Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production

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Autotaxin (ATX) is a tumor cell motility–stimulating factor, originally isolated from melanoma cell supernatants. ATX had been proposed to mediate its effects through 5′-nucleotide pyrophosphatase and phosphodiesterase activities. However, the ATX substrate mediating the increase in cellular motility remains to be identified. Here, we demonstrated that lysophospholipase D (lysoPLD) purified from fetal bovine serum, which catalyzes the production of the bioactive phospholipid mediator, lysophosphatidic acid (LPA), from lysophosphatidylcholine (LPC), is identical to ATX. The Km value of ATX for LPC was 25-fold lower than that for the synthetic nucleoside substrate, *p*-nitrophenyl-tri-monophosphate. LPA mediates multiple biological functions including cytoskeletal reorganization, chemotaxis, and cell growth through activation of specific G protein–coupled receptors. Recombinant ATX, particularly in the presence of LPC, dramatically increased chemotaxis and proliferation of multiple different cell lines. Moreover, we demonstrate that several cancer cell lines release significant amounts of LPC, a substrate for ATX, into the culture medium. The demonstration that ATX and lysoPLD are identical suggests that autocrine or paracrine production of LPA contributes to tumor cell motility, survival, and proliferation. It also provides potential novel targets for therapy of pathophysiological states including cancer.

Introduction

Lysophosphatidic acid (1- or 2-acyl-lysophosphatidic acid; LPA)* is an autacoid-like lipid mediator with multiple biological functions including induction of platelet aggregation, smooth muscle contraction, and stimulation of cell proliferation and chemotaxis (Moolenaar, 1999; Contos et al., 2000). LPA evokes its multiple effects through G protein–coupled receptors, with subsequent activation of PLC and phospholipase D, Ca^{2+} mobilization, inhibition of adenylyl cyclase, activation of MAPK, and transcription of serum response element–regulated genes, such as c-*fos*. LPA mediates its activity through a series of G protein–coupled receptors of the endothelial differentiation gene (EDG) family (Hecht et al., 1996; An et al., 1998; Bandoh et al., 1999).

In contrast to the extensive analysis of mechanisms underlying LPA signaling mediated by the LPA receptor family, the enzymes regulating LPA production and degradation have not been characterized fully. LPA can be produced by a variety of cells including platelets, fibroblasts, adipocytes, and ovarian cancer cells (Gerrard and Robinson, 1989; Eichholtz et al., 1993; Shen et al., 1998). LPA is also produced by the action of extracellular lysophospholipase D (lysoPLD) on lysophosphatidylcholine (LPC; Tokumura

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*Abbreviations used in this paper: ATX, autotaxin; EDG, endothelial differentiation gene; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; lysoPLD, lysophospholipase D; pNP-TMP, *p*-nitrophenyl-tri-monophosphate.

Key words: lysoPLD; EDG receptor; lysophosphatidylcholine; chemotaxis; cell proliferation

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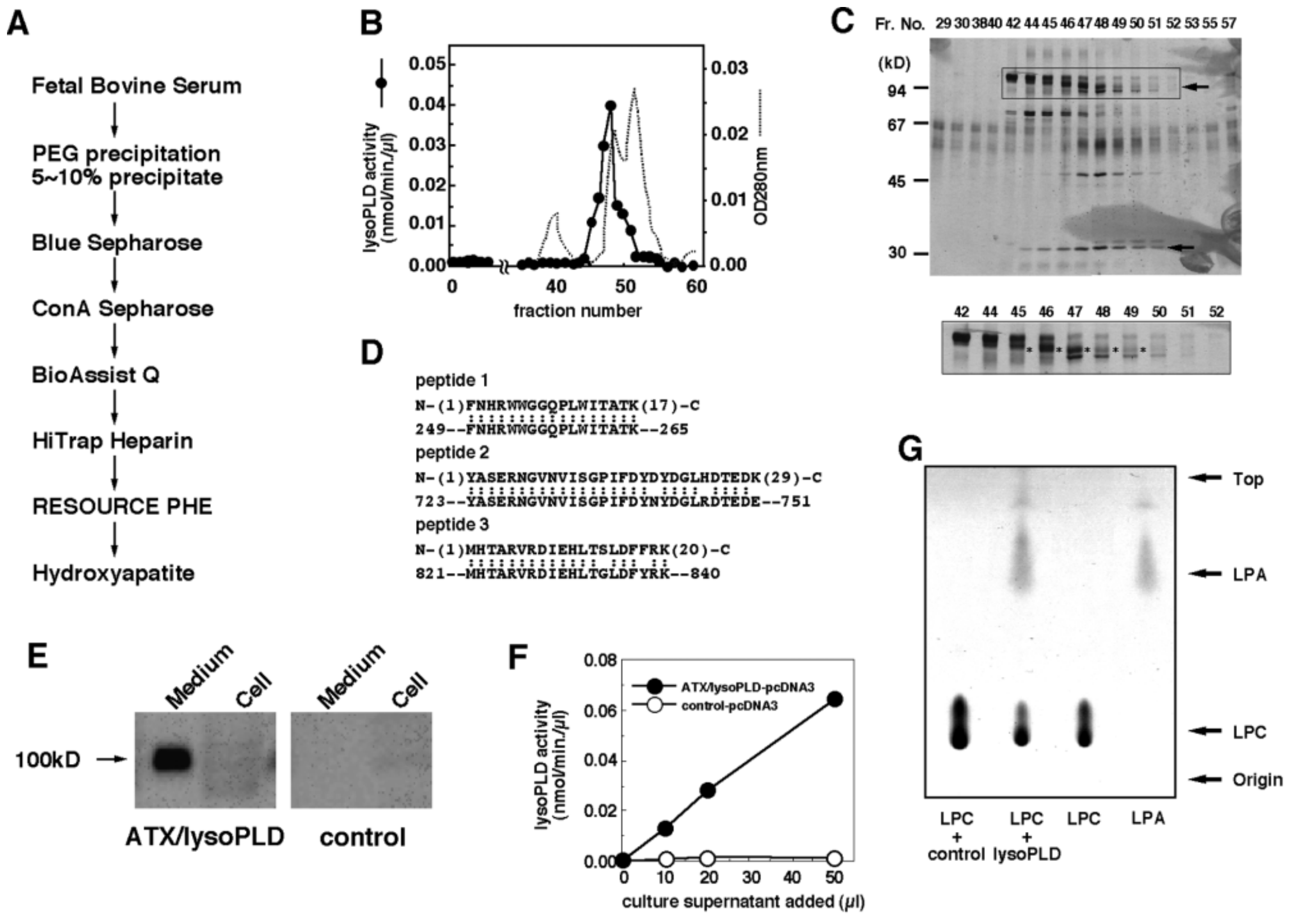

Figure 1. **Purification of lysoPLD and identification as ATX.** (A) Strategy used for purification of lysoPLD. (B) Elution profile of lysoPLD activity on hydroxylapatite column chromatography (CHT-II). The flow-through fractions of the RESOURCE® PHE hydrophobic column were applied to a CHT-II column, and the absorbed proteins were eluted with a linearly increasing gradient of KH_2PO_4 (0–0.4 M). 1.5 ml-fractions were collected, examined for lysoPLD activity (closed circle), and protein concentration was monitored by absorbance at 280 nm (dashed line). (C) The active fractions from the CHT-II column were subjected to SDS-PAGE and the proteins were detected by silver staining. Arrows indicate the protein bands that co-migrate with lysoPLD activity. The lower band (~30 kD) was a degradation product of the 100-kD band as judged by mass spectrometry and sequence analyses (unpublished data). In the bottom panel, an enlarged picture of the active fractions (fractions 42–52) is shown. The 100-kD band is indicated by an asterisk. (D) Alignment of peptide sequences obtained from amino acid sequence analysis of bovine lysoPLD with the amino acid sequence of rat autotaxin. Numbers in the second lines correspond to the amino acid numbers of rat ATX. Identical amino acids were indicated by colons. (E) Expression of ATX/lysoPLD recombinant protein in CHO-K1 cells. Culture supernatant or cells from myc epitope–tagged ATX/lysoPLD-pcDNA3 or control pcDNA3 vector transfected CHO-K1 were subjected to Western blot analysis using anti-myc mAb (9E11). ATX/lysoPLD protein was recovered almost exclusively from culture supernatant of ATX/lysoPLD-pcDNA3–transfected cells. (F) Recombinant ATX shows lysoPLD activity as assessed by choline release. The culture supernatant of ATX/lysoPLD-pcDNA3 (closed circle) or control pcDNA3 vector–transfected CHO-K1 (open circle) was subjected to a lysoPLD assay. (G) Formation LPA by lysoPLD. Phospholipids in the lysoPLD reaction (F) were analyzed by TLC after the lipids in the reaction mixture were extracted by organic solvents. LPC or LPA is included to indicate migration on the TLC plate.

et al., 1986), which is present at high micromolar levels in plasma (Okita et al., 1997; Tokumura et al., 1999; Croset et al., 2000). LPA can be detected in various biological fluids such as serum, plasma, ascites, and saliva (Tokumura et al., 1986, 1999; Tigyi and Miledi, 1992), and its levels are elevated in diverse physiological and pathological conditions such as pregnancy, high cholesterol diet, and ovarian cancer (Xu et al., 1995; Tokumura et al., 2000, 2002). Plasma lysoPLD appears to mediate the production of LPA in plasma (Tokumura et al., 1986), potentially contributing to the aberrant LPA levels in pathophysiological states. To characterize the as yet uncloned lysoPLD and to further elucidate the biological function of LPA, we purified lysoPLD from biological fluids.

Results and discussion

Purification and identification of lysoPLD as autotaxin

As assessed by the ability to liberate choline from LPC, lysoPLD activities are widely distributed in various biological fluids including serum and cerebrospinal fluid, with the highest activity being present in FBS. Thus, we purified lysoPLD from FBS (Fig. 1, A–C), resulting in an ~10,000-fold increase in specific activity. In each chromatographic step, lysoPLD activity eluted as a single peak (unpublished data), suggesting the presence of a single lysoPLD enzyme in FBS. Gel filtration and Con A column chromatography demonstrated that lysoPLD is a glycoprotein with an apparent molecular mass of 100 kD (unpublished data). Matrix-assisted laser desorption ionization mass spectrometry and Edman

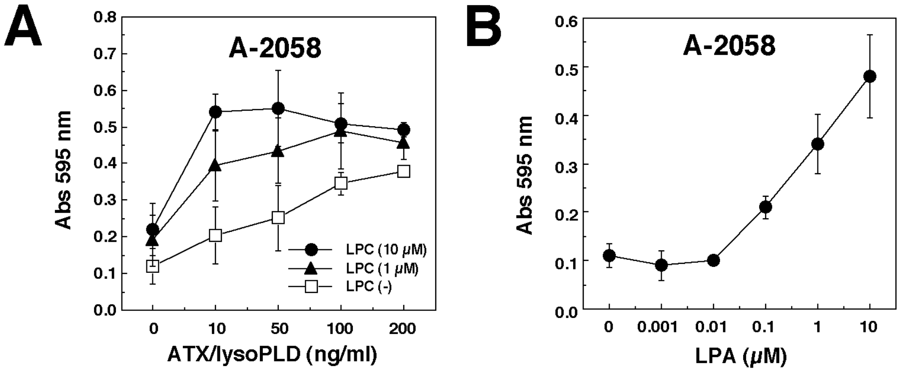

Figure 2. **ATX/lysoPLD-induced chemotaxis is enhanced by lysophosphatidylcholine in A-2058 melanoma cells.** (A) Lysophosphatidylcholine (LPC) enhances chemotaxis of A-2058 cells induced by recombinant ATX/lysoPLD. ATX/lysoPLD-induced chemotaxis was assessed in the presence or absence of LPC (1-oleoyl, 1 μM; closed triangle) or 10 μM (closed circle; mean ± SEM, $n = 4$). ATX/lysoPLD was produced by baculovirus (see Materials and methods). (B) Dose dependency of LPA (1-oleoyl)-induced chemotaxis of A-2058 and CHO-K1 cells (mean ± SEM, $n = 4$; see Materials and methods).

degradation of a lysylendopeptidase digest of the 100-kD protein after hydroxyapatite column chromatography (Fig. 1, B and C) showed three polypeptide sequences: FNHRW-WGGQPLWITATK, YASERNGVNVISGPIFDYDYDGL-HDTEDK, and MHTARVRDIEHLTSLDFFRK. These three sequences showed high (>95% identity) homology to rat, murine, and human autotaxin (ATX; Fig. 1 D). ATX is an autocrine motility factor that stimulates pertussis toxin–sensitive cell motility in human melanoma cells and possesses phosphodiesterase and pyrophosphatase activities (Stracke et al., 1992, 1997; Murata et al., 1994; Clair et al., 1997). LysoPLD catalyzes the hydrolysis of LPC phosphodiester bonds, which is compatible with it belonging to the same phosphodiesterase family as ATX.

ATX, a type II transmembrane ectoenzyme with multiple domains, is released from the cell by cleavage of the 12th amino acid external to the transmembrane domain (Stracke et al., 1997). Recombinant ATX was detected almost exclusively in culture supernatants of transfected CHO-K1 cells (Fig. 1 E) indicative of efficient extracellular cleavage. Similarly, lysoPLD activity, as assessed by choline production from LPC, was detected at high levels in the supernatants of ATX cDNA–transfected CHO-K1 cells but not in vector-transfected cells (Fig. 1 F), which is compatible with ATX possessing lysoPLD activity. Consistent with ATX possessing lysoPLD activity, LPA was readily detected by TLC after the addition of recombinant ATX to LPC (Fig. 1 G). ATX has been previously demonstrated to exhibit phosphodiesterase activity toward various nucleotide substrates including nucleotide triphosphate (NTP), nucleotide diphosphate (NDP), and a synthetic substrate, *p*-nitrophenyl-tri-monophosphate (pNP-TMP; Clair et al., 1997). The V_{max} and K_m values of recombinant ATX calculated from Lineweaver-Burk plots are 9.0 nmol/min·μg and 250 μM for LPC, and 11.0 nmol/min·μg and 14.1 mM for pNP-TMP, indicating a much higher affinity of ATX for LPC than that for pNP-TMP. Similar results were obtained when lysoPLD purified from FBS was assessed. Combined with the observation that LPC is present at high micromolar levels in biological fluids (Tokumura et al., 1999; Croset et al., 2000), LPC is likely to serve as a physiologically relevant substrate for ATX. Both purified lysoPLD and recombinant ATX failed to hydrolyze phosphatidylcholine with two long acyl chains, indicating that lysoPLD is selective for the lyso form of phospholipids (unpublished data).

ATX/lysoPLD stimulates cell motility particularly in the presence of LPC

The cell motility–stimulating activity of ATX on tumor cells requires an intact catalytic domain (Lee et al., 1996), however, the ATX substrate essential for the ability of ATX to induce cell motility has yet to be identified. In the following experiments, we investigated whether the cell motility–stimulating activity of ATX (Stracke et al., 1992; Fig. 2 A) could be explained by its lysoPLD activity because of the observation that LPA, the product of lysoPLD, is an effective inducer of chemotaxis in multiple cell lineages (Imamura et al., 1993; Stam et al., 1998; Sturm et al., 1999; Manning et al., 2000). Consistent with previous observations (Stracke et al., 1992), recombinant ATX produced in Sf9 cells stimulated a dose-dependent chemotaxis of the human melanoma A-2058 cell line using a Boyden chamber assay (Fig. 2 A). Interestingly, the ability of recombinant ATX to induce motility was dramatically increased by the addition of LPC, a substrate for lysoPLD (Fig. 2 A). LPC alone was insufficient to alter cell motility (Fig. 2 A). The addition of nucleotide substrates of ATX, including ATP, ADP, and adenosine (up to 100 μM) failed to alter the effects of ATX on cell motility (unpublished data). In contrast, exogenous LPA was sufficient to stimulate motility of A-2058 melanoma cells (Fig. 2 B). ATX in the absence of exogenous LPC was sufficient to modestly increase cellular motility (Fig. 2 A). If the effects of ATX on cellular motility are due to the hydrolysis of LPC and the subsequent action of LPA on cells, LPC must be present in the cells or cell supernatants. One possible explanation is that cancer cells can release LPC into culture media, which may in turn serve as substrate for ATX, resulting in the production of LPA which, in turn, induces cellular motility (see Fig. 4 A and next paragraph).

ATX/lysoPLD stimulates cell proliferation of multiple cancer cell lines

LPA has been demonstrated to be a potent inducer of cell proliferation (van Corven et al., 1989, 1992), an activity that had not previously been attributed to ATX. If ATX mediates LPA production from LPC, ATX could manifest proliferation-stimulating activity, particularly in the presence of exogenous LPC. As shown in Fig. 3 A, recombinant ATX stimulated proliferation of multiple cell lines including A-2058 melanoma cells, the breast cancer cell line MDA-MB-231, and CHO-K1 cells. As expected, LPA also stimulated

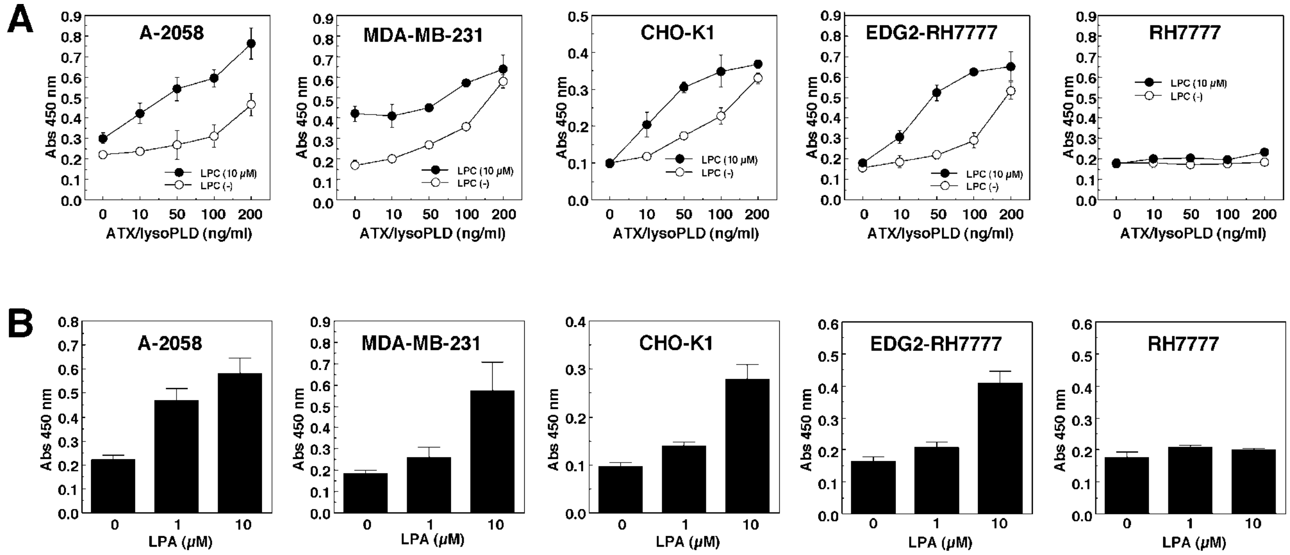

Figure 3. **ATX/lysoPLD stimulates cell proliferation.** (A) ATX/lysoPLD stimulates cell growth of cancer cell lines and LPA receptor–expressing rat hepatoma cells. Cells were starved for 48 h. Cell proliferation induced by the addition of recombinant ATX/lysoPLD both in the presence or absence of 10 μM LPC (1-oleoyl) was evaluated by MTT hydrolysis (mean ± SEM, *n* = 3). The following cells were used: A-2058 (melanoma, human), MDA-MB-231 (breast cancer, human), CHO-K1 (ovary-derived fibroblast, hamster), RH7777-EDG2, and parental RH7777 (hepatoma, rat). (B) Dose dependency of LPA (1-oleoyl)-induced cell growth. LPA-induced cell growth was assessed by MTT hydrolysis as in A (mean ± SEM, *n* = 3).

the proliferation of these cells (Fig. 3 B). The proliferation-stimulating activity of ATX was, once again, markedly increased by the addition of LPC to the media (Fig. 3 A). To further ascertain whether the activity of ATX can be attributed to its ability to produce LPA, we determined whether the ability of recombinant ATX to induce cellular proliferation required the expression of functional LPA receptors. RH7777 cells, which show no or little expression of functional LPA receptors (Fukushima et al., 1998), were minimally stimulated by LPA, whereas stable expression of the EDG2/LPA1 LPA receptor rendered RH7777 responsive to LPA-induced cellular proliferation (Fig. 3 B). Similarly, recombinant ATX exhibited minimal (if any) effect on the proliferation of parental RH7777 cells. Once again, expression of EDG2/LPA1 in RH7777 cells rendered the cells responsive to recombinant ATX, a process that was augmented by exogenous LPC (Fig. 3 A). As noted above, ATX was sufficient to modestly increase the proliferation of A-2058, MDA-MB-231, CHO-K1, and EDG2-RH7777 cells, which could be explained if these cell lines produce LPC. As demonstrated in Fig. 4 A, LPC (which is a substrate for ATX) could be readily detected in culture media of A-2058, MDA-MB-231, CHO-K1, and EDG2-RH7777 cells, suggesting that the effects of ATX in the absence of exogenous LPC could be mediated by the conversion of endogenously produced LPC to LPA.

Exogenous LPC was not sufficient to increase the proliferation of CHO-K1 or EDG2-RH7777 cells (Fig. 3 A). In contrast, exogenous LPC did increase the proliferation of A-2058 and MDA-MB-231 cells (Fig. 3 A). These results coincided with the observation that lysoPLD activity was readily detectable in the supernatant of A-2058 and MDA-MB-231 cells, but not CHO-K1 or EDG2-RH7777 cells (Fig. 4 B). As assessed by RT-PCR, A-2058 and MDA-MB-231 cells, but not CHO-K1 or EDG2-RH7777 cells, express ATX mRNA (unpublished data). Thus, the conversion of exogenously added LPC to LPA by endogenously expressed ATX appears sufficient to result in the proliferation of A-2058 and MDA-MB-231 cells.

Here, we have demonstrated that lysoPLD is identical to ATX and provided evidence that LPA, the product of ATX/lysoPLD, mediates chemotaxis and proliferation of cancer cells. LPA is known to stimulate both chemotaxis (Imamura et al., 1993; Stam et al., 1998; Sturm et al., 1999; Manning et al., 2000) and proliferation (van Corven et al., 1989, 1992) of multiple cell lineages. The LPA receptors, in particular the EDG2 receptor analyzed here, can couple with pertussis toxin–sensitive G_i (Hecht et al., 1996), which is consistent with the effect of pertussis toxin on cell motility induced by ATX. As assessed by quantitative PCR, all of the cancer cell lines (with the exception of RH7777) used here express EDG2 (unpublished data). Some tumor cells appear to be capable of secreting factors that stimulate their own motility, survival, and growth. Although autocrine secretion of motility factors might play a role in the initiation of tumor cell invasion, autocrine secretion of growth factors by tumor cells might contribute to the proliferation and survival of metastatic colonies. Both chemoattractant and proliferative activities can be stimulated by LPA, which can be produced by the action of ATX/lysoPLD on LPC. LPC is present at high levels in plasma, providing a potential source for paracrine or endocrine action of ATX. In the microenvironment of the tumor, LPC secreted by tumor cells may play an important role in ATX-mediated autocrine motility and proliferation of tumor cells.

LPC was detected in the culture media of various cell types (Fig. 4 A). Indeed, LPC levels in culture supernatants of RH7777 cells were ~1 μM. The release of LPC (Fig. 4 A) from cells was dependent on the presence of BSA in the

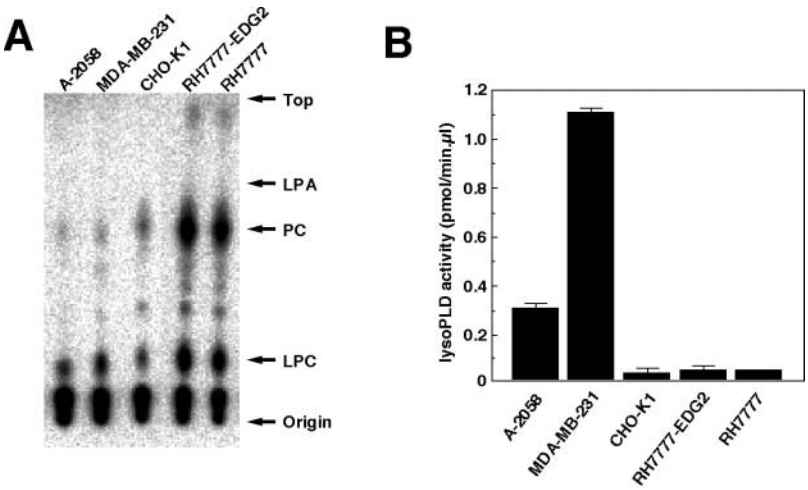

Figure 4. **Secretion of LPC and expression of lysoPLD activity by a variety of cell lineages.** (A) Monolayers of cell lines were preincubated with [^{32}P]orthophosphoric acid for 12 h. Lipids in the culture medium were extracted with chloroform and methanol, and then subjected to TLC. Radioactivity of each lipid spot was detected and visualized using an image analyzer. Identification of each lipid spot was confirmed by using standard lipids both in one- and two-dimensional TLC (unpublished data). (B) Cancer cells secrete lysoPLD. Cells were cultured for 48 h in serum-free medium, and the lysoPLD activity of the culture medium was determined (see Materials and methods). The following cells were used: A-2058, MDA-MB-231, CHO-K1, RH7777-EDG2, and parental RH7777.

media (unpublished data), which is capable of removing LPC from the outer membrane leaflet. LPC is also present at significant levels in intact cells (unpublished data). Thus, LPC released from cells or in the extracellular leaflet of the cell membrane could be a source of substrate for lysoPLD. A similar mechanism for LPA production was proposed by van Dijk et al. (1998) using bacterial phospholipase D. Indeed, when cell supernatants of RH7777 cells, which release high levels of LPC, were incubated with lysoPLD, LPA was readily detectable (unpublished data). ATX/lysoPLD was also present in the supernatant of a number of cancer cell lines. Thus, there is the potential for an autocrine loop where both ATX/lysoPLD and LPC are produced by the same cells, leading to LPA production. Alternatively, paracrine loops may occur where cells produce one (but not both) of ATX/lysoPLD or LPC.

ATX/lysoPLD (also called NPP-2) shares significant homology (~45% in amino acid level) with members of the nucleotide pyrophosphatase/phosphodiesterase (NPP) family, which includes PC-1/NPP-1 and gp130^{RB13-6}/NPP-3. PC-1/NPP-1 and gp130^{RB13-6}/NPP-3 have been implicated in multiple processes, including bone mineralization (Okawa et al., 1998; Nakamura et al., 1999) and signaling by insulin and by nucleotides (Bollen et al., 2000). As with ATX/lysoPLD, the biological effects of other NPP family members require a functional catalytic site. However, the physiological substrates for these two enzymes still remain to be identified. Indeed, it is possible that similar to ATX/lysoPLD, their substrates may be lipidlike molecules and potentially lysophospholipids.

Materials and methods

Materials

LPC (1-oleoyl) and LPA (1-oleoyl) were obtained from Avanti Polar Lipids, Inc. FBS was purchased from JRH Biosciences, Inc. Other chemicals were purchased from Sigma-Aldrich.

Purification of lysoPLD

5–10% polyethleneglycol precipitate of FBS was loaded onto blue Sepharose 6 Fast Flow (Amersham Biosciences), and eluted with a linear gradient of NaCl (0–2 M). The active fractions were loaded onto Con A Sepharose (Amersham Biosciences) and eluted with 5 mM α-methylmannopyranoside at room temperature. The active fractions from the eluate were sequentially loaded onto a BioAssist Q ion exchange column (TOSOH) and a HiTrap™ heparin column (Amersham Biosciences), and were eluted with a linear gradient of NaCl (0–0.5 M). Active fractions were loaded onto a RESOURCE® PHE hydrophobic column (Amersham Biosciences). Active fractions (flow-through fractions) were loaded onto an Econo-Pac CHT-II hydroxyapatite column (Bio-Rad Laboratories), and eluted with a linear gradient of Na_2HPO_4 (0–0.15 M). All column chromatography was performed at a neutral pH (7.5). The latter four-column chromatography steps were performed using ÄKTA™ (Amersham Biosciences). Amino acid sequence analysis of purified lysoPLD was performed as described previously (Takeda et al., 2001).

LysoPLD assay

1–50-µl samples were incubated with 1 mM LPC (from egg) in the presence of 100 mM Tris-HCl, pH 9.0, 500 mM NaCl, 5 mM $MgCl_2$, and 0.05% Triton X-100 for 1 h at 37°C. The liberated choline was detected by an enzymatic photometric method using choline oxidase (Asahi Chemical), horseradish peroxidase (Toyobo), and TOOS reagent (*N*-ethyl-*N*-(2-hydoroxy-3-sulfoproryl)-3-methylaniline; Dojindo Molecular Technologies, Inc.) as a hydrogen donor (Imamura and Horiuti, 1978; Tamaoku et al., 1982).

Plasmids and recombinant enzyme

Rat cDNA for ATX (corresponding to human autotaxin-T) was amplified by RT-PCR using a rat liver cDNA library as template DNA based on the sequence information in the database (*Rattus norvegicus* ectonucleotide pyrophosphatase/phosphodiesterase 2; GenBank/EMBL/DDBS accession no. NM_057104). A myc-tag was added at the COOH terminus. Transient transfection into CHO-K1 cells was performed using LipofectAMINE™ (Invitrogen). The rat cDNA for ATX was also introduced into the baculovirus transfer vector pFASTBac-1 (Invitrogen), and recombinant baculovirus was prepared according to the manufacturer's protocol. Purification of recombinant ATX/lysoPLD protein was performed as described above from 1 liter of culture supernatant of Sf9 insect cells infected with ATX/lysoPLD recombinant baculovirus.

Chemotaxis assay

A-2058 cells were maintained in RPMI 1640 with 5% heat-inactivated FBS at 37°C and 5% CO_2. Polycarbonate filters with 8-µm pores (Neuro Probe, Inc.) were coated with 13.3 mg/ml fibronectin (Sigma-Aldrich) in PBS for 60 min. A dry coated filter was placed on a 96-blind well chamber (Neuro Probe, Inc.) containing the indicated amounts of LPA (18:1; Avanti Polar Lipids, Inc.) or ATX/lysoPLD recombinant protein both in the presence or absence of 1 or 10 µM LPC (1-oleoyl; Avanti Polar Lipids, Inc.), and cells (200 µl, 8 × 10^4 per well) were added to the top wells. The ligand solution and cell suspension were prepared in the same buffer (serum-free RPMI 1640 medium containing 0.1% BSA). After incubation at 37°C in 5% CO_2 for 4 h, the filter was disassembled. The cells on the filter were fixed with methanol and stained with a Diff-Quick staining kit (International Reagents Corp.). The top side of the filter was scraped free of cells. The number of cells that migrated to the bottom side was determined by measuring optical densities at 595 nm using a 96-well microplate reader (model 3550; Bio-Rad Laboratories). When LPC or LPA was added to the cells, it was suspended in serum-free media containing 0.1% BSA.

Proliferation assay

Human EDG2 cDNA (in pcDNA3 expression vector) was cloned as described previously (Bandoh et al., 1999). Rat hepatoma RH7777 cells stably expressing human LPA1/EDG2 were established as described previously (Fukushima et al., 1998). Cancer cell lines were obtained from American Type Culture Collection. MDA-MB-231 cells were maintained in RPMI 1640 with 5% heat-inactivated FBS at 37°C and 5% CO_2. CHO-K1 and RH7777 cells were maintained in Ham's F12 and DME, respectively, with 10% heat-inactivated FBS at 37°C and 5% CO_2. Cells were seeded in 96-well plates and cultured for 24 h. Cells were starved for 48 h by replacing the media with serum-free media (DME for RH7777 cells, Ham's F12 for CHO-K1 cells, and RPMI 1640 for other cancer cells) containing 0.1% BSA, followed by addition of the indicated amount of LPA or recombinant ATX/lysoPLD in the presence or absence of LPC (10 μM). The cells were further cultured for 48 h. Cell proliferation was evaluated by MTT hydrolysis using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.). When LPC or LPA was added to the cells, it was suspended in serum-free media containing 0.1% BSA.

Lipid analysis

Phospholipids in reaction mixture, cells, or culture media were extracted by the method of Bligh and Dyer (1959) under acidic conditions by adjusting the pH to 3.0 with 1 N HCl to recover LPA efficiently. Lipids in the aqueous phase were reextracted and pooled with the previous organic phase. The extracted lipids were dried, dissolved in chloroform/methanol (1:1), and used for thin TLC analysis using chloroform/methanol/formic acid/H_2O (60:30:7:3, vol/vol) for 1-D TLC, and chloroform/methanol/formic acid/H_2O (60:30:7:3, vol/vol) and chloroform/methanol/28% ammonia/H_2O (50:40:8:2, vol/vol) for 2-D TLC as organic solvents. Phospholipids were detected using iodine vapor. For detection of LPC in cultured cells, cells were cultured for 12 h in the presence of [^{32}P]orthophosphate (50 μCi/ml) and serum-free media containing 0.1% BSA in 12-well plates before the lipid extraction. The radioactivities were detected using an image analyzer (model BAS 2000; Fuji Film). The recovery of lipids was monitored by the addition of trace amounts of 1-[^{3}H]oleoyl–LPC or 1-[^{3}H]oleoyl–LPA to the samples. Under the described conditions, recoveries of 1-[^{3}H]oleoyl–LPC and 1-[^{3}H]oleoyl–LPA were always >95%. Concentration of LPC was determined by an enzyme-linked fluorometric method. After the lipids in the samples were extracted and concentrated, LPC concentration was determined by fluorometry of H_2O_2 using 3-(4-hydroxyphenyl) propionic acid (7.5 mM) as a peroxidase donor (Tamaoku et al., 1982). H_2O_2 was generated by reaction of LPA with 500 U/ml monoglyceride lipase (Asahi Chemical Industry Co. Ltd.), 10 U/ml phosphocholine phosphodiesterase (GPCP; Asahi Chemical Industry Co. Ltd.), and 500 U/ml glycero-3-phosphate oxidase (Asahi Chemical Industry Co. Ltd.) in a buffer containing 50 mM Hepes, 2 mM $CaCl_2$, 0.2% Triton X-100, and 100 U/ml peroxidase (Toyobo), pH 7.5, in a total volume of 1,500 μl. Fluorescence intensity at excitation at 320 nm/emission at 404 nm was measured by a fluorometer (Hitachi) 5 min after mixing the samples. LPC concentrations as low as 0.1 nmol could be readily detected. The assay was linear up to 10 nmol.

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