outer membrane, apocytochrome *c* can be translocated across the lipid bilayers. Our previous report, which suggests that the phase separation of acidic phospholipids can occur spontaneously in the PC matrix in a concentration- and headgroup-dependent manner (*12*), also might explain the requirement of relatively high PS content for passing of apocytochrome *c* across model membranes. However, our results indicate that, in the absence of PS, PE itself or PE-induced change of membrane dynamics does not have a significant effect on the interaction of apocytochrome *c* with membranes. In related studies, it has been shown that PE is important for

the association of protein kinase C and other cytoplasmic proteins with model membranes containing PS as an acidic phospholipid in the presence of Ca^{2+} (*27*) and PE molecules restrict the dissipation of clustered phospholipids such as phosphatidic acid in membranes (*28*).

Clearly the explanations of phase separation given above are not the only explanations for the PE effect of membrane binding and insertion of apocytochrome *c*. Other possible effects of PE have been suggested in relation to membrane incorporation of protein (*29*, *30*). The membrane binding/insertion of alamethicin, a voltage-gated channel-forming peptide, decreases with increasing mole fraction of PE in the PC/PE system (*29*, *30*). These results were largely explained as a PE effect producing the difference in headgroup size between PE and PC and the spontaneous curvature, which is a stress within the bilayer resulting from the presence of PE preferring inverted phases such as inverted hexagonal phases. From the former reason, membranes containing PE prefer to accommodate the peptide adsorption in the headgroup region in lieu of hydrocarbon regions due to the smaller headgroup size of PE than PC. The latter focuses on the idea that membrane curvature stress is a source of the PE-induced difference in binding free energy necessary to expand the bilayer upon peptide insertion. Both effects of headgroup size and curvature seem to be a result of a localized thinning of the bilayer promoted by the peptide. However, as the binding and insertion of apocytochrome *c* to the membrane were enhanced only in the presence of PS (Figures 2 and 3), a more likely effect of PE on PS is the change of acidic membrane properties induced by PE. The headgroup size and curvature of PE seem not to be the explanations for the PE effects on the binding and insertion of apocytochrome *c* to the membrane. Even more, in the case of alamethicin, binary components containing PC and PE were used (*29*, *30*), and therefore it would not be easily predictable whether PE will provide the same effect on membrane properties in PC/PE and PC/PE/anionic phospholipid (such as PS) systems. More detailed studies of PE-producing change of membrane properties in the ternary system will be needed to fully account for the PE effects.

Besides the PE-induced lateral clustering of PS, the difference of headgroup size, and spontaneous curvature of PE, we should also consider the PE propensity of forming nonbilayer structure, which is also related to the spontaneous curvature of PE (*31*). Models about the involvement of nonbilayer lipid structures in the translocation of proteins through the membrane have been suggested (*32*, *33*). Taken together, the following process concerning the passage of apocytochrome *c* across membranes could be suggested as the most probable mechanism. The first step will be the binding of apoprotein to negatively charged phospholipids and/or their domains on membrane surface. After binding, the insertion of apocytochrome *c* into the lipid bilayer takes place, followed by extensive hydrophobic interaction between the protein and lipids which is promoted by PE. This hydrophobic interaction could cause deeper penetration of the apocytochrome *c* into bilayers and eventually translocation across membranes.

As we suggested previously (*12*), the mixing property of PS in the PC matrix is not random. The phase separation occurs in the liquid-crystalline phase in a PS concentration-dependent manner. From this result, it can be hypothesized that at even low content of PS in PC/PS membranes (for example, 20% of PS) the PS-enriched domains are formed spontaneously. However, the relative portion of the PS clustering is too small to be detected. We suggest that this kind of microdomain of PS can act as a binding site for the binding/insertion of apocytochrome *c* upon interacting with membranes. At low L/P ratio ($\leq$200), the absolute number of binding sites for apocytochrome *c* would be lower in the PC/PS system than that of PC/PE/PS membranes because of PE-induced phase separation of PS. As a consequence, the binding efficiency will be decreased, resulting in reduced F/F_0 ratio upon interaction with PC/PS vesicles. However, at high L/P ratio (about 400), the number of binding sites for apocytochrome *c* is sufficient to accommodate apocytochrome *c* molecules at the membrane surface even in PC/PS vesicles so the PE effect, the phase separation of PS and resulting increase of binding sites for the protein, is not observable (Figure 1).

The lipid-dependent binding, insertion, and translocation of apocytochrome *c* might be related to the structural change of apocytochrome *c* induced by phospholipids. Apocytochrome *c*, which in aqueous solution is largely unstructured, acquires a highly α-helical structure upon interaction with lipid. It has been shown that α-helix content induced in apocytochrome *c* depends on the lipid system, and formation of the α-helical structure of apocytochrome *c* precedes membrane insertion (*34*).

In conclusion, we propose that the local concentration of acidic phospholipids is as well important to regulate the binding and the insertion of apocytochrome *c* into vesicles. In view of lipid dynamics, our findings also suggest that the lipid domain enriched acidic phospholipids can be formed by lipid component(s) itself such as PE as well as charge neutralization of the acidic lipid species (*21*, *35*). It might have resulted from the formation of hydrogen bonding between PE molecules and thereby the nonrandom mixing property of membranes as recently we suggested (*36*). We speculate, therefore, that the balance of PC and PE in membranes might be critical to modulating the translocation of apocytochrome *c*. On the basis of our observations, other component(s), which can induce the formation of acidic lipid domains, might promote the translocation event of apocytochrome *c* in vivo.

REFERENCES

1. Hannavy, K., Rospert, S., and Schatz, G. (1993) *Curr. Opin. Cell Biol. 5*, 694−700.
2. Kranz, R., Lill, R., Goldman, B., Bonnard, G., and Merchant, S. (1998) *Mol. Microbiol. 29*, 383−296.
3. Nicholson, D. W., Hergersberg, C., and Neupert, W. (1988) *J. Biol. Chem. 263*, 19034−19042.
4. Mayer, A., Neupert, W., and Lill, R. (1995) *J. Biol. Chem. 270*, 12390−12397.
5. Stuart, R. A., Nicholson, D. W., and Neupert, W. (1990) *Cell 60*, 31−43.
6. Rietveld, A., Sijens, R., Verkleij, A. J., and de Kruijff, B. (1983) *EMBO J. 2*, 907−913.
7. Rietveld, A., Jordi, W., and de Kruijff, B. (1986) *J. Biol. Chem. 261*, 3846−3856.
8. Demel, R. A., Jordi, W., Lambrechts, H., van Damme, H., Hovius, R., and de Kruijff, B. (1989) *J. Biol. Chem. 264*, 3988−3997.
9. Baranska, J. (1982) *Adv. Lipid Res. 19*, 163−184.
10. Boggs, J. M. (1980) *Can. J. Biochem. 58*, 755−770.
11. Bergelson, L. D. (1977) *Science 197*, 224−230.

12. Ahn, T., and Yun, C-H. (1998) *J. Biochem. (Tokyo) 124*, 622−627.
13. Somerharju, P. J., Virtanen, J. A., Eklund, P., and Kinnunen, P. K. J. (1985) *Biochemistry 24*, 2773−2781.
14. Fisher, W. R., Taniuchi, H., and Anfinsen, C. B. (1973) *J. Biol. Chem. 248*, 3188−3195.
15. Hennig, B., and Neupert, W. (1983) *Methods Enzymol. 97*, 261−274.
16. Ahn, T., Guengerich, F. P., and Yun, C.-H. (1998) *Biochemistry 37*, 12860−12866.
17. Vaskovsky, V. E., Kostetsky, E. Y., and Vasendin, I. M. (1975) *J. Chromatogr. 114*, 129−141.
18. Subbarao, N. K., and MacDonald, R. C. (1993) *Analyst 118*, 913−916.
19. Berkhout, T. A., Rietveld, A., and de Kruijff, B. (1987) *Biochim. Biophys. Acta 897*, 1−4.
20. Ahn, T., and Kim, H. (1998) *J. Biol. Chem. 273*, 21692−21698.
21. Galla, H. J., and Sackmann, E. (1974) *Biochim. Biophys. Acta 339*, 103−115.
22. Galla, H. J., and Hartmann, W. (1980) *Chem. Phys. Lipids 27*, 199−219.
23. Graham, J., Gagné, J., and Silvius, J. R. (1985) *Biochemistry 24*, 7123−7131.
24. Johnson, I. D., Kang, J. C., and Haugland, R. P. (1991) *Anal. Biochem. 198*, 228−237.
25. Pilon, M., Jordi, W., de Kruijff, B., and Demel, R. A. (1987) *Biochim. Biophys. Acta 902*, 207−216.
26. Silvius, J. R. (1990) *Biochemistry 29*, 2930−2938.
27. Bazzi, M. D., Youakim, M. A., and Nelsestuen, G. L. (1992) *Biochemistry 31*, 1125−1134.
28. Bazzi, M. D., and Nelsestuen, G. L. (1992) *Biochemistry 31*, 10406−10413.
29. Heller, W. T., He, K., Ludtke, S. J., Harroun, T. A., and Huang, H. W. (1997) *Biophys. J. 73*, 239−244.
30. Lewis, J. R., and Cafiso, D. S. (1999) *Biochemistry 38*, 5932−5938.
31. Dan, N., and Safran, S. A. (1998) *Biophys. J. 75*, 1410−1414.
32. Nesmeyanova, M. A. (1982) *FEBS Lett. 142*, 189−193.
33. de Kruijff, B., and Cullis, P. R. (1980) *Biochim. Biophys. Acta 602*, 477−490.
34. Bryson, E. A., Rankin, S. E., Carey, M., Watts, A., and Pinheiro, T. J. T. (1999) *Biochemistry 38*, 9758−9767.
35. Ikeda, T., Yamaguchi, H., and Tazuke, S. (1990) *Biochim. Biophys. Acta 1026*, 105−112.
36. Ahn, T., and Yun, C-H. (1999) *Arch. Biochem. Biophys. 369*, 288−294.

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