— **Rapid Communication** —

Characterization of a 62-Kilodalton Acidic Phospholipid-Binding Protein Isolated from the Edible Mushroom *Pleurotus ostreatus*

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Many lipid-binding proteins such as pleurotolysin and ostreolysin have been isolated from the edible mushroom *Pleurotus ostreatus*. In this study, we detected a novel lipid-binding protein with a molecular weight of 62 kDa by measuring via centrifugation the association of aqueous extracts of the mushroom with lipid vesicles composed of various phospholipids. The 62-kDa protein (p62) was purified by sedimentation of the mixture of protein extracts and acidic phospholipid-containing lipid vesicles. The purified p62 bound to phosphatidylglycerol (PG)/phosphatidylcholine/cholesterol (5 : 45 : 50) vesicles but not to vesicles composed of other phospholipids including phosphatidylserine (PS), phosphatidylinositol, phosphatidic acid, lysoPS, and lysophosphatidylinositol. The p62 protein specifically associated with the PG-containing vesicles but not with other polyglycerophospholipid vesicles consisting of cardiolipin, bis(monoacylglycero)phosphate, monolysocardiolipin, or dilysocardiolipin, suggesting that p62 recognized a precise molecular structure of PG. Intrinsic tryptophan fluorescence of p62 was changed by incubation of p62 with PG-containing vesicles. Staining of giant unilamellar vesicles with fluorescence-labeled p62 showed that p62 bound to PG-containing vesicles but not PS-containing vesicles. These observations signify the potential usefulness of p62 as a tool for studying the functions of PG molecules in biological membranes.

Key words —— *Pleurotus ostreatus*, lipid-binding protein, phosphatidylglycerol, acidic phospholipid, mushroom

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INTRODUCTION

Little is known about where particular phospholipids are localized in membranes and how they participate in cellular functions, mainly because of the lack of appropriate methodologies for manipulating and tracing the functions of membrane phospholipids. Various probes for studying the molecular motion of membrane phospholipids have been developed, such as lysenin specific to sphingomyelin (SM),[1–4] a small cyclic peptide specific to phosphatidylethanolamine,[5,6] and monoclonal antibodies against phosphatidylcholine (PC),[7] phosphatidylserine (PS),[8,9] and phosphatidylinositol 4,5-bisphosphate.[10,11]

Several cytolytic proteins have also been isolated from the basidiocarps of both toxic and edible mushrooms, and their lipid-binding specificities as well as pore-forming properties have been studied.[12–16] A 2-component hemolysin, pleurotolysin A (17 kDa) and pleurotolysin B (59 kDa) from *Pleurotus ostreatus* (*P. ostreatus*), has been purified and characterized.[13,17] Pleurotolysin A specifically bound to SM/cholesterol vesicles and formed a transmembrane pore in concert with pleurotolysin B. Ostreolysin was also isolated as a 16-kDa hemolytic protein from the same mushroom, which was suggested to specifically recognize a cholesterol-rich lipid domain.[18–20]

In the present study, we detected a 62-kDa protein (p62) from *P. ostreatus* and showed that p62 binds to lipid vesicles containing acidic phospholipids, especially phosphatidylglycerol (PG).

MATERIALS AND METHODS

Materials —— Egg yolk PC, 1-palmitoyl-2-oleoyl

PC (POPC), egg yolk phosphatidylethanolamine, bovine brain SM, bovine brain PS, bovine liver phosphatidylinositol, egg yolk phosphatidic acid, egg yolk PG, and bovine heart cardiolipin were purchased from Sigma (St. Louis, MO, U.S.A.). Bis(monoacylglycero)phosphate, monolysocardiolipin, and dilysocardiolipin were kindly provided by Dr. T. Kobayashi of RIKEN (Wako, Saitama, Japan). Cholesterol, lysoPS, and lysophosphatidylinositol were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). 3-[(3-Cholamidopropyl) dimethylammonio]propanesulfonate (CHAPS) was obtained from Dojindo Laboratories (Tokyo, Japan). A fluorescein isothiocyanate (FITC) labeling kit was purchased from Thermo Fisher Scientific K.K. (Yokohama, Japan). All other chemicals used in this study were of analytical grade.

Purification of p62 —— All purification steps were carried out at 0–4°C, and p62 fractions were treated with protease inhibitors at each step. Basidiocarps of *P. ostreatus* (100 g, wet weight) were homogenized in 100 ml of 50 mM Tris-HCl buffer (pH 8.5). The homogenate was centrifuged at 15000 × *g* for 30 min, and ammonium sulfate was added to the supernatant to 35% saturation. After 30 min, the mixture was centrifuged at 15000 × *g* for 30 min, and ammonium sulfate was added to the supernatant to 70% saturation. The resultant precipitates were dialyzed against 50 mM Tris-HCl buffer (pH 8.5).

Lipid vesicles composed of PS and cholesterol (1 : 1, molar ratio; 0.1 mmol of lipids) were incubated with the ammonium sulfate fraction proteins (100 mg of proteins precipitating between 35–70% saturation) in 20 ml of 50 mM Tris-HCl buffer (pH 8.5) containing 2 mM EDTA at 4°C for 60 min on ice. The liposome fractions were collected by centrifugation at 16000 × *g* for 10 min at 4°C and washed twice with 50 mM Tris-HCl buffer (pH 8.5) containing 0.1% (w/v) CHAPS. The washed liposomes were solubilized in 0.5% (w/v) CHAPS at 37°C for 10 min and were centrifuged at 16000 × *g* for 30 min. The supernatants were used as a source of the purified p62 protein in subsequent lipid-binding assays using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentrations were determined spectrophotometrically by using the BCA Protein Assay Reagent (Thermo Fisher Scientific K.K.).

Binding Assay of *P. ostreatus* Proteins to Vesicles —— The multilamellar vesicles consisting of 50 mol% PC, SM, PS, or phosphatidylinositol and 50 mol% cholesterol were prepared for the binding studies. Lipids that were in chloroform were placed in Pyrex glass tubes, and the organic solvent was removed by using a rotary evaporator. The lipid film was thoroughly dried to remove residual organic solvent by placing the samples on a vacuum pump overnight. The dried lipid film was dispersed in 100 μl of Tris-HCl (pH 8.5) at room temperature and agitated vigorously by using a vortex mixer. The test tubes containing the lipid suspension were placed in a bath sonicator and gently sonicated for 15 min. The suspension was transferred to a microcentrifuge tube and centrifuged for 10 min at 16000 × *g*. The supernatant was removed, and the pellet, resuspended in Tris-HCl (pH 8.5), was used for the binding studies. Each of the lipid vesicles was incubated with ammonium sulfate fraction proteins from *P. ostreatus* at 37°C for 30 min. The vesicles were washed twice with Tris-HCl (pH 8.5), and subjected to SDS-PAGE. Protein bands were stained with Coomassie brilliant blue R-250.

To assess the binding activity of purified p62 to acidic phospholipids in membranes, lipid vesicles composed of 5 mol% PG, PS, phosphatidylinositol, lysoPS, lysophosphatidylinositol, or phosphatidic acid, 45 mol% PC from egg yolk, and 50 mol% cholesterol were prepared. Then, the binding assay was performed as described above.

The binding activities of p62 to the polyglycerophospholipids, namely bis(monoacylglycero)phosphate, monolysocardiolipin, and dilysocardiolipin in membranes were examined by incubating p62 with lipid vesicles composed of 10 mol% polyglycerophospholipids, 40 mol% egg yolk PC, and 50 mol% cholesterol, which were prepared as described above.

Fluorescence Measurements —— Steady-state intrinsic fluorescence of p62, either alone or in combination with lipids, was measured at 37°C in a Fluoromax spectrofluorimeter (HORIBA, Kyoto, Japan) equipped with a thermostatically controlled cell holder and a magnetic stirrer. Excitation and emission slits were set at 5 nm as described.[2,3] Samples were excited at 280 nm. Fluorescence emission spectra of 5 μg/ml of p62 were recorded over the range of 300–400 nm. Intrinsic tryptophan fluorescence signals were corrected for the dilution factor, and the background was subtracted by using appropriate blanks. Small unilamellar vesicles composed of 10 mol% PG, PS, or cardiolipin, 40 mol% PC, and 50 mol% cholesterol were prepared by 10-min pulsed sonication with a Sonifier (Branson Ul-

trasinic Corp., Danbury, CT, U.S.A.), using output scale 4 and 50% duty cycle at room temperature. All the fluorescence measurements were taken in 50 mM Tris-HCl buffer (pH 8.5).

Preparation of Giant Unilamellar Vesicles and Observation of p62 Binding under Fluorescent Microscopy —— The ratio between the components comprising the vesicles (PG/POPC/cholesterol or PS/POPC/cholesterol) was fixed at 10 : 40 : 50. Giant unilamellar vesicles were prepared by the gentle hydration method as previously described.[21] The vesicle suspension was incubated at room temperature with FITC-conjugated p62, which was prepared as reported,[22] and then transferred to a glass plate. The samples were observed with an inverted fluorescence microscope.

RESULTS

Detection of the Phospholipid-binding Proteins from *P. ostreatus*

To detect the phospholipid-binding proteins, aqueous extracts of *P. ostreatus* (acquired from 35–70% ammonium sulfate fractionation) were incubated with lipid vesicles composed of SM, PC, PS, or phosphatidylinositol and cholesterol at a molar ratio of 1 : 1. After incubation at 37°C for 30 min, the solutions were centrifuged. The resultant pellets containing lipid vesicle-associated proteins were subjected to SDS-PAGE (Fig. 1). A protein band with a molecular weight of 17 kDa, which corresponds to pleurotolysin,[13,17] was observed by sedimentation of the aqueous extracts with SM-containing vesicles. It was reported that pleurotolysin A specifically bound to SM-cholesterol vesicles.[13] Interestingly, the pellets of acidic-phospholipid vesicles, namely PS and phosphatidylinositol, showed a single protein band with an apparent molecular weight of 62 kDa. In contrast, no protein band was observed with PC vesicles under the same conditions.

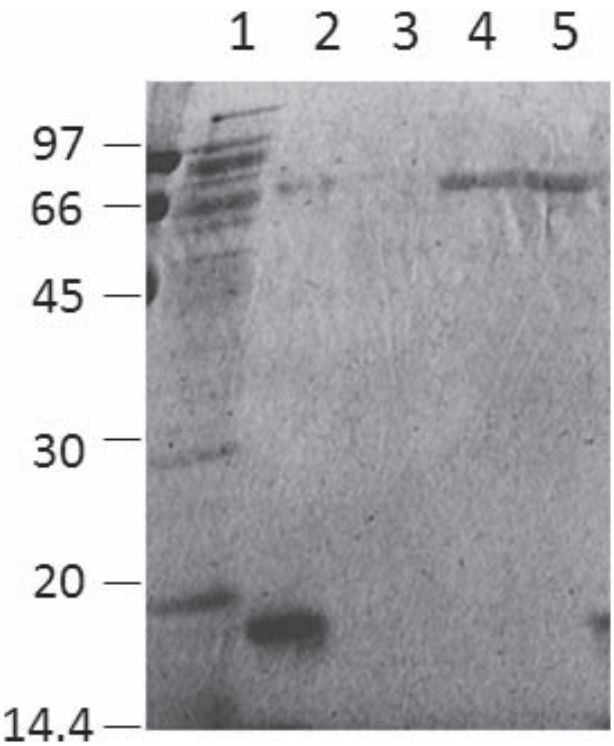

Fig. 1. SDS-PAGE of Lipid Vesicle-associated Proteins from *P. ostreatus*

P. ostreatus aqueous extracts separated by 35–70% ammonium sulfate fractionation were incubated with lipid vesicles composed of various phospholipids and cholesterol at 1 : 1 molar ratio at 37°C for 30 min; SM/cholesterol (lane 2), PC/cholesterol (lane 3), PS/cholesterol (lane 4), or phosphatidylinositol/cholesterol (lane 5). The samples were centrifuged and the resulting pellets were analyzed by SDS-PAGE. Protein bands were stained with Coomassie brilliant blue R-250. The ammonium sulfate fraction from *P. ostreatus* is shown in lane 1.

Purification of p62 from *P. ostreatus*

The protein p62 was purified from basidiocarps of *P. ostreatus* by ammonium sulfate precipitation, followed by sedimentation with lipid vesicles composed of PS and cholesterol at a 1 : 1 molar ratio. Efficient enrichment of p62 was observed by sedimentation of the *P. ostreatus* aqueous extract with the PS-cholesterol vesicle (Fig. 2, lane 2). The pellet was washed with 0.1% CHAPS to remove nonspecific binding proteins, and purified p62 was further solubilized in 0.5% CHAPS (Fig. 2, lane 3). The supernatant was loaded onto a POROS® HQ anion exchange column to remove CHAPS.

Tryptic peptides of the p62 band were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry by using peptide mass matching. Mass spectra database search indicated that there were no high scoring homologous proteins. This nearly purified p62 was used for the subsequent experiments.

Binding Specificity of p62 to Acidic Phospholipids

The interaction of purified p62 with various acidic phospholipids was examined by measuring its association with lipid vesicles via centrifugation of the p62 extract mixed with vesicles containing 5 mol% acidic phospholipids, 45 mol% PC, and

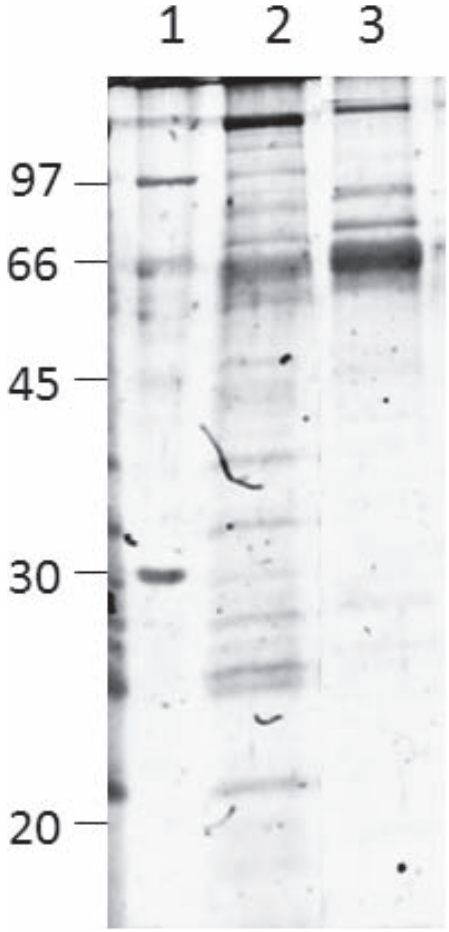

Fig. 2. Purification of p62 by Sedimentation with PS/cholesterol Vesicles

Lipid vesicles composed of PS and cholesterol (50 : 50, molar ratio) were incubated with ammonium sulfate fraction proteins (35–70%) at 4°C for 60 min. The samples were centrifuged to precipitate lipid vesicles. The pellets were washed twice with a buffer containing 0.1% CHAPS and further treated with 0.5% CHAPS to solubilize p62. The samples were electrophoresed on 12% SDS-PAGE, and protein bands were stained with Oriole fluorescent gel stain (Bio-Rad). Lane 1, molecular weight marker; lane 2, pellet of lipid vesicles after incubation with *P. ostreatus* aqueous extract; lane 3, supernatant after 0.5% CHAPS elution of vesicle-bound proteins from vesicles.

50 mol% cholesterol. The amount of bound p62 in the lipid vesicle-containing pellet was determined by SDS-PAGE.

The p62 protein was efficiently recovered by sedimentation of the *P. ostreatus* aqueous extract with PG-containing vesicles, but not with other acidic phospholipids, namely PS, phosphatidylinositol, and phosphatidic acid (Fig. 3A). No association of p62 with monoacyl analogs of phospholipids such as lysoPS and lysophosphatidylinositol was observed. As shown in Fig. 3B, the binding of p62 with lipid vesicles that were composed of a variety of polyglycerophospholipids was also compared. The p62 protein specifically bound to the PG-containing vesicles but did not bind to other lipid vesicles containing cardiolipin, bis(monoacylglycero)phosphate, monolysocardiolipin, or dilysocardiolipin.

Intrinsic tryptophan fluorescence is widely used to estimate the local environment of tryptophan residues in proteins. We compared the fluorescence emission spectra of tryptophan in the absence or presence of one of the following liposomes: PG/PC/cholesterol, PS/PC/cholesterol, cardiolipin/PC/cholesterol, and PC/cholesterol (Fig. 4). The largest increase in tryptophan fluorescence and a slight blue shift from 356 nm to 354 nm in the maximum emission wavelength was observed after incubation with PG/PC/cholesterol vesicles. Moderate changes in fluorescence were observed in the presence of PS/PC/cholesterol or cardiolipin/PC/cholesterol vesicles. These results suggest the migration of the residual tryptophans of p62 to a less polar environment after incubation with PG-containing vesicles.

The binding of fluorescence-labeled p62 to PG in giant unilamellar vesicles was also examined. After incubation of the PG/PC/cholesterol (10 : 40 : 50) giant vesicles with FITC-labeled p62, vesicles tended to aggregate larger than the PS/PC/cholesterol vesicles as shown in Fig. 5. In addition, PG/PC/cholesterol giant vesicles were labeled fluorescently by incubation with FITC-p62, whereas PS/PC/cholesterol vesicles were not. These observations suggest the potential usefulness of p62 as a tool for studying the functions of PG molecules in biological membranes.

DISCUSSION

In the present study, we detected a 17 kDa-protein, which corresponds to pleurotolysin A, by sedimentation with SM/cholesterol vesicles. Pleurotolysin is a SM-specific cytolysin that consists of A (17 kDa) and B (59 kDa) components from *P. ostreatus*.[13,17] It is known that pleurotolysin A alone specifically binds to SM/cholesterol vesicles, which is consistent with our data, and forms transmembrane pore complex in concert with pleurotolysin B.[13]

The p62 protein was efficiently enriched as a lipid vesicle-associated protein by incubation of the *P. ostreatus* extract with PS/cholesterol (50 : 50) or phosphatidylinositol/cholesterol (50 : 50) vesi-

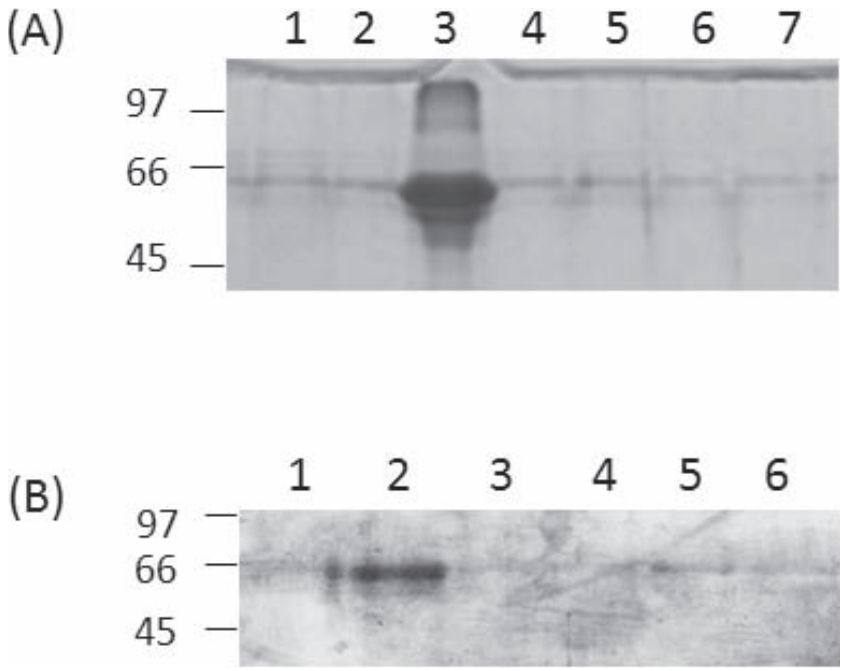

Fig. 3. Phospholipid Specificity of p62 Binding to Lipid Vesicles

(A) Purified p62 was mixed with lipid vesicles composed of phospholipid/PC/cholesterol (5 : 45 : 50), and the mixture was incubated at 37°C for 30 min. After centrifugation, the precipitates were analyzed by SDS-PAGE, followed by Coomassie brilliant blue staining. Lane 1, PC; lane 2, phosphatidic acid; lane 3, PG; lane 4, PS; lane 5, lysoPS; lane 6, phosphatidylinositol; lane 7, lysophosphatidylinositol. (B) Purified p62 was incubated at 37°C for 30 min with vesicles composed of various polyglycerophospholipids to be tested, PC, and cholesterol (5 : 45 : 50). Lane 1, PC as control; lane 2, PG; lane 3, cardiolipin; lane 4, bis(monoacylglycero)phosphate; lane 5, monolysocardiolipin; lane 6, dilysocardiolipin.

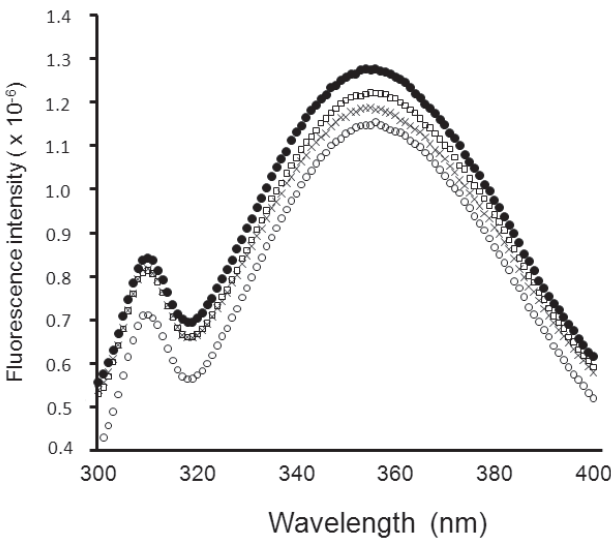

Fig. 4. Changes in Intrinsic Fluorescence Emission Spectra of p62 after Interaction with PG-containing Vesicles

p62 was incubated with PG/PC/cholesterol (10 : 40 : 50, ●), PS/PC/cholesterol (10 : 40 : 50, □), cardiolipin/PC/cholesterol (10 : 40 : 50, ×), PC/cholesterol (50 : 50, ○) for 30 min at 37°C. Fluorescence spectra were obtained at the excitation wavelength of 280 nm.

cles. By exploiting the binding specificity of p62 to PS/cholesterol, p62 was purified to near homogeneity. The purified p62 specifically bound to PG/PC/cholesterol (5 : 45 : 50) vesicles, but not to PS/PC/cholesterol (5 : 45 : 50) vesicles. Since the purified p62 could bind to PS/cholesterol vesicles (50 : 50, data not shown), the possibility of alterations in binding specificity of p62 due to the purification procedures is excluded. It was suggested that a high density of PS in liposomal membranes was required for binding with p62.

Although p62 specifically bound to PG/PC/cholesterol (5 : 45 : 50) vesicles, lipid vesicles composed of other acidic phospholipids, such as PS, phosphatidylinositol, or phosphatidic acid/PC/cholesterol (5 : 45 : 50), did not interact with p62. Among the polyglycerophospholipid analogs examined, only PG-containing vesicles were able to bind to p62. These data suggest that p62 may recognize a molecular structure on the unesterified glycerol moiety of PG.

PG is found in almost all bacterial membranes as well as in the mitochondrial inner membranes of eukaryotic cells,[23] and is the second most abundant of the surfactant lipids.[24,25] It is also known that PG is required for the formation and function of thylakoid membranes in cyanobacteria and plants.[26–28] Although appreciable evidence for the essential role of PG in these cells is now accumu-

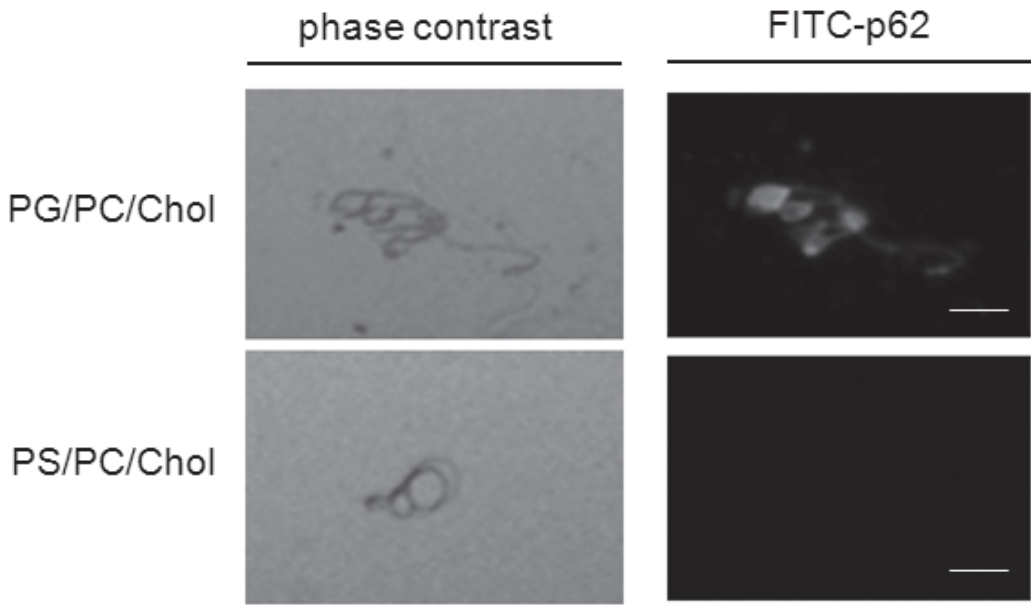

Fig. 5. Fluorescence Microscope Images of the Binding of FITC-labeled p62 to Giant Unilamellar Vesicles Containing PG

After incubation of giant unilamellar vesicles consisting of PG/POPC/cholesterol (10 : 40 : 50) or PS/POPC/cholesterol (10 : 40 : 50) with the FITC-labeled p62 at room temperature, p62-bound vesicles were observed under a fluorescence microscope. The scale bar represents 10 μm.

lating, it is still insufficient to determine the exact role of PG in the membranes. We believe that p62 may be useful to define the precise roles of PG in biological membranes.

It is reported that tryptophan residues are involved in the interaction between several lipid-binding toxins and membranes. The tryptophan fluorescence of lysenin increases, and the maximum emission wavelength undergoes a blue shift after incubation with SM-containing vesicles.[2,3] In the present study, we observed similar changes in tryptophan fluorescence of p62 in the presence of PG-containing membranes. This result suggests that tryptophan residues of p62 migrate to a less polar environment by interacting with PG molecules in membranes.

Giant unilamellar vesicles, which are readily observed under a microscope, serve as useful cell models. In this study, we observed that the interaction of FITC-labeled p62 with PG-containing giant liposomes but not with PS-containing giant liposomes. Membrane disruption activity of p62 was not observed under the conditions used. Although further analyses of the binding properties between p62 and PG are required, our findings suggest that p62 is a useful tool to probe the motion and function of PG in biological membranes.

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