Biochemical and Molecular Characterization of a Novel Choline-specific Glycero phosphodiester Phosphodiesterase Belonging to the Nucleotide Pyrophosphatase/Phosphodiesterase Family

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The mammalian NPP family comprises seven members, but only three of these (NPP1–3) have been studied in some detail. Previously we showed that lysophospholipase D, which hydrolyzes lysophosphatidylcholine (LPC) to produce lysophosphatidic acid, is identical to NPP2. More recently an uncharacterized novel NPP member (NPP7) was shown to have alkaline sphingomyelinase activity. These findings raised the possibility that other members of the NPP family act on phospholipids. Here we show that the sixth member of the NPP family, NPP6, is a choline-specific glycero phosphodiester phosphodiesterase. The sequence of NPP6 encodes a transmembrane protein containing an NPP domain with significant homology to NPP4, NPP5, and NPP7/alkaline sphingomyelinase. When expressed in HeLa cells, NPP6 was detected in both the cells and the cell culture medium as judged by Western blotting and by enzymatic activity. Recombinant NPP6 efficiently hydrolyzed the classical substrate for phospholipase C, p-nitrophenyl phosphorylcholine, but not the classical nucleotide phosphodiester substrate, p-nitrophenyl thymidine 5′-monophosphate. In addition, NPP6 hydrolyzed LPC to form monoacylglycerol and phosphorylcholine but not lysophosphatidic acid, showing it has a lysophospholipase C activity. NPP6 showed a preference for LPC with short (12:0 and 14:0) or polyunsaturated (18:2 and 20:4) fatty acids. It also hydrolyzed glycero phosphorylcholine and sphingosylphosphorylcholine efficiently. In mice, NPP6 mRNA was predominantly detected in kidney with a lesser expression in other organs.

Nucleotide pyrophosphatases/phosphodiesterases (NPPs) are ubiquitous membrane-associated or secreted ectoenzymes that release nucleoside 5′-monophosphate from a variety of nucleotides and nucleotide derivatives. The mammalian NPP family comprises seven members, but only three of these (NPP1–3) have been studied in some detail. Previously we showed that lysophospholipase D, which hydrolyzes lysophosphatidylcholine (LPC) to produce lysophosphatidic acid, is identical to NPP2. More recently an uncharacterized novel NPP member (NPP7) was shown to have alkaline sphingomyelinase activity. These findings raised the possibility that other members of the NPP family act on phospholipids. Here we show that the sixth member of the NPP family, NPP6, is a choline-specific glycero phosphodiester phosphodiesterase. The sequence of NPP6 encodes a transmembrane protein containing an NPP domain with significant homology to NPP4, NPP5, and NPP7/alkaline sphingomyelinase. When expressed in HeLa cells, NPP6 was detected in both the cells and the cell culture medium as judged by Western blotting and by enzymatic activity. Recombinant NPP6 efficiently hydrolyzed the classical substrate for phospholipase C, p-nitrophenyl phosphorylcholine, but not the classical nucleotide phosphodiester substrate, p-nitrophenyl thymidine 5′-monophosphate. In addition, NPP6 hydrolyzed LPC to form monoacylglycerol and phosphorylcholine but not lysophosphatidic acid, showing it has a lysophospholipase C activity. NPP6 showed a preference for LPC with short (12:0 and 14:0) or polyunsaturated (18:2 and 20:4) fatty acids. It also hydrolyzed glycero phosphorylcholine and sphingosylphosphorylcholine efficiently. In mice, NPP6 mRNA was predominantly detected in kidney with a lesser expression in brain and heart, and in human it was detected in kidney and brain. The present results suggest that NPP6 has a specific role through the hydrolysis of polyunsaturated LPC, glycero phosphorylcholine, or sphingosylphosphorylcholine in these organs.

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1 The abbreviations used are: NPP, nucleotide pyrophosphatase/phosphodiesterase; hNPP, human NPP; mNPP, mouse NPP; GDE, glycero phosphodiester phosphodiesterase; lsoPLC, lysophospholipase C; lsoPLD, lysophospholipase D; PLC, phospholipase C; LPC, lysophosphatidylcholine; LPA, lysophosphatidic acid; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; SPC, sphingosylnlyosphosphorylcholine; GPC, glycero phosphorylcholine; pNPP, p-nitrophenyl phosphorylcholine; pNP-TMP, p-nitrophenyl thymidine 5′-monophosphate; pNPPC, p-nitrophenyl phosphorylcholine; MG, monocacylglycerol; alk-SMase, alkaline sphingomyelinase; PAF, platelet-activating factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; and AQP, aquaporin.
Choline-specific Glycerophosphodiester Phosphodiesterase

The finding that NPP2 has lysoPLD activity provides a mechanism by which NPP2/autotaxin stimulates the cell motility. In addition, it was recently revealed that intestinal alk-SMase, which is predominantly expressed in gut, is a new member of NPP family (we call the enzyme NPP7 in this study) (4). These findings raised the possibility that other members of the NPP family act on phospholipids. In this study, we biochemically characterized a novel member of the NPP family, NPP6, and show that it is a choline-specific glycerophosphodiester phosphodiesterase (choline-GDE).

EXPERIMENTAL PROCEDURES

Materials—Egg LPC, 1- lauroyl (12:0) LPC, 1-myristoyl (14:0) LPC, 1-palmitoyl (16:0) LPC, 1-stearoyl (18:0) LPC, 1-arachidyl (20:0) LPC, 1-oleyl (18:1) LPC, 1-oleoyl LPA, 1-oleoyl lysophosphatidylethanolamine (LPE), 1-oleoyl lysophosphatidylserine (LPS), dioleoyl phosphatidylcholine (PC), dioleoyl phosphatic acid, dioleoyl phosphatidylethanolamine, and dioleoyl phosphatidylserine were purchased from Avanti Polar Lipids (Alabaster, AL). 1-Linoleoyl (18:2) LPC, porcine liver lysophosphatidylinositol (LPI), porcine liver phosphatidylinositol, dioleoyl phosphatidylglycerol, and sphingomyelin (SM) were from Doerfler Laboratories (Plymouth Meeting, PA). P-Nitrophenyl thymidine (P-NP), nitrophenyl thymidine (pNPPP), nitrophenyl thymidine 5'-monophosphate (pN-TP), nitrophenyl phosphorylcholine (pNPC), bis(p-nitrophenyl) phosphate, and anti-Myc mouse IgG monoclonal antibody (9E10) were from Sigma.

LysoPLD, LysoPLC, and GDE Assays—LysoPLD assay was performed as described previously (20). LysoPLC activity was assayed as follows. LPC (2 μM) was incubated with recombinant NPP6 protein in buffer containing 500 mM NaCl, 0.05% Triton X-100, and 100 μM Tris-HCl (pH 9.0) for 2 h at 37 °C, and the produced 2′-OH-choline oxidase as described previously (20). The lysoPLC activity of NPP6 toward choline-containing phospholipids, such as SPC, platelet-activating factor (PAF), lysOPAF, PC, and SM, and the GDE activity toward glycerophosphorylcholine (GPC) were expressed as the amount of choline released. Choline was measured using the same method as that used to measure LPC. To examine lysoPLC activity against LPE, LPS, and LPI, phosphatase activity against LPA, and PLC activity against PC, phosphatidylethanolamine, phosphatidylserine, and phosphatidylycerol, the formation of monoacylglyceride (MG) or diacylglyceride in the reaction mixture was measured using a Triglyceride E-Test Wako (Wako Pure Chemical Industries, Ltd.) in which the amount of glycerol released by lipase was quantified. The concentration of LPA was determined as described previously (20). The determination of LPI activity was performed as described previously (24). In brief, LPC was hydrolyzed by lysophospholipase (EC 3.1.1.5) and glycerophosphocholine phosphodiesterase (EC 3.1.4.2), and released choline was measured as described above.

Western Blotting—Protein samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes using the Bio-Rad transfer system. The membranes were blocked with Tris-buffered saline (TBS) containing 0.05% Tween 20, incubated with anti-hNPP6 monoclonal antibody, and then treated with anti-rat IgG-horseradish peroxidase. Proteins bound to the antibodies were visualized with an enhanced chemiluminescence kit (ECL, Amersham Biosciences).

Immunofluorescent Staining—HeLa cells transfected with hNPP6 cDNA were grown on coverslips, fixed with 3.7% (w/v) formaldehyde in phosphate-buffered saline. When intracellular organelles were stained with organelle markers, cells were additionally permeabilized with 0.1% Triton X-100 in phosphate-buffered saline. After incubating the cells with antibodies (rat anti-hNPP6 monoclonal antibody and mouse anti-caveolin-2 monoclonal antibody (BD Biosciences), mouse anti-p230 polyclonal antibody (BD Biosciences), or rabbit anti-calnexin) and incubated with anti-hNPP6 monoclonal antibody, and then treated with anti-rat IgG-horseradish peroxidase. Proteins bound to the antibodies were visualized with an enhanced chemiluminescence kit (ECL, Amersham Biosciences).

Purification of Recombinant Protein—Cell culture supernatant of HeLa cells (~500 ml) was extracted with either NHP6-ex or mNPP6-ex cDNAs was loaded onto a Mono Q ion exchange chromatography column (Amersham Biosciences) and eluted with a linear gradient of NaCl (0–2 M) using the AKTA system (Amersham Biosciences).

Monoclonal Antibody to hNPP6—A polyclonal antibody corresponding to the 80 amino acids of hNPP6 (444EVLGIIVTFYCTLEYGNVTDPNFLANASDALSFSFKRGLAAYHERIDVEHYGHYPSQPBDKALAVTVLYMTK) was expressed in Escherichia coli as a glutathione S-transferase fusion protein. W3YK2zm rats were immunized via the hind footpads with the recombinant protein using Freund’s complete adjuvant. The sera of the rats were used for cell fusion with mouse myeloma cells, PAL. The antibody-secretory hybridoma cells were selected by screening with enzyme-linked immunosorbent assay, immunofluorescence, and Western blotting. In this study we established four hybridoma cell lines, and we used 7C5 for Western blotting and immunofluorescence analyses.

Characterization of NPP Activity of NPP6—The phosphodiesterase activity of NPP6 was examined by using four classical NPP substrates (pNPPP, pNP-TP, pNPC, and bis(p-nitrophenyl) phosphate). The recombinant NPP6 protein was used as an enzyme source. Substrates (2 mM each) were incubated with recombinant NPP6 in buffer containing 500 mM NaCl, 0.05% Triton X-100, and 100 μM Tris-HCl (pH 9.0) for 1 h at 37 °C, and the production of p-nitrophenol was kinetically analyzed by measuring the optical density at 405 nm every 5 min for 2 h in a microplate reader (Bio-Rad, model 550).

Expression of NPP6 in HeLa Cells—The cDNA clones A535867 (hum) and AK046881 (mouse) were identified as NPP6 by searching the GenBankTM database using the amino acid sequence of either NPP6 or NPP7. For in vitro transfection, the full-length (mNPP6) was amplified by PCR using primers 5′-CCATAGATCTGTGGCAGACCT-3′ and 5′-CAATATCTGCAGTATGCGAC-3′ and human kidney cDNA as a template DNA. cDNA for the extracellular domain of hNPP6 (hNPP6-ex, amino acids 1–421) was amplified by PCR using primers 5′-CCATAGATCTGTGGCAGACCT-3′ and 5′-ATCAGCTCGAG-3′ and human kidney cDNA as a template DNA. cDNA for the extracellular domain of mNPP6 (mNPP6-ex) was amplified by PCR using primers 5′-CCATAGATCTGTGGCAGACCT-3′ and 5′-CAATATCTGCAGTATGCGAC-3′ and mouse kidney cDNA as a template DNA. cDNA for the extracellular domain of mNPP6 was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

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The enlarged medial iliac lymph nodes from the rats were used for cell culture. The cells were cultured in DMEM supplemented with antibiotics, glutamine, and 10% (v/v) fetal bovine serum in a humidified atmosphere at 37 °C in 5% CO2. The culture media were collected 72 h after the transfection. To reduce the serum content, the medium change was performed 24 h after the transfection to serum-free media. The cells were cultured for another 48 h, and the culture medium was used as an enzyme source.

Quantitative Real Time Reverse Transcription-PCR—Total RNA from cells was extracted using Isogen (Nippongene, Toyama, Japan) and reverse transcribed using the SuperScript first strand synthesis system for reverse transcription-PCR (Invitrogen). Oligonucleotide primers for PCR were designed using Primer Express Software (Ap-
FIG. 1. Sequence analyses of NPP6. A, nucleotide and amino acid sequence of hNPP6. The first and second lines indicate the nucleotide and the deduced amino acid sequence, respectively. Nucleotide and amino acid positions are shown on both sides. The putative catalytic serine (Ser71) is shown in reverse. The consensus sequences for Asn-linked glycosylation sites are boxed. The amino-terminal signal peptide is underlined. The double underlined sequences indicate the carboxyl-terminal hydrophobic peptide.

B, sequence alignment of hNPP6, hNPP7/alk-SMase, hNPP4, hNPP5, hNPP1, hNPP2, and hNPP3. Residues conserved among more than four members are shown in reverse. Residues marked by an asterisk are the putative catalytic centers. C, phylogenetic relationship of members of the hNPP family. A phylogenetic tree was generated from Genetyx-Mac Version 10.1.6 (Software Development Co. Ltd., Tokyo, Japan). The sequence divergence between any pair of sequences is equal to the sum of lengths of horizontal branches connecting the two sequences. D, domain structure and membrane orientation of seven NPP members. NPP1–3 are type II membrane proteins, and NPP4–7 are type I membrane proteins. Ex, extracellular; In, intracellular.
RESULTS

Identification of NPP6—Previously NPP4 and NPP5 were identified in the GenBank™ data base as close homologues of the NPP family members. Recently another member of the NPP family, NPP6, was registered. hNPP6 (GenBank™ accession number AY358676) is on human genome 4q35.1 and mNPP6 (GenBank™ accession number AY358676) is on chromosome 11. The deduced amino acid sequence had identities of 86% amino acid identity with mNPP6 and confirmed the nucleotide sequences. hNPP6 shows 86% amino acid identity with mNPP6 (Fig. 1C and D). The deduced amino acid sequence had identities of 33% with NPP1/PC-1, 25% with NPP2/autotaxin/lysoPLD, 29% with NPP3/gp130, 32% with NPP4, 34% with NPP5, and 31% with NPP7/alk-SMase. The phylogenetic tree in Fig. 1C showed that NPP6, NPP4, NPP5, and NPP7/alk-SMase form a subfamily within the NPP family. Thr238 residues in NPP1/PC1 and Thr210 in NPP2/autotaxin/lysoPLD have been shown to be the catalytic centers of these enzymes and are completely conserved within the NPP family. It is noted that the Thr residue is replaced by Ser71 in both hNPP6 and mNPP6 (Fig. 1, A and B).

Expression and Cellular Distribution of NPP6—To determine the biochemical characteristics and cellular distribution of the protein, we first tried to express NPP6 in mammalian cells. When hNPP6 was expressed in HeLa cells by transiently transfecting cDNA for hNPP6, we detected it as a protein band with an apparent molecular mass of 50 kDa on Western blotting analysis using anti-hNPP6 monoclonal antibody (Fig. 2A). Most of the protein was recovered in cells, but a small portion was detected in the cell culture medium (Fig. 2A). The fluorescence image of recombinantly expressed hNPP6 shows that it is localized in plasma membrane, although cytoplasmic and perinuclear staining is also apparent (Fig. 2B). To further examine the cellular localization of hNPP6 in detail we stained hNPP6 with endogenous organelle markers. Although the images did not completely merge, the fluorescence images of hNPP6 mainly overlapped with those of a plasma membrane marker, caveolin-2, indicating that most of the cell-associated hNPP6 protein is localized to the plasma membrane (Fig. 2C). A cluster of hydrophobic amino acid residues is present at the carboxyl terminus of both hNPP6 (Fig. 1A) and mNPP6 (data not shown) that is probably a transmembrane region of NPP6. To confirm this we constructed a truncated form of hNPP6 (hNPP6-ex) in which the carboxyl terminal hydrophobic sequence (amino acids 422–440) was removed. As shown in Fig. 2A, most of the hNPP6-ex protein expressed in HeLa cells was recovered from culture supernatant, showing that the hydrophobic sequence is required for membrane association of hNPP6. Similar cellular distribution of NPP6 was observed in CHO-K1 cells (data not shown). Thus, NPP6 is a membrane protein, and a small portion is secreted from cells as a soluble form as is often the case with membrane proteins.

NPP6 Hydrolyzes a Classical PLC Substrate, p-Nitrophosphorylcholine—To characterize the enzymatic activity of NPP6, we next prepared hNPP6 recombinant protein. To do this we transfected HeLa cells with cDNA for the truncated form of hNPP6 (hNPP6-ex) or mNPP6 (mNPP6-ex), and the
**Expression and cellular distribution of NPP6 protein in HeLa cells.** A. HeLa cells were transfected with cDNA for hNPP6, extracellular domain of hNPP6 (hNPP6-ex), or empty vector (mock), and the cellular distribution of hNPP6 protein was determined by Western blotting using anti-hNPP6 monoclonal antibody. B. HeLa cells transfected with either hNPP6 or mock plasmids were fixed with 3.7% formaldehyde in phosphate-buffered saline and were stained with anti-hNPP6 monoclonal antibody. C. Intracellular localization of hNPP6 and organelle markers in transfected HeLa cells. Monolayers of HeLa cells were transfected with hNPP6 plasmids. Twenty-four hours post-transfection cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and stained with anti-hNPP6 (green) and organelle markers (red or blue, anti-caveolin-2 (plasma membrane marker), anti-p230 (Golgi), anti-calcinexin (endoplasmic reticulum marker), and 4,6-diamidino-2-phenylindole (DAPI) (nuclear marker)). Overlaid images are also shown.

Recombinant proteins were purified using ion exchange chromatography (Mono Q) (data not shown). Using the recombinant proteins we first tested classical phosphodiesterase substrates. We chose pNPPP (25), pNP-TMP (26), pNPPC (27), and bis(p-nitrophenyl) phosphate (28) (Fig. 3A). pNPPC is a classical substrate for phospholipase C. The hydrolysis of these substrates was monitored by the formation of p-nitrophenol (optical density at 405 nm). Among the substrates, pNPPC and pNPPP were found to be hydrolyzed efficiently by both hNPP6 (Fig. 3B) and mNPP6 (not shown) recombinant proteins. We also examined the catalytic activity of NPP6 using pNPPC as a substrate and culture supernatants from HeLa cells transfected with various NPP6 cDNAs. The pNPPC hydrolyzing activity was detected in all the cell culture supernatants from HeLa cells transfected with NPP6 cDNAs (hNPP6, mNPP6, hNPP6-ex, and mNPP6-ex) but not from HeLa cells transfected with mock cDNA (Fig. 3C). This analysis confirmed that NPP6 is actually enzymatically active and that NPP6 is secreted from cells like other members of NPP family (20, 29).

**NPP6 Shows LysoPLC Activity Toward LPC—**As NPP6 hydrolyzed pNPPC, we next tried to identify an endogenous substrate using recombinant NPP6 proteins. We first tested whether LPC can be a substrate of NPP6. To detect both lysophospholipase C (lysoPLC) and lysoPLD activities we first tested classical phosphodiester substrates. The hydrolysis of these substrates was monitored by the formation of p-nitrophenol (optical density at 405 nm). Among the substrates, pNPPC and pNPPP were found to be hydrolyzed efficiently by both hNPP6 (Fig. 3B) and mNPP6 (not shown) recombinant proteins. We also examined the catalytic activity of NPP6 using pNPPC as a substrate and culture supernatants from HeLa cells transfected with various NPP6 cDNAs. The pNPPC hydrolyzing activity was detected in all the cell culture supernatants from HeLa cells transfected with NPP6 cDNAs (hNPP6, mNPP6, hNPP6-ex, and mNPP6-ex) but not from HeLa cells transfected with mock cDNA (Fig. 3C). This analysis confirmed that NPP6 is actually enzymatically active and that NPP6 is secreted from cells like other members of NPP family (20, 29).

**NPP6 Requires Divalent Cations for Catalytic Activity—**We further characterized the lysoPLC activity of NPP6. Like other members of the NPP family, the optimal pH range was pH 8.5–9.5 (data not shown). In addition, the enzyme activities against 14:0-LPC, LPA, and pNPPC were readily inhibited by EDTA and EGTA (Fig. 6), indicating that a divalent cation is essential for catalytic activity. Interestingly the catalytic activity of NPP6 toward pNPPC was partially inhibited by the divalent cations. We also tried to recover the activity of EDTA-treated NPP6 by adding various divalent cations. However, none of the divalent cations tested (Ca2+, Mg2+, Zn2+, Ni2+).
Co²⁺, Fe²⁺, and Cu²⁺) rescued the lost activity (data not shown). Thus, depriving NPP6 of divalent cation(s) may cause it to undergo gross conformational changes, which lead to an inactivation of the enzyme.

NPP6 Hydrolyzes Extracellular LPC and Determines Its Level—It is possible that NPP6 determines the extracellular LPC level by hydrolyzing LPC. To test this possibility we determined the LPC concentration in the culture cell supernatants of HeLa cells either transfected with hNPP6 cDNA or mock cDNA. As shown in Fig. 7, culture media used in this study contain ~80 μM LPC, mainly derived from fetal bovine serum, which decreased more rapidly in NPP6-expressing cells than mock-transfected cells. The result indicates that cell-associated NPP6 hydrolyzes LPC in the cell culture media and determines its level.

Expression of NPP6—We examined the tissue distribution of human and mouse NPP6 mRNA by a quantitative real time PCR analysis. Unlike other members of the NPP family, which showed ubiquitous tissue expression patterns (1), NPP6 mRNA was expressed predominantly in kidney in mouse (Fig. 8B). A similar tissue distribution pattern was observed by Northern blot analysis (data not shown). In human, it was expressed predominantly in brain and kidney (Fig. 8A). These expression profiles completely agree with those in the Unigene data base. We further performed immunochemical analysis using anti-hNPP6, 7C5, to identify NPP6-expressing cells in human kidney (Fig. 9, A and E). We also used antibodies to several segment markers to identify individual nephron segments. The proximal tubules and thin descending limbs of Henle were identified as AQP1-positive tubules in the cortex and the medulla, respectively (30). The thick ascending limbs of Henle in the medulla were distinguished by the expression of Tamm-Horsfall protein (31). The collecting tubules were reported to be positive for AQP2 (32). NPP6 was expressed only in AQP1-positive tubules in the cortex (Fig. 9A) and medulla (Fig. 9E). The results suggest that NPP6 is expressed specifically in the proximal tubules and thin descending limbs of Henle in the human kidney. It should be noted that signals are intensively detected on the inner surface or inside of tubules.

**DISCUSSION**

**NPP6, a Unique Glycerophosphodiester Phosphodiesterase with LysoPLC Activity Specific to Choline**—We previously showed that NPP2/autotaxin has a lysoPLD activity and propose that cell motility-stimulating activity of NPP2/autotaxin is mediated by the LPA produced (20). This conclusion was further supported by our recent finding that cells lacking in one LPA receptor subtype, LPA₁, did not show a migratory response to NPP2/autotaxin (33). In addition, recently identified alk-SMase/NPP7 is an NPP family member (4). These notions raised the possibility that some members of the NPP family act on phospholipids. In this study we identified a novel member belonging to the NPP family, NPP6, and showed that it has a lyosphospholipase C activity toward LPC (Fig. 5). NPP6 hydrolyzed GPC efficiently (Fig. 5C). Thus, NPP6 is a GDE. Recently,...
Mir16, a mammalian homologue of a bacterial GDE, was identified as a protein interacting with RSG16 (regulator of G-protein signaling 16) (34, 35). Recombinant Mir16 selectively hydrolyzes glycerophosphoinositol, suggesting that it is involved in phosphatidylinositol metabolism. NPP6 does not show any homology to Mir16, indicating that the two GDEs have different functions. NPP6 is classified into a subgroup of the NPP family together with uncharacterized NPP4, NPP5, and recently identified alk-SMase/NPP7. Interestingly, NPP6 showed a marked preference for choline-containing phospholipids or phosphodiesters such as LPC, SPC, lysoPAF, PAF, and GPC, whereas it did not hydrolyze diacylphospholipids such as PC and SM appreciably (Fig. 5). We previously showed that NPP2 (lysoPLD/autotaxin) has broad substrate specificity. It did not show a preference for a choline residue and hydrolyzed LPC, LPE, LPS, LPI, and SPC (23, 36). In addition, NPP2 did not hydrolyze GPC. Thus, NPP2 and NPP6 appear to show quite different substrate specificities. It can be speculated that, unlike NPP2, NPP6 has a domain that specifically recognizes a choline residue. We also observed that neither LPA nor choline was formed in the reaction mixture of NPP6. LPC was mixed with purified mNPP6-ex in the absence or presence of calf intestinal alkaline phosphatase (CIP), and the formation of LPA, MG, and choline was determined by colorimetric assays as described under "Experimental Procedures." The results are mean ± S.E. from three separate experiments.

![Diagram](image)

**Fig. 4.** NPP6 has a lysoPLC activity toward LPC. The catalytic activity of NPP6 toward 14:0-LPC is shown. A, structures of 14:0-LPC. The two arrows indicate the phosphodiester bonds that are possibly hydrolyzed by lysoPLC and lysoPLD, respectively. The two arrows indicate the resulting products. B, detection of LPA, MG, and choline in the reaction mixture of NPP6. 14:0-LPC was mixed with purified mNPP6-ex in the absence or presence of calf intestinal alkaline phosphatase (CIP), and the formation of LPA, MG, and choline was determined by colorimetric assays as described under "Experimental Procedures." The results are mean ± S.E. from three separate experiments.

![Diagram](image)

**Fig. 5.** NPP6 is a choline-specific phosphodiesterase. The substrate specificity of NPP6 toward various lysophospholipids and diacylphospholipids is shown. A, effect of head groups on the catalytic activity of NPP6. The substrates used are LPC (1-oleoyl), LPI (from porcine liver), LPE (1-oleoyl), LPS (1-oleoyl), LPA (1-oleoyl), PC (dioleyl), phosphatidylinositol (PI) (from porcine liver), phosphatidylethanolamine (PE) (dioleyl), phosphatidylerine (PS) (dioleyl), phosphatic acid (PA) (dioleyl), and phosphatidylglycerol (PG) (dioleyl). Catalytic activities were evaluated by MG or diacylglycerol (DG) formation using colorimetric assays as described under "Experimental Procedures." B, substrate specificity of NPP6 toward choline-containing phospholipids. The substrates used are LPC (1-oleoyl), PC (dioleyl), SPC, SM, lysoPAF, and PAF. Catalytic activities were evaluated by choline release. C, effect of the fatty acid moiety of LPC on the lysoPLC activity of NPP6. The substrate specificity of NPP6 with regard to the acyl group of LPC was determined using LPC with various acyl groups. Each substrate was subjected to the NPP6 reaction, and the activities were evaluated by choline release. The substrates used are LPC (12:0, 14:0, 16:0, 18:0, 18:1, 18:2, 20:0, 20:4, and egg) and GPC. The results are mean ± S.E. from three separate experiments.
It was revealed that the recently identified intestinal alk-SMase belongs to the NPP family (4). It is classified into the subgroup of the NPP family consisting of NPP4, NPP5, and NPP6. Thus, based on its structural and functional characteristics, the alk-SMase should have another name, NPP7, because it is the seventh member of the NPP family. Duan et al. (4) showed that NPP7/alk-SMase has SMase activity toward SM in the presence of bile salts and also possesses a lysoPLC activity toward LPC, although they have not determined whether the enzyme hydrolyzes SPC or GPC. The facts suggest that other members of the NPP family may have such activities. We recently showed that both NPP1/PC-1 and NPP3/ gp130 have neither lysoPLD (37) nor lysoPLC activity.2 In addition, our preliminary data show that NPP4 and NPP5 possess neither lysoPLD nor lysoPLC activity toward LPC.3 They also did not show phosphodiesterase activity toward GPC.3 The present study clearly demonstrated that catalytic activity of NPP6 is significantly affected by the head groups (Fig. 5A) and the fatty acid moiety of lysophospholipids (Fig. 5C). This indicates that various phospholipids with different head groups and fatty acids need to be tested to identify substrates for other members of NPP family.

Possible Role of NPP6 in Kidney—We show that NPP6 is expressed predominantly by certain cell types in human kid-

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<th>Substrate</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (nmol/min/mg)</th>
<th>Km (μM)</th>
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<tbody>
<tr>
<td>GPC</td>
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<td>344</td>
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<tr>
<td>12:0-LPC</td>
<td>463</td>
<td>296</td>
</tr>
<tr>
<td>14:0-LPC</td>
<td>367</td>
<td>440</td>
</tr>
<tr>
<td>16:0-LPC</td>
<td>89</td>
<td>165</td>
</tr>
<tr>
<td>18:2-LPC</td>
<td>285</td>
<td>434</td>
</tr>
<tr>
<td>20:4-LPC</td>
<td>470</td>
<td>563</td>
</tr>
</tbody>
</table>

FIG. 6. NPP6 requires divalent cation(s) for the catalytic activity. The catalytic activity of NPP6 toward 14:0-LPC (A), pNPPC (B), and GPC (C) were determined in the presence or absence of divalent cation chelators EDTA and EGTA. Catalytic activities were evaluated by choline release. The results are mean ± S.E. from three separate experiments.

FIG. 7. Recombinant NPP6 determines the extracellular LPC level. The amount of LPC in the culture medium of HeLa cells transfected with either NPP6 or mock cDNA was determined. The closed and open circles indicate NPP6- and mock cDNA-transfected cells, respectively.

FIG. 8. Expression of NPP6 mRNA in human (A) and mouse (B) tissues. The NPP6 mRNA levels in human and mouse tissues were measured by quantitative real time reverse transcription-PCR and are expressed as a relative value to GAPDH mRNA. The results are mean ± S.E. from three separate experiments.

The proximal tubules and thin descending limbs of Henle (Fig. 9). NPP6 may have specific functions in these tubules. Interestingly NPP6 is predominantly localized to the inner side of these tubules, and it is likely that in these tubules NPP6 is exposed to primary urine. Because NPP6 shows specificity to choline it may have some role in resorption of choline by degrading choline-containing compounds such as LPC, SPC, and GPC. This idea is supported by the fact that choline concentration is high in plasma but is low in urine.

The preferable substrates of NPP6 identified in this study are LPC with unsaturated fatty acids (18:2 and 20:4) and SPC (Figs. 5 and 9). LPC is abundantly present in blood. Indeed the concentration of LPC in plasma of mammals ranges from 100 to 800 μM depending on mammalian species. Plasma LPC is potentially an endogenous substrate for NPP6 because the LPC level significantly decreased in NPP6-expressing cells (Fig. 7). NPP6 may protect cells from LPC, which is sometimes toxic, or it may supply choline, which is an essential factor for various mammalian cells. Other than LPC, GPC is present in kidney at a high level (38). In the renal medulla of mammals, GPC plays an important role as an organic osmolyte to maintain intracellular osmotic pressure (39). The concentrations of NaCl and urea are elevated in the renal medulla as part of the renal concentrating mechanism. To remain in osmotic equilibrium, cells in the renal medulla contain high levels of GPC (38). Interestingly several studies have suggested that the activity of GPC-hydrolyzing enzymes is high in the cortex of kidney where GPC contents are low and low in the papilla where GPC contents are usually high (40, 41). Thus, NPP6 may have a role in regulating the concentration of GPC in kidney (Figs. 5C and 9). It is also possible that NPP6 is a producing enzyme for bioactive lipids. We showed that NPP6 converts C(18:2) to LPA. These possibilities are being tested in our laboratory.
Biochemical and Molecular Characterization of a Novel Choline-specific Glycerophosphodiester Phosphodiesterase Belonging to the Nucleotide Pyrophosphatase/Phosphodiesterase Family

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