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Phospholipids and Their Derivatives as Mitogen and Motogen of Budding Tunicates

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In order to discover novel invertebrate cytokines from the budding tunicate, *Polyandrocarpa misakiensis*, we treated the water-insoluble fraction of tunicate homogenates with trypsin. The extracts showed remarkable activities to promote the growth and motility of tunicate cells. The activities were heat-stable and proteinase K-resistant. After anion exchange chromatography, the activities were eluted with detergents such as 0.1% deoxycholic acid. The Fourier transform infrared spectrum indicated large amounts of fatty acids and phospholipids instead of polypeptides in the extracts. Consistently, the activities were extractable with organic solvents such as chloroform. Long chains of n-3 polyunsaturated free fatty acids (FFA), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) were the major components in the lipid-soluble fraction. A cDNA for FFA-releasing enzyme phospholipase A_2 (PLA_2) was cloned. The expression of this gene could be seen in epidermal cells during budding. The recombinant protein, as in the case of the authentic PLA2, preferred PC and PE as substrates, followed by PS and PI. The resultant FFAs only promoted cell growth, while the remaining lysophospholipids stimulated cell motility. The former contained unsaturated fatty acids (C18:1, C20:5, and C22:6) while the latter did not, suggesting that unsaturated fatty acids are responsible for mitogenic activity in tunicate cells. These results show for the first time that phospholipids and their derivatives are bio-mediators promoting cell growth and cell motility in invertebrates.

Key words: budding, cell growth, cell motility, fatty acid, phospholipase A_2, phospholipid, tunicate.

Cell growth and differentiation continue throughout the life span of living organisms. In mammals, for example, they contribute to the homeostasis of hematopoietic tissues, small intestinal epithelium and epidermal skin (for review, Refs. *1* and *2*). There are examples in which tissue homeostasis plays a role in morphallactic regeneration of the body (*3*). Some marine and freshwater invertebrates have such a remarkable potential that a piece of tissue can reconstruct the entire body. In those animals, somatic cells are expected to possess a capacity to grow and differentiate comparable to embryonic cells.

Polyandrocarpa misakiensis is a budding tunicate that propagates by asexual reproduction (*4*). Buds have a simple architecture, comprising outer and inner epithelia with coelomic cells between them (*3*). After bud formation, the inner epithelium enters mitosis and forms most tissues and organs of a new asexual animal, including the gut, pharynx and brain (*5–7*). We have disclosed several genes and proteins that regulate cell growth and differentiation of the multipotent inner epithelium, including C-type lectins, serine protease, and serine protease inhibitor (*8–10*).

In this study, we aimed to discover novel cytokines from *Polyandrocarpa* homogenates. Based on findings from mammals (*11–13*), we expected that some kinds of growth factor would be bound to the extracellular matrix (ECM), and that they might be liberated by protease. We challenged the isolation of growth factors by trypsinizing water-insoluble tunicate fractions. This paper describes first that lipid-soluble substances comprise the major source of the trypsinized fraction. Secondly, we show that phospholipids and their derivatives are potent mitogen and motogen for cultured tunicate cells. Thirdly, we characterized their structures. It turns out that tunicates have unique n-3 polyunsaturated fatty acids. Finally, we show that phospholipase A_2, a free fatty acid-releasing enzyme, is expressed during budding, and that free fatty acids are responsible for the mitogenic activity of phospholipids. This is the first report demonstrating in invertebrates that fat-soluble factor(s) act as mitogen and motogen.

MATERIALS AND METHODS

Animals—Asexual individual *P. misakiensis* were reared in culture boxes settled in Uranouchi Inlet near the Usa Marine Biological Institute, Kochi University.

Cell culture—Cell lines have been established from the multipotent epithelium of *P. misakiensis* (*14*). They were

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kept in growth medium containing 3% fetal bovine serum (FBS) in a basal medium consisting of synthetic seawater and Dulbecco's modified Eagle's Medium (5:1).

Preparation of Trypsinized Homogenates—Colonies (about 10 g) were powdered in liquid nitrogen and then extracted for 30 min with 100 ml of 7.5 mM phosphate buffer (PB, pH 7.0) in an ice bath. After centrifugation at 15,000 ×*g* for 20 min, the pellet was resuspended in 20 ml of PB, to which trypsin (Merck) was added at a final concentration of 0.1%. The suspension was incubated at 35°C for 1 h in a shaker bath, centrifuged as described above, and the supernatant was stored for chromatography.

Lipid Extraction—Total lipids were extracted from colonies of *P. misakiensis* by the conventional method (*15*). In brief, animals (about 100 mg, wet weight) were homogenized in a mixture of 1 ml of chloroform/methanol (2:1) and 0.5 ml of phosphate-buffered saline (PBS). Lipids were extracted three times with the same solution. The chloroform layer was collected, dried by flushing with N_2 gas and kept at –80°C until use.

Proteinase K Digestion—After dialysis against distilled water, samples were concentrated by lyophilization, and incubated with 200 μg/ml proteinase K (Gibco BRL) in 7.5 mM PB (pH7.0) at 37°C for 24 h. Proteinase K was inactivated by heating at 80°C for 10 min.

Fourier Transform Infrared Spectroscopy—Samples were lyophilized, set in a CaF_2 disc, and analyzed by a Fourier transform infrared spectrometer (FTIR, JEOL JIR-100).

Chemicals—Organic solvents (methanol, chloroform, hexane, diethyl ether, acetic acid, sulfuric acid, and ammonium hydroxide) were purchased from Nacalai Tesque Inc. (Japan). Phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidic acid (PA), lysophosphatidylethanolamine (LPE), lysophosphatidylcholine (LPC), cardiolipin (CL), cerebroside (Cer), and sphingomyelin (SM) were purchased from Doosan Serdary Research Laboratories (USA). Mammalian fatty acids and their methyl esters were purchased from Cayman Chemical Company (USA). Silica gel 60 plates (20 × 20 cm, Merck, Germany) for thin-layer chromatography (TLC) were activated by heating before use.

Column chromatography—For gel filtration chromatography, the sample was dialyzed against 0.1 M ammonium acetate and passed through a column (3 × 100 cm) of Sephacryl S-200 (Amersham Pharmacia, USA) equilibrated with the same buffer. For anion exchange chromatography, the sample was dialyzed against 10 mM PB (pH 8.0) and applied to a column (1.6 × 4 cm) of DEAE-Toyopearl-650M (Tosoh Co., Japan). The column was eluted with a linear gradient of 0–0.5 M NaCl at a flow rate of 0.5 ml/min, and finally with 0.1% deoxycholic acid (DOC) in the same buffer (10 mM PB pH 8.0, 0.5 M NaCl).

Thin-Layer Chromatography—In the first TLC, samples were developed for about 1 hour with a solvent system of hexane/diethyl ether/acetic acid (70:30:1). This was done to separate phospholipids from neutral lipids. The second TLC was done to separate phospholipid species, using chloroform/methanol/28% NH_4OH (65:35:8). The plates were dried and visualized by spraying with 50% sulfuric acid and baking. In order to isolate and extract native substances after TLC, samples were spotted onto two neighboring points. After development, one lane was visualized as described, and the other was scratched to collect silica gel at positions corresponding to colored spots. Substances adsorbed to the silica gel were extracted three times with 1 ml of chloroform/methanol/3% NH_4OH (6:5:1) and dried with flushing N_2 gas.

Gas-Liquid Chromatography and GC/MS—Phospholipids were saponified to cleave the ester bonds. The liberated free fatty acids were then methylated with 5% HCl/methanol at 100°C for 2 h under N_2 gas. Fatty acid methyl esters (FAMEs) were extracted with hexane, and analyzed by GLC according to Yoshida and Takeshita (*16*). A GLC system (Model 163, Hitachi) was equipped with a BPX70 capillary column (25 m × 0.53 mm I.D., Australia). A GC/MS system (Agilent Technology, USA) consisted of a gas chromatograph (6890N) and a Mass Selective Detector (5973). The oven temperature was raised from 100°C to 220°C at 4°C/min.

cDNA Cloning for Phospholipase A_2—cDNAs were prepared from *Polyandrocarpa* colonies and ligated to the arms of the phage vector λgt 11, as described previously (*9*). Based on the partial nucleotide sequence of *P. misakiensis* phospholipase A_2 (PmPLA2) obtained by EST analysis (*9*), primers were designed: PL1, 5′-atcgggattagtgttgctc-3′; PL2, 5′-ccacagtagcaaccgtagtc-3′. A full length PmPLA2 cDNA was amplified by polymerase chain reaction (PCR) [94°C (0.5 min), 55°C (1 min), 72°C (1.5 min), 30 cycles], and ligated to a TA cloning vector (pGEM-T, Promega, USA).

In Situ Hybridization—RNA probes were labeled with digoxigenin (DIG) (Roche). Specimens were fixed in 4% paraformaldehyde in PBS for 12 h at 4°C, washed three times with chilled PBS containing 0.1% Tween 20 (PBST), dehydrated with serial methanol, and dewaxed in cold xylene for 15 min before hybridization. Prehybridization and hybridization were performed at 65°C for 1 h and 12 h, respectively. Subsequent procedures are described elsewhere (*8*). Some specimens were dehydrated and embedded in plastic resin, followed by serial cutting with glass knives and mounting on glass slips for microscopy (*10*).

Recombinant PmPLA2 Protein—Primers with artificial restriction enzyme sites were designed: PL3, 5′-gaattcttccaggaaggctggatg-3′; PL4, 5′-aagcttagcacacactattcctgtg-3′. A cDNA for PmPLA2 was amplified, as indicated above, cut with *Eco*RI and *Hin*dIII, and ligated to a pMAL-c2X vector (NEB, USA) cut with the same restriction enzymes. The recombinant protein was induced using 0.1 mM isopropyl-β-D-thiogalactopyranoside for 6 h at 37°C, and purified with amylose resin.

Hydrolysis of PLA2 Phospholipids—Dried phospholipids were dissolved in 2 ml ethanol/diethyl ether (5:95), diluted with hydrolysis buffer [80 mM Tris-HCl (pH 8.0), 20 mM $CaCl_2$], and incubated with bee venom phospholipase A_2 (Sigma Chemical Co., USA) at a final concentration of 0.4 mg/ml for more than 2 h at room temperature. Hydrolysis products were dried and developed on TLC to separate free fatty acids from lysophospholipids.

Bioassay—Lipid samples extracted from 0.1 g wet weight of *Polyandrocarpa* colonies were dried and dissolved in 100 μl of dimethyl sulfoxide (DMSO), after which samples (5 μl each) were diluted with 1 ml of the

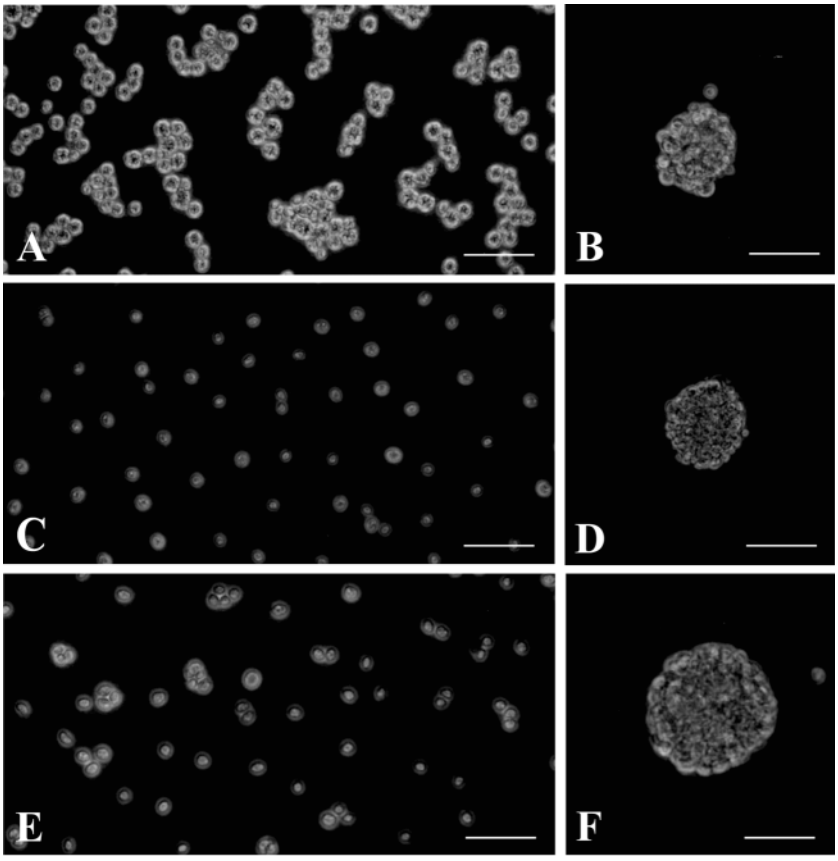

Fig. 1. **Effects of trypsinized extracts of tunicate homogenates on cultured cells from *P. misakiensis*.** (A) Cells treated with trypsinized extracts in serum-free culture medium. The cells proliferated and scattered. (B) Cells in serum-free culture medium. The cells aggregated. (C) Cells treated with the chloroform layer of trypsinized extracts. (D) Cells treated with the water layer of trypsinized extracts. (E) Cells treated with lysophospholipids prepared from PLA2-digested phospholipids. Cell scattering activity was evident. (F) Cells treated with free fatty acids that were prepared from PLA2-digested phospholipids. Cell aggregates became larger. Bars, 50 μm.

basal medium for cell culture. Cells in the growth medium were harvested and washed twice with basal medium (-FBS), and resuspended in basal medium in the presence or absence of lipids at a density of 1.5×10^5 cells/ml. Aliquots (100 μl) were plated in 96-well multiplates. Cell number was estimated everyday after plating by a hemocytometer.

RESULTS

Cell Growth-Promoting Activity of Trypsinized Supernatant—Polyandrocarpa colonies (10 g) were homogenized and the water-insoluble pellet was extracted with 20 ml of 0.1% trypsin. The extract was diluted one hundred-fold with basal medium for cell culture. The extract showed remarkable activities to promote cell growth and cell scattering (Fig. 1A). As a control, cells were cultured only in basal medium. They formed aggregates immediately and proliferated very slowly (Fig. 1B).

Both the cell growth and cell scattering activities were eluted in the void volume upon gel filtration chromatography (Fig. 2A). When applied to anion exchange chromatography, the activities were not eluted by 0–0.5 M NaCl but were by subsequent elution with 0.1 M DOC (Fig. 2B). On SDS-PAGE, the DOC fraction gave no bands, even after silver staining (not shown).

Both the cell growth and cell scattering activities in the DOC fraction were resistant to heat treatment (80°C, 10 min) or proteinase K treatment (37°C, 24 h). The FTIR spectrum showed trace amounts of peptide bonds but, instead, large amounts of fatty acids and phospholipids (Fig. 2C). The DOC fraction (10 ml) was mixed with the same volume of organic solvents (chloroform/methanol/3% NH_4OH = 6:5:1). After centrifugation, both the chloroform layer and water layer were dried separately and prepared for bioassay. Only the former promoted cell growth and cell scattering (Fig. 1, C and D).

Biochemical Analysis of the Chloroform Layer—The chloroform layer was analyzed by TLC. In the first TLC (hexane/diethyl ether/acetic acid = 70:30:1), at least five spots were evident. One remained at the origin and the others corresponded to cholesterol (Cho), free fatty acid (FFA), triacylglycerol (TG), and cholesterol ester (ChoE) (Fig. 3A). Essentially, the same components were found in *Polyandrocarpa* body fluid untreated with trypsin (Fig. 3B). Unmovable substances in the first TCL were further separated using a second solvent system (chloroform/methanol/28% NH_4OH, 65:35:8) (Fig. 3C). From their R_f values, spots were identified as phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL). The unmovable substances in the second TLC might include lysophosphatidic acid (LPA).

The fatty acid components were identified by GC/MS. They consisted mainly of C16:0 (8%), C18:0 (13%), C18:1n-9 (14%), C20:5n-3 (18%), C22:6n-3 (15%) (Table 1). C20:5n-3 and C22:6n-3 are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively. In order to know more about the fatty acids within phospholipids, tunicate phospholipids were treated with bee venom phospholipase A2 (PLA2, FFA-releasing enzyme). The major components were long chain unsaturated FFAs

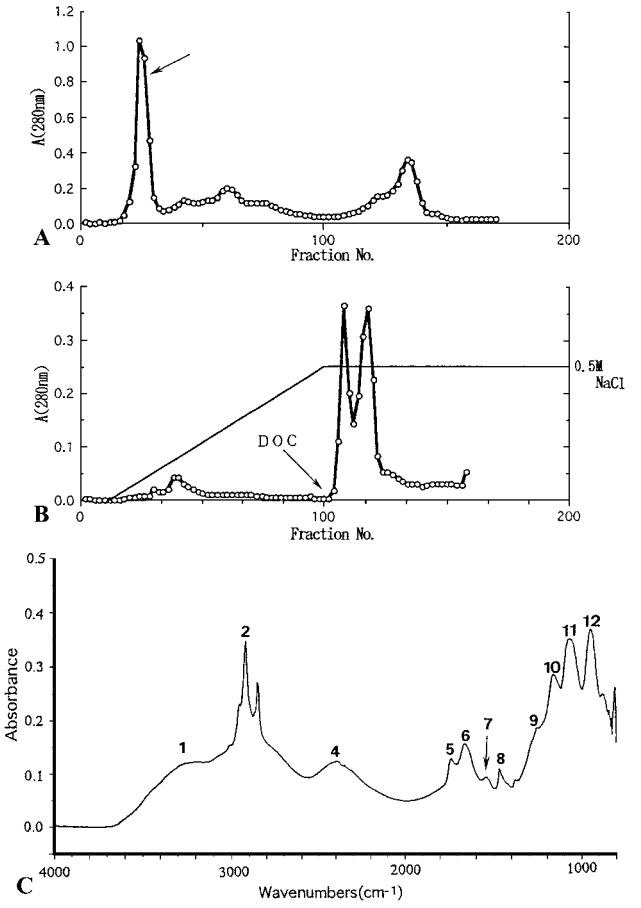

Fig. 2. **Chromatographic and spectroscopic analysis of trypsinized extracts of tunicate homogenates.** (A) Elution profile of gel filtration chromatography. Cell growth-promoting activity was eluted in the void volume (arrow). (B) The elution profile from anion exchange chromatography. The activity was eluted by 0.1% DOC after a 0–0.5 M NaCl gradient (arrow). (C) FTIR spectrum of the active fraction after anion exchange chromatography. Peaks (2,3,5,8) indicate the presence of fatty acids. Peaks (10,11) indicate phospholipids. Peak (12) indicates carotenoids. Peak (7) indicates peptide bonds.

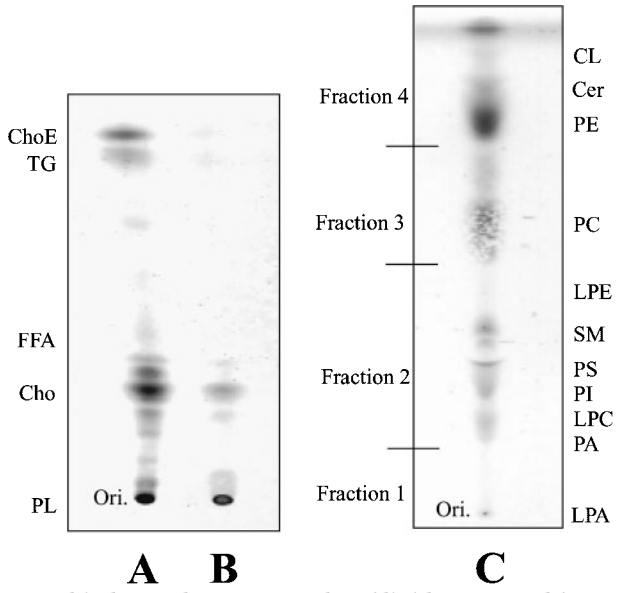

Fig. 3. **Thin layer chromatography of lipids extracted from *P. misakiensis*.** The first TLC (A,B) was developed with a solvent system of hexane/diethyl ether/acetic acid (70:30:1). The second TLC (C) was developed with a solvent system of chloroform/methanol/28% NH_4OH (65:35:1). Spots were visualized by heating after spraying with 50% sulfuric acid. (A) Samples were prepared from the chloroform layer of trypsinized extracts. (B) Samples were prepared from body fluid without trypsin treatment. (C) Substances remaining at the origin in the first TLC were subjected to the second TLC. For convenience, the spots were grouped into four fractions. Cer, cerebroside; Cho, cholesterol; ChoE, cholesterol ester; CL, cardiolipin; FFA, free fatty acid; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; SM, sphingomyelin; TG, triacylglycerol.

such as C18:1n-9 (13.6%), C20:5n-3 (26.2%), and C22:6n-3 (20.4%) (Table 1, Fig. 4A), consistent with previous findings in sessile and pelagic tunicates (*17–19*). The remaining phospholipids, including lysophospholipids, comprised mainly saturated fatty acids such as C16:0 and C18:0 (compare Fig. 4A with 4B). For comparison, mammalian phospholipids were treated with PLA2. The FFAs comprised mainly of C16:0 (14%), C18:0 (25%), C18:1n-9 (17%), C18:2n-6 (17%) (Table 1). Unlike tunicate fatty acids, the amounts of C20 and C22 were residual.

In Situ Hybridization of PmPLA2 and Its Substrate Specificity—In *P. misakiensis*, the amounts of TG and FFAs increase during budding (Yubisui et al., in preparation). Concerning the latter, fatty acid-liberating enzymes, such as phospholipase, may be expressed during budding. A cDNA for PmPLA2 has been cloned (accession number, AB107990). It comprises 702 nucleotides with a deduced ORF encoding 167 amino acids. Like secretory PLA2 in other species, there is a highly hydrophobic domain at the N-terminus (Fig. 5A). Both a

Table 1. **Fatty acid composition of total lipids and phospholipids from *P. misakiensis* with reference to mammalian fatty acids.**

Fatty acids	Amount in total tunicate lipids (%)*	Amount in tunicate PL (%)	Amount in mammalian PL (%)
C14:0	0.4 ± 0.4	0.7 ± 0.4	ND
C15:1	3.4 ± 0.5	1.9 ± 0.5	ND
Unknown peak	2.3 ± 1.2	ND	0.9 ± 0.5
C16:0	8.0 ± 1.2	5.5 ± 0.4	14.3 ± 0.6
C16:1n-9	3.5 ± 0.7	2.7 ± 0.5	ND
C17:0	0.4 ± 0.4	ND	ND
C17:1	1.7 ± 0.5	ND	ND
C18:0	13.4 ± 2.2	7.4 ± 1.3	25.3 ± 0.7
C18:1n-9	13.5 ± 1.1	13.6 ± 0.4	17.1 ± 0.4
C18:2n-6	2.5 ± 0.2	2.7 ± 0.4	16.9 ± 0.3
C18:3n-3	0.4 ± 0.4	1.5 ± 0.2	ND
C20:0	2.3 ± 0.6	ND	ND
C20:4n-6	4.2 ± 0.0	6.9 ± 0.2	0.8 ± 0.8
C20:5n-3	17.9 ± 1.0	26.2 ± 1.2	ND
C22:0	ND	ND	0.7 ± 0.4
C24:0	ND	ND	0.3 ± 0.3
C24:1n-9	ND	ND	2.6 ± 0.2
C22:6n-3	14.6 ± 1.0	20.4 ± 1.7	1.5 ± 0.0
Unknown peak	0.6 ± 0.6	ND	ND
Others	11.5 ± 1.3	9.2 ± 1.6	9.2 ± 1.6

*mean ± S.D.; ND, not detected.

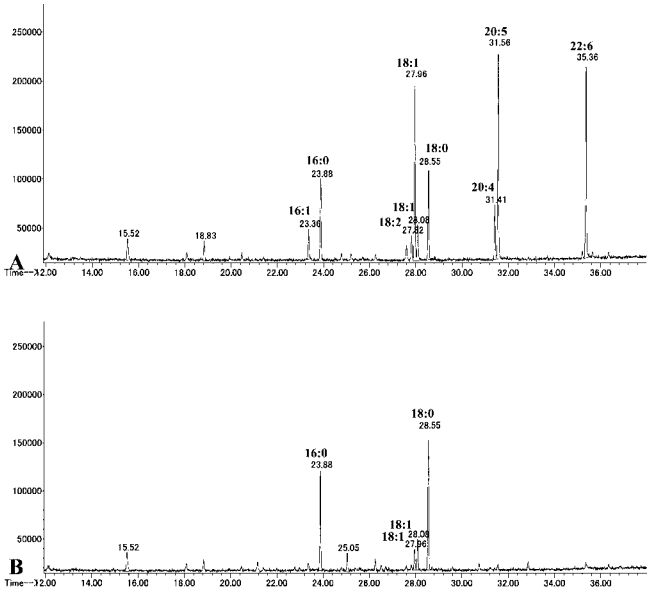

Fig. 4. **GC/MS of fatty acid components in tunicate phospholipids.** (A) Free fatty acids cut by bee venom PLA2. (B) Fatty acids remaining in lysophospholipids. Note that unsaturated long chains (C18:1, C20:5, C22:6) were characteristic in the free fatty acids.

histidine active site and aspartic acid active site are conserved (Fig. 5A boxes). PmPLA2 from which the N-terminal signal peptide and proenzyme domain (39 amino acids) were removed was prepared as an MBP fusion protein using a bacterial expression system (Fig. 5B). Aliquots (50 μg/500 μl) were incubated with authentic PC, PE, PI, or PS (100 μg each) for 4 h at room temperature. The FFAs produced were 1.85 μg from PC, 1.77 μg from PE, 1.47 μg from PS, and 0.97 μg from PI, respectively.

Gene expression of PmPLA2 was examined by *in situ* hybridization. Figure 6A shows a growing bud still attached to the parent animal. Adult animals had no signals (Fig. 6B), while buds showed specific signals on the epidermis (Fig. 6C). The apical area of epidermal cells was stained heavily (Fig. 6D). Buds enter morphogenetic stages after isolated from the parent. From two days after bud isolation onward, signals disappeared from the epidermis of developing buds as well as juvenile animals (Fig. 6E).

Biological Activities of Phospholipids and Their Derivatives—After separation by the first TLC (Fig. 4A), neither of the activities to promote cell growth and cell scattering could be found in the neutral lipids (Cho, ChoE, and TG), while they were present at the origin (not shown). After the second TLC of substances in the origin (Fig. 3C), fractions 2 and 3 showed those biological activities. In respective fractions, PI and PC spots of 5 μg/ml were sufficient to promote cell growth (Fig. 7) and cell scattering (not shown). For comparison, authentic substances were added to cultured cells. Mammalian PI and PC showed both mitogenic and scattering activities at concentrations of more than 2 μg/ml, but their activities were not so strong as tunicate phospholipids (not shown).

FFAs (4 μg/ml) were prepared from tunicate phospholipids for bioassay, as described (Fig. 4A). They were found to promote cell growth strongly (Fig. 7), but, because of the lack of cell scattering activity, cell aggregates became larger and larger during cell culture (Fig. 1F). The remaining phospholipids including lysophospholipids (3.6 μg/ml) were prepared for bioassay. As described, they contained mainly saturated fatty acids (Fig. 4B). They induced cell scattering, although they did not promote cell growth (Figs. 1E and 7).

DISCUSSION

Lipids are the growth-promoting factor dissolved in trypsinized supernatant—In this work, we found a striking mitogenic activity in the trypsinized supernatant of water-insoluble fractions of *Polyandrocarpa* homogenates. This activity was resistant to both heating (80°C, 10 min) and proteinase K digestion. Peptide bonds were scarcely detected in the Fourier transform infrared spectrum (FTIR); instead, large amounts of fatty acids and phospholipids were found. The mitogenic activity could be re-extracted by organic solvents such as chloroform. All these facts indicate that the mitogenic activity should be attributable to fat-soluble substances instead of proteins.

The question arises as to how trypsin liberated bioactive lipids from water-insoluble fractions. In mammals,

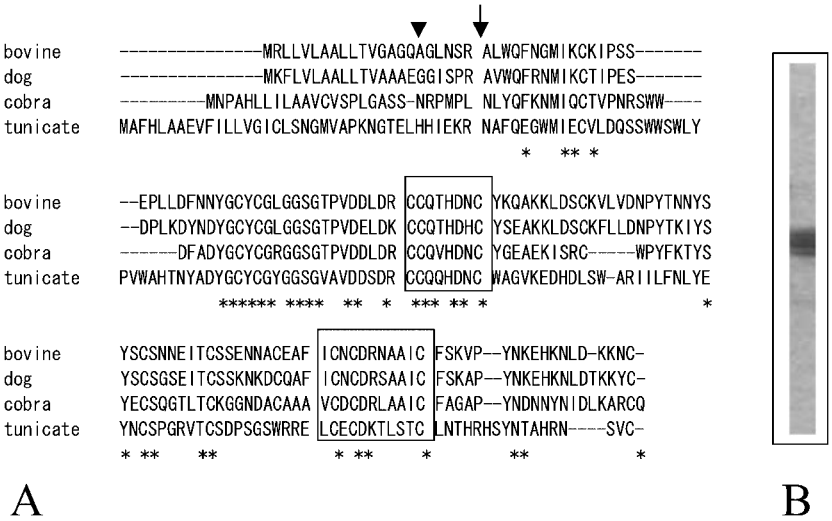

Fig. 5. **Deduced amino acid sequence of PmPLA2.** (A) Multiple alignment of PLA2s from several species. Signal peptides were cut off at positions marked by the arrowhead. Proenzymes were further cut (arrow) to become mature proteins. Boxes show a histidine active site and an aspartic acid active site. (B) Recombinant MBP-PmPLA2 fusion protein, SDS-PAGE.

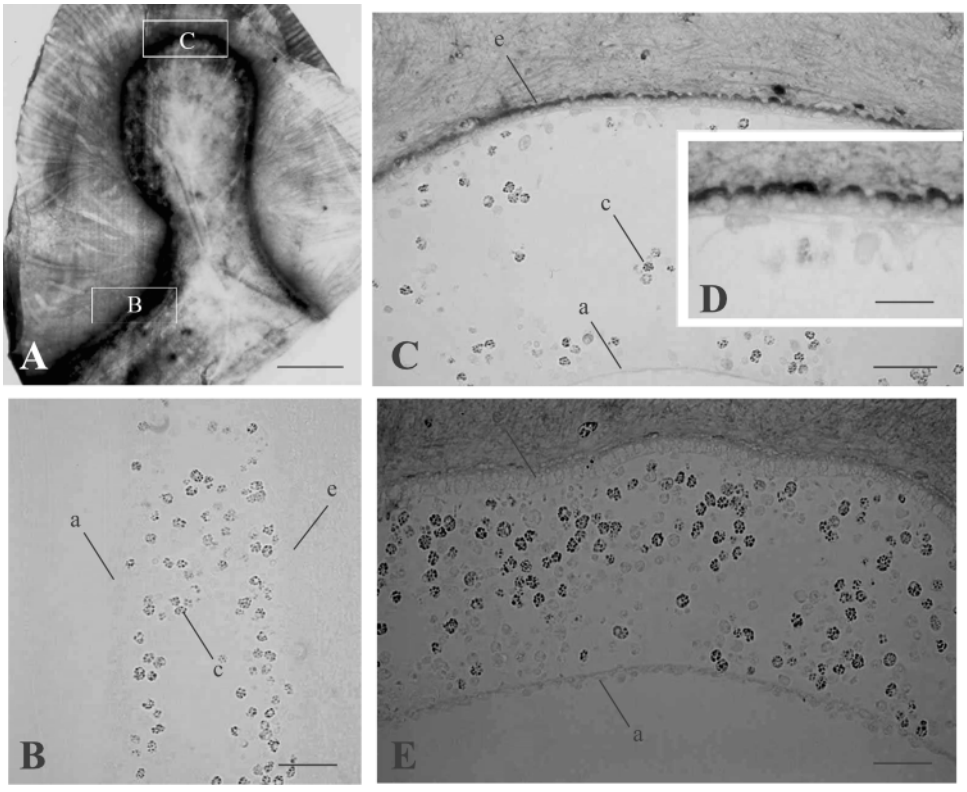

Fig. 6. **Gene expression of PLA_2 during budding of *P. misakiensis*.** (A) Whole mount in situ hybridization, a piece of parent animal and a protruding bud. The boxed area 'B' is shown in (B). The boxed area 'C' is shown in (C). Bar, 0.5 mm. (B) A section of parental tissue. Bar, 25 μm. (C) Distal area of a protruding bud. Bar, 50 μm. (D) Higher magnification of (C). Bar, 25 μm. (E) Distal area of a developing bud one day after isolated from the parent. Bar, 25 μm. a, atrial epithelium; c, coelomic cells; e, epidermis.

lysophospholipids are found associated with oxidized low density lipoproteins (LDL) and bind to the extracellular matrix (ECM) (*20*, *21*), where they are involved in various cellular functions (*22*). In *P. misakiensis*, both apolipoprotein, a component of LDL, and LDL receptor-related protein have been found in the EST project (*9*). Phospholipids and cholesterol exist in tunicate body fluid (this issue). Therefore, it is probable that in the body fluid of *P. misakiensis*, phospholipids and cholesterol bind LDL, and the complex is then trapped by the ECM owing to LDL-ECM interactions. The treatment of tissue homogenates with trypsin may have liberated phospholipids from the ECM by digesting ECM-LDL complexes (Fig. 7).

In *P. misakiensis*, the in vivo expression of trypsin-like serine proteases is upregulated at least twice (*8*, *9*). The first occurrence is during budding, when the gene expression of serine proteases increases three-fold over the level in the pre-budding stage (*9*). The second increase occurs at an early stage of bud development, during which the gene expression of tunicate retinoic acid-inducible modular protease (TRAMP) is upregulated (*8*). TRAMP has LDL receptor class A domains at its N-terminus and the catalytic domain of a trypsin-like serine protease at its C-terminus. These proteases may serve as in vivo phospholipid-releasing factors (Fig. 7).

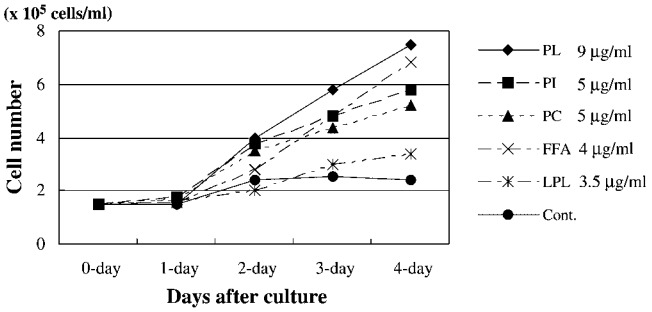

Fig. 7. **Effects of phospholipids and their derivatives on the cell proliferation of *P. misakiensis*.** Cells (1.5×10^5 cell/ml) were cultured for four days in 1 ml of basal medium containing PL or their derivatives dissolved in 5 μl DMSO. As a control, DMSO was added to the basal culture medium. FFA, free fatty acid; LPL, lysophospholipids; PC, phosphatidylcholine; PI, phosphatidylinositol; PL, phospholipid.

Phospholipids as Mitogen and Motogen—The present study shows that in *P. misakiensis*, phospholipids have activities to promote both cell growth and cell motility. After fractionation, PI and PC show strong activities. Authentic chemicals gave similar results, but their activities were not as strong as tunicate phospholipids. As discussed later, this may arise from differences in the fatty acid components.

In mammals, phospholipids become lipid mediators such as LPA and PAF after undergoing metabolic conversion to some extent. Actually, LPA acts as an intercellular messenger to trigger a variety of cellular functions, including cell growth and differentiation (*23*) and chemotaxis (*24*). It also contributes to the formation of the cytoskeletal network via the activation of the low molecular weight G protein, Rho (*25*). In *P. misakiensis*, unlike in mammals, LPA does not promote cell growth, although it does promote cell motility.

Some of bioactive lipids are known to activate PI3 kinase (*26*), and PI, PIP, and PIP_2 are possible substrates for PI3 kinase (*27*, *28*). Activated substrates are then involved in cell growth (*29–31*) and cell scattering (*32*). In *P. misakiensis*, wortmannin, a specific inhibitor of PI3 kinase (*33*), blocks cell growth induced by tunicate phos-

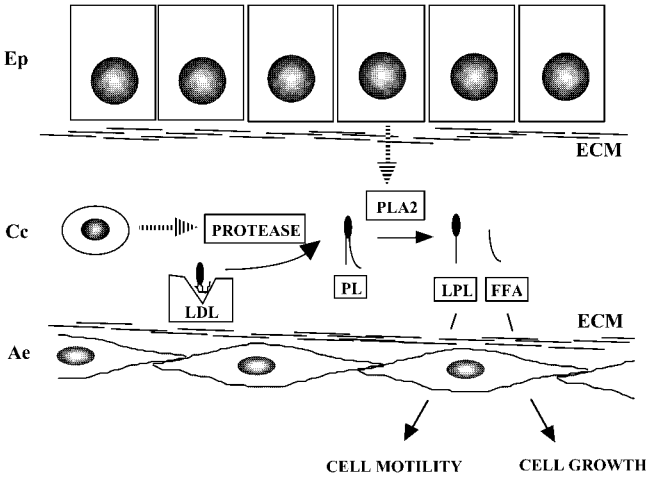

Fig. 8. **Scheme of the possible relationship among PL, protease, and PLA_2 in *P. misakiensis*.** PL(s) in the body fluid bind to LDL and are trapped by the ECM. Protease(s) secreted by coelomic cells attack the LDL-ECM complex to liberate PL. During budding, the epidermis secretes PLA2, which digests PL to give rise to FFA and LPL. FFA and LPL play roles in cell growth and cell scattering, respectively. Ae, atrial epithelium; Cc, coelomic cell; ECM, extracellular matrix; Ep, epidermis; FFA, free fatty acid; LDL, low density lipoprotein; LPL, lysophospholipids; PL, phospholipids; PLA_2, phospholipase A_2.

pholipids (our unpublished data). It is possible, therefore, that in *P. misakiensis* PI3 kinase might mediate the growth signal of PI.

Possible Role of Unsaturated Fatty Acids in Cell Growth—In *P. misakiensis*, the amounts of TG and FFAs increase during budding (Yubisui et al., in preparation). The increase in TG has been discussed in the context of the budding-specific expression of cytochrome b_5, which is well known as a participant in fatty acid metabolism in vertebrates (*34*). In the present study, TG as well as Cho and ChoE had no apparent effect on cell growth or cell motility.

PLA2 produces FFAs and LPAs from phospholipids (Fig. 8). Therefore, the expression of the $PmPLA_2$ gene during budding may be responsible for the increase in amount of FFAs. Results of in situ hybridization showed that the epidermis is a major source of PmPLA2 during budding (Fig. 7). Interestingly, the PLA2 mRNA is localized in the apical area of epidermal cells, although it is uncertain at present what this localization means. So far, two types of PLA_2 are known, one cytosolic and the other secretory (*35*). From the deduced amino acid sequence, PmPLA2 is supposed to be a secretory form. The recombinant PmPLA2 protein prefers PC and PE as substrates (PC = PE > PS > PI), as is the case for authentic PLA2.

The fatty acid components of *Polyandrocarpa* phospholipids differ from those of mammalian phospholipids in two points: very long chains of polyunsaturated fatty acids, and some odd-numbered chains (Table 1). Similar results have been reported for both sessile and pelagic tunicates (*17–19*). FFAs isolated from tunicate phospholipids by PLA2 were able to promote cell growth, although they did not support cell motility. These FFAs included both saturated (C16:0, C18:0) and unsaturated fatty acids (C18:1, C20:5, C22:6). Saturated fatty acids were also found among the remaining lysophospholipids after PLA2 treatment, and these stimulated cell motility only. These results suggest that C18:1, C20:5, and/or C22:6 are responsible for the mitogenic activity of fatty acids. In mammals, EPA (C20:5n-3) and DHA (C22:6n-3) do not stimulate cell growth of Swiss 3T3 fibroblasts (*36–38*). In tunicate cells, on the other hand, both exhibit cell growth-promoting activity (our unpublished data).

CONCLUSION

The present study is the first report that phospholipids act as mitogen and motogen in invertebrates. They are found in the supernatant of trypsinized tunicate homogenates. *In vivo*, budding-specific proteases (*6*, *7*) may serve as phospholipid-releasing factors from the ECM. The fatty acid components of phospholipids have unique structures and show cell growth-promoting activity when applied to cultured tunicate cells. They are believed to be released by PmPLA2 and act on multipotent tunicate cells. In *P. misakiensis*, budding involves cell division and dedifferentiation of the multipotent epithelium. Knowledge about mitogens will facilitate our understanding of how efficiently the developmental flexibility of somatic cells is achieved.

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REFERENCES

1. Fuchs, E. and Segre, J.A. (2000) Stem cells: A new lease on life. *Cell* **100**, 143–155
2. Weissman, I.L. (2000) Stem cells: Units of development, units of regeneration, and units in evolution. *Cell* **100**, 157–168
3. Kawamura, K. and Nakauchi, M. (1991) Homeostatic integration of stem cell dynamics during palleal budding of ascidians. *Zool. Sci.* **8**, 11–22
4. Watanabe, H. and Tokioka, T. (1972) Two new species and one possibly new race of social styelids from Sagami Bay, with remarks on their life history, especially the mode of budding. *Publ. Seto Mar. Biol. Lab.* **19**, 327–345
5. Kawamura, K. and Fujiwara, S. (1995) Cellular and molecular characterization of transdifferentiation in the process of morphallaxis of budding tunicates. *Semin. Cell Biol.* **6**, 117–126
6. Kawamura, K. and Fujiwara, S. (1994) Transdifferentiation of pigmented multipotent epithelium during morphallactic development of budding tunicates. *Int. J. Dev. Biol.* **38**, 369–377
7. Kawamura, K. and Sugino, Y.M. (1999) Cell adhesion in the process of asexual reproduction of tunicates. *Micro. Res. Tech.* **44**, 269–278
8. Ohashi, M., Kawamura, K., Fujii, N., Yubisui, T., and Fujiwara, S. (1999) A retinoic acid-inducible modular protease in budding ascidians. *Dev. Biol.* **214**, 38–45
9. Kawamura, K., Hayata, D., Fujiwara, S., and Yubisui, T. (1998) Serine protease inhibitors expressed in the process of budding of tunicates as revealed by EST analysis. *J. Biochem.* **124**, 1004–1012
10. Matsumoto, J., Nakamoto, C., Fujiwara, S., Yubisui, T., and Kawamura, K. (2001) A novel C-type lectin regulating cell growth, cell adhesion and cell differentiation of the multipotent epithelium in budding tunicates. *Development* **128**, 3339–3347
11. Baird, A. and Ling, N. (1987) Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: Implications for a role of heparins-like enzymes

in the neovascular response. *Biochem. Biophys. Res. Commun.* **142**, 428–435
12. Folkman, J., Klagsbrun, M., Sasse, J., Wadzinski, M., Ingber, D., and Vlodavsky, I. (1988) A heparin-binding angiogenic protein-basic fibroblast growth factor-is stored within basement membrane. *Amer. J. Pathol.* **130**, 393–400
13. Saksela, C. and Rifkin, D.B. (1990) Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity. *J. Cell Biol.* **110**, 767–775
14. Kawamura, K. and Fujiwara, S. (1995) Establishment of cell lines from multipotent epithelial sheet in the budding tunicate, *Polyandrocarpa misakiensis*. *Cell Struct. Funct.* **20**, 97–106
15. Brigh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917
16. Yoshida, S. and Takeshita, M. (1987) Analysis of the condensation step in elongation of very-long chain saturated and tetraenoic fatty acyl-CoAs in swine cerebral microsomes. *Arch. Biochem. Biophys.* **254**, 170–179
17. Miura, T., Okabe, M., Satake, M., Nakanishi, T., Inada, A., Fujimoto, Y., Hata, F., Matsumura, Y., and Ikekawa, N. (1986) Fatty acids and sterols of tunicate, *Salpa thompsoni*, from the Antarctic Ocean: chemical composition and hemolytic activity. *Chem. Pharm. Bull.* **34**, 4562–4568
18. Vysotskii, M.V., Ota, T., and Takagi, T. (1992) n-3 polyunsaturated fatty acids in lipids of ascidian *Halocynthia roretzi*. *Nippon Suisan Gakkaishi* **58**, 953–956
19. Carballeira, N.M., Shalabi, F., Stefanov, K., Dimitriv, K., Popov, S., Kujumgiev, A., and Andreev, S. (1995) Comparison of the fatty acids of the tunicate *Botryllus schlosseri* from the black sea with two associated bacterial strains. *Lipids* **7**, 677–679
20. Kaplan, M. and Aviram, M. (1997) Oxidized LDL binding to a macrophage-secreted extracellular matrix. *Biochem. Biophys. Res. Commun.* **237**, 271–276
21. Wheeler, D.C. and Chana, R.S. (1993) Interactions between lipoproteins, glomerular cells and matrix. *Miner. Electrolyte Metab.* **19**, 149–164
22. Hla, T., Lee, M.J., Ancellin, N., Paik, J.H., and Kluk, M.J. (2001) Lysophospholipids-receptor revelations. *Science* **294**, 1875–1878
23. Lee, M.J., Thangada, S., Liu, C.H., Thompson, B.D., and Hla, T. (1998) Lysophosphatidic acid stimulates the G-protein-coupled receptor EDG-1 as a low affinity agonist. *J. Biol. Chem.* **273**, 22105–22112
24. Gerrard, J.M., Clawson, C.C., and White, J.D. (1980) Lysophosphatidic acid : III Enhancement of neutrophil chemotaxis. *Amer. J. Pathol.* **100**, 609–618
25. Nobes, C.D. and Hall, A. (1995) Rho, rac, and cdc42 GTPase regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53–62
26. Rameh, L.E. and Cantley, L.C. (1999) The role of phosphoinositide 3-kinase lipid products in cell function. *J. Biol. Chem.* **274**, 8347–8350
27. Cantley, L.C. (2002) The phosphoinositide 3-kinase pathway. *Science* **296**, 1655–1657
28. Laffargue, M., Raynal, P., Yart, A., Peres, C., Wetzker, R., Roche, S., Payrastre, B., and Chap, H. (1999) An epidermal growth factor receptor/Gab1 signaling pathway is required for activation of phosphoinositide 3-kinase by lysophosphatidic acid. *J. Biol. Chem.* **274**, 32835–32841
29. Carpenter, C.L., Duckworth, B, C., Auger, K.R., Cohen, B., Schaffhausen, B.S., and Cantley, L.C. (1990) Purification and characterization of phosphoinositide 3-kinase from rat liver. *J. Biol. Chem.* **265**, 19704–19711
30. Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991) Oncogenes and signal transduction. *Cell* **64**, 281–302
31. Stephens, L.R., Jackson, T.R., and Hawkins P.T. (1993) Agonist-stimulated synthesis of phosphatidylinositol(3, 4, 5)-triphosphate: a new intracellular signaling system? *Biochim. Biophys. Acta* **1179**, 72–75
32. Wennstrom, S., Siegbahn, A., Yokote, K., Arvidsson, A.K., Heldin, C.H., Mori, S., and Claesson-Welsh, L. (1994) Membrane ruffling and chemotaxis transduced by the PDGF beta-receptor require the binding site for phosphatidylinositol 3′ kinase. *Oncogene* **9**, 651–660
33. Okada, T., Sakuma, L., Fukui, Y., Hazeki, O., and Ui, M. (1994) Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. *J. Biol. Chem.* **269**, 3563–3567
34. Yubisui, T., Takahashi, F., Takabayashi, T., Fujiwara, S., and Kawamura, K. (2001) Characterization of cytochrome b_5 in the ascidian *Polyandrocarpa misakiensis* and budding-specific expression. *J. Biochem.* **129**, 709–716
35. Dennis, E.A. (1997) The growing phospholipase A_2 superfamily of signal transduction enzyme. *Trends Biochem. Sci.* **22**, 1–2
36. Danesch, U., Weber, P.C., and Sellmayer, A. (1996) Differential effects of n-6 and n-3 polyunsaturated fatty acids on cell growth and early gene expression in Swiss 3T3 fibroblasts. *J. Cell Physiol.* **168**, 618–624
37. Chen, Z.Y. and Istfan, N.W. (2001) Docosahexaenoic acid, a major constituent of fish oil diets, prevents activation of cyclin-dependent kinases and S-phase entry by serum stimulation in HT-29 cells. *Prostagrandins Leukot. Essent. Fatty Acid* **64**, 67–73
38. Colquhoun, A., Ramos, K.L., and Schumacher, R.I. (2001) Eicosapentaenoic acid and docosahexaenoic acid effects on tumor mitochondrial metabolism, acyl CoA metabolism and cell proliferation. *Cell Biochem. Funct.* **9**, 97–105