Discovery and Characterization of a Ca\textsuperscript{2+}-independent Phosphatidylethanolamine N-Acyltransferase Generating the Anandamide Precursor and Its Congeners*

Received for publication, July 5, 2006, and in revised form, November 9, 2006. Published, JBC Papers in Press, December 7, 2006, DOI 10.1074/jbc.M606369200

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\textsuperscript{*} The abbreviations used are: NAEs, \textsuperscript{1}NAE-PLD, \textsuperscript{2}NAPE, \textsuperscript{2}N-acylphosphatidylethanolamines (NAPEs) are precursors of bioactive N-acylethanolamines, including the endocannabinoid anandamide. In animal tissues, NAPE is formed by transfer of a fatty acyl chain at the sn-1 position of glycerophospholipids to the amino group of phosphatidylethanolamine (PE), and this reaction is believed to be the principal rate-limiting step in N-acylenethanolamine synthesis. However, the Ca\textsuperscript{2+}-dependent, membrane-associated N-acyltransferase (NAT) responsible for this reaction has not yet been cloned. In this study, on the basis of the functional similarity of NAT to lecithin-retinol acyltransferase (LRAT), we examined a possible PE N-acylation activity in two rat LRAT homologous proteins. Upon overexpression in COS-7 cells, one protein, named rat LRAT-like protein (RLP)-1, catalyzed transfer of a radioactive acyl group from phosphatidylcholine (PC) to PE, resulting in the formation of radioactive NAPE. However, the RLP-1 activity was detected mainly in the cytosolic rather than membrane fraction and was little stimulated by Ca\textsuperscript{2+}. Moreover, RLP-1 did not show selectivity with respect to the sn-1 and sn-2 positions of PC as an acyl donor and therefore could generate N-arachidonoyl-PE (anandamide precursor) from 2-arachidonoyl-PC and PE. In contrast, under the same assay conditions, partially purified NAT from rat brain was highly Ca\textsuperscript{2+}-dependent, membrane-associated, and specific for the sn-1-acyl group of PC. RLP-1 mRNA was expressed predominantly in testis among various rat tissues, and the testis cytosol exhibited an RLP-1-like activity. These results reveal that RLP-1 can function as a PE N-acyltransferase, catalytically distinguishable from the known Ca\textsuperscript{2+}-dependent NAT.

N-Acylethanolamines (NAEs)\textsuperscript{2} are ethanolamides of long-chain fatty acids and have received considerable attention because of their presence in a variety of organisms and their biological activities (1, 2). For example, anandamide (N-arachidonoyl ethanolamine) acts as a ligand of cannabinoid receptors (3) and the vanilloid receptor (4) in mammalian tissues. N-Palmitoylethanolamine has been reported to be an anti-inflammatory and analgesic substance (5, 6), and N-oleoylethanolamine (7) and N-stearoylethanolamine (8) have been shown to be anorexigenic mediators.

It is generally accepted that, in animal tissues, NAEs are principally biosynthesized from membrane glycerophospholipids by two enzyme reaction steps (1, 2, 9–12). The first reaction is the transfer of a fatty acyl group at the sn-1 position of phosphatidylcholine (PC) and other glycerophospholipids to the primary amine of phosphatidylethanolamine (PE), forming N-acylphosphatidylethanolamine (NAPE) (Fig. 1) (1, 2, 9–11). This reaction is attributable to catalysis by Ca\textsuperscript{2+}-dependent N-acyltransferase (NAT) (1, 2, 9–11). The second reaction is hydrolysis of NAPE to generate NAE and phosphatidic acid catalyzed by NAPE-hydrolyzing phospholipase D (NAPE-PLD) (1, 2, 9–11). Recently, we cloned the cDNA of NAPE-PLD (13) and showed that the enzyme is structurally and functionally a member of the metallo-\beta-lactamase family (14). Very recently, analysis of gene-disrupted mice revealed that NAPE-PLD plays an important role in the conversion of NAPEs to NAE, although the involvement of another enzyme or pathway was also suggested (15). On the other hand, NAT has not yet been cloned. So far, with crude preparations (16–23) or partially purified enzyme (24), NAT has been shown to be membrane-associated, stimulated by Ca\textsuperscript{2+}, and specific for the sn-1-fatty acyl chain of glycerophospholipids as an acyl donor. In degenerating tissues and cells resulting from myocardial infarction (25, 26), glutamate-induced neuronal cytotoxicity (27), post-decapping brain ischemia (28, 29), CdCl\textsubscript{2}-administered testicular inflammation (30), and irradiation of epidermal cells by UV light (31), both NAPEs and NAEs markedly accumulate. These findings suggest that the Ca\textsuperscript{2+}-dependent NAT reaction is the principal rate-limiting step in NAE synthesis and that the intracellular free Ca\textsuperscript{2+} concentration regulates the activity of NAT (2, 9).

Lecithin-retinol acyltransferase (LRAT) is an enzyme that catalyzes the transfer of an acyl group at the sn-1 position of PC to retinol, leading to the formation of retinyl ester (32). Its cDNA has already been cloned (33, 34). Recently, LRAT was reported to transfer the acyl group to retinolamine, resulting in
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the formation of N-retinylamides (35). One the basis of the similarity of the LRAT reaction to the NAT reaction, we assumed that the primary structure of NAT is homologous to that of LRAT. In this study, we isolated the cDNAs of three rat proteins with homology to rat LRAT and investigated the ability of two of the proteins to transfer a fatty acyl group from PC to the amino group of PE, leading to the generation of NAPE. One protein (tentatively named rat LRAT-like protein (RLP)-1) actually showed such an activity when overexpressed in COS-7 cells. However, our present results strongly suggest that RLP-1 is a novel PE N-acyltransferase catalytically distinguishable from the known NAT.

**EXPERIMENTAL PROCEDURES**

**Materials**—[1-\textsuperscript{14}C]Palmitic acid (2.06 GBq/mmol), [1-\textsuperscript{14}C]palmitoyl-CoA (2.035 GBq/mmol), and [1,2-\textsuperscript{14}C]di-palmitoyl-sn-glycero-3-phosphocholine (4.107 GBq/mmol) were purchased from PerkinElmer Life Science. 1-Palmitoyl-2-[1-\textsuperscript{14}C]oleoyl-PC (2.22 GBq/mmol), 1-palmitoyl-2-[1-\textsuperscript{14}C]oleoyl-PC (2.07 GBq/mmol), 1-palmitoyl-2-[1-\textsuperscript{14}C]arachidonoyl-PC (1.962 GBq/mmol), 1-palmitoyl-2-[1-\textsuperscript{14}C]linoleoyl-sn-glycero-3-phosphoethanolamine (2.04 GBq/mmol), [1-\textsuperscript{14}C]oleic acid (2.22 GBq/mmol), [1-\textsuperscript{14}C]arachidonic acid (2.07 GBq/mmol), HiTrap Q, horseradish peroxidase-linked anti-mouse IgG, Hybond P, and an ECL Plus kit were from Amersham Biosciences. Palmitic acid, palmitoyl-CoA, linoleic acid, [1-\textsuperscript{14}C]palmitoyl-PE, 1,2-dioleoyl-PE, 1,2-dipalmitoyl-PC, 1-palmitoyl-2-oleoyl-PC, 1-palmitoyl-2-arachidonoyl-PC, 1-palmitoyl-2-lino-

**DNA Cloning**—The cDNA containing the coding region of rat LRAT and cDNAs containing the putative full-length coding regions of RLP-1, RLP-2, and RLP-3 were generated by reverse transcription (RT)-PCR using the forward primers 5’-AAGCTT-TAGAAGAACTCAATGCTGAGGC-3’ (LRAT), 5’-AAGCTT-TATGATCCCGGGCACCAGGACCC-3’ (RLP-1), 5’-AAGCTT-GGTTTGGGTCT-3’ (RLP-2), and 5’-AAGCTTATGCCCATACCAGACCAAGCTT-3’ (RLP-3) and the reverse primers 5’-GAATTCCTAGGATGCTTCTC-3’ (LRAT), 5’-GAATTCCTAGGATGCTTCTC-3’ (RLP-1), 5’-GAATTCCTAGGATGCTTCTC-3’ (RLP-2), and 5’-GAATTCCTAGGATGCTTCTC-3’ (RLP-3). These primers were designed based on the cDNA sequences of rat LRAT (GenBank\textsuperscript{\textregistered} accession number AF255060), RLP-1 (BC099084), RLP-2 (XM_213590), and RLP-3 (XM_579390). cDNAs used as templates for PCR were prepared from total RNA (5 μg) of adult rat brain or testis using Moloney murine leukemia virus reverse transcriptase and random hexamer. PCR amplification by Pyrobest DNA polymerase was performed at a denaturing temperature of 94 °C for 30 s, followed by annealing at 58 °C for 30 s and extension at 72 °C for 1 min (35 cycles). These PCR products were subcloned into the pCR2.1-TOPO vector. The RLP-1 cDNA was then digested with HindIII and EcoRI and inserted into the eukaryotic expression vector pcDNA3.1(+). The cDNAs of N-terminally FLAG-tagged LRAT, RLP-1, and RLP-3 were generated by PCR using the forward primers 5’-AAGCTT-TATGATCCCGGGCACCAGGACCC-3’ (LRAT), 5’-GAATTCCTAGGATGCTTCTC-3’ (RLP-1), 5’-GAATTCCTAGGATGCTTCTC-3’ (RLP-2), and 5’-GAATTCCTAGGATGCTTCTC-3’ (RLP-3) and the above-mentioned reverse primers. PCR amplification by Pyrobest DNA polymerase was performed as described above using the pCR2.1-TOPO vector harboring the respective full-length cDNA as a template. The PCR products were ligated into pcDNA3.1(+). All constructs were sequenced in both directions using an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA).

**Overexpression of Recombinant Proteins in COS-7 Cells**—COS-7 cells were grown at 37 °C to 70% confluency in a 100-mm dish containing Dulbecco’s modified Eagle’s medium with 10% fetal calf serum in a humidified 5% CO\textsubscript{2} and 95% air incubator. The cells were then treated with 8 μg of the expres-
sion vector harboring rat LRAT, RLP-1, or RLP-3 cDNA and Lipofectamine and cultured at 37 °C for 48 h, with one change of medium at 12 h. Control COS-7 cells were prepared in the same way, except that the insert-free vector was used for transfection. The harvested cells were sonicated three times for 3 s each in 20 mM Tris-HCl (pH 7.4), and the homogenates were centrifuged at 105,000 × g for 55 min at 4 °C. The resultant supernatant was used as the cytosolic fraction, whereas the pellet was suspended in 20 mM Tris-HCl (pH 7.4) and used as the membrane fraction. Recombinant rat NAPE-PLD highly purified from Escherichia coli (14), the membrane fraction of COS-7 cells overexpressing rat fatty acid amide hydrolase (39), and group IB secretory phospholipase A2 purified from rat stomach (40) were prepared as described previously.

**Purification of Recombinant RLP-1**—The cytosol of COS-7 cells overexpressing FLAG-tagged RLP-1 was prepared from 10 dishes (100 mm) as described above, except that 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 0.05% Nonidet P-40 (buffer A) was used. To the cytosol was added 1 ml of anti-FLAG M2 affinity gel pre-equilibrated with buffer A, and the mixture was then incubated overnight with gentle mixing to allow FLAG-tagged RLP-1 to bind to the gel. The gel was packed into a column and washed three times each with 12 ml of buffer A. The elution was eluted with buffer A containing 0.1 mg/ml FLAG peptide, and every 0.5-ml fraction was collected.

**Enzyme Preparation from Rat Tissues**—Adult Wistar-ST rats (Japan SLC Inc.) were anesthetized with diethyl ether and killed by cervical dislocation. Various tissues were removed, cut into small pieces, and then homogenized in 5 volumes (v/w) of 20 mM Tris-HCl (pH 7.4) containing 0.32 mM sucrose using a Polytron homogenizer. The cell-free extracts were first centrifuged at 800 g for 55 min. The resultant clear supernatant was used as the cytosolic fraction, whereas the pellet was suspended in 20 mM Tris-HCl (pH 7.4) containing 200 mM NaCl, an active fraction was eluted with 6 ml of buffer B containing 100 mM NaCl, and 6 ml of buffer B containing 200 mM NaCl, an active fraction was eluted with 6 ml of buffer B containing 500 mM NaCl. This fraction was used as partially purified NAT. The protein concentration was determined by the method of Bradford (41) with bovine serum albumin as the standard.

**Enzyme Assays**—For the transacylation assay, the enzyme was incubated with 40 µM 1,2-[14C]dipalmitoyl-PC (45,000 cpm) and 75 µM 1,2-dioleoyl-PE in 100 µl of 50 mM glycine/NaOH (pH 9.0), 2 mM (±)-dithiothreitol, and 0.05% Nonidet P-40 at 37 °C for 30 min. To the reaction mixture was added 5 mM CaCl₂ or 5 mM EDTA as indicated. For the LRAT assay, the enzyme was incubated with 100 µM 1,2-[14C]dipalmitoyl-PC (45,000 cpm) and 20 µM all-trans-retinol (dissolved in 1 µl of dimethyl sulfoxide) in 100 µl of 100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 2 mM (±)-dithiothreitol, and 0.6% (w/v) fatty acid-free bovine serum albumin at 37 °C for 120 min. A mixture of chloroform/methanol (2:1, v/v; 0.3 ml) containing 5 mM (23)-butyl-4-hydroxyanisole was added to the reaction mixture to terminate the reaction. After centrifugation, 100 µl of the lower layer was spotted on a silica gel thin-layer plate (10-cm height) and developed in solvent system A at 4 °C for 25 min. The distribution of radioactivity on the plate was quantified using a BAS1500 bioimaging analyzer (FUJIX Ltd., Tokyo).

**Western Blotting**—After separation by SDS-PAGE on a 14% gel, proteins were electrotransferred to a hydrophobic polyvinylidene difluoride membrane (Hybond P). The membrane was blocked with phosphate-buffered saline containing 5% dried milk and 0.1% Tween 20 (buffer C) and then incubated with anti-FLAG antibody (1:2000 dilution) in buffer C at room temperature for 1 h, followed by incubation with horseradish peroxidase-labeled secondary antibody (1:4000 dilution) in buffer C at room temperature for 1 h. Finally, FLAG-tagged proteins were visualized using the ECL Plus kit and analyzed using a LAS1000plus lumino-imaging analyzer (FUJIX Ltd.).

**RT-PCR**—Total RNA was isolated from various organs of Wistar-ST rats using Trizol. cDNAs were then prepared from total RNA (5 µg) using Moloney murine leukemia virus reverse transcriptase and random hexamer and subjected to PCR amplification by Ex Taq DNA polymerase. The primers used for RLP-1 mRNA were 5′-ATGGGCTTGAGCCCCAGCCAG-3′ and 5′-TCAACGCTTATGGGTGTTGGTCT-3′ (nucleotides 73–96 and 841–864, respectively, in GenBank accession number BC099084), and those for rat glycerolaldehyde-3-phosphate dehydrogenase mRNA were 5′-AAGGTCTGTTGACCCGATTTGGTGGTCT-3′ and 5′-ACAAACATGGGCGATCAGC-3′ (nucleotides 7–29 and 370–389, respectively, in NM_017008). The PCR conditions used were as follows: for RLP-1, denaturation at 94 °C for 30 s, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min (30 or 40 cycles); and for glycerolaldehyde-3-phosphate dehydrogenase, denaturation at 95 °C for 30 s, annealing at 58 °C for 15 s, and extension at 72 °C for 30 s (27 cycles).

**RESULTS**

**Isolation of cDNAs of Rat LRAT and Its Related Proteins**—Using BLAST, we found three rat cDNAs (GenBank accession numbers BC099084, XM_213590, and XM_579390) with homology to rat LRAT (AF255060) at the amino acid sequence level. These rat genes appear to be homologs of human genes (HRLP5 (H-REV107-like protein 5), A-CI, and H-REV107, respectively). H-REV107 (42, 43) and A-C1 (44) have been shown to act as tumor suppressors, but have not been reported as enzymes. The function of HRLP5 remains unclear. In this work, we tentatively refer to BC099084, XM_213590, and XM_579390 as RLP-1, RLP-2, and RLP-3, respectively. We prepared PCR primers based on their nucleotide sequences and isolated cDNAs from rat testis (RLP-1, RLP-2, and RLP-3) and brain (LRAT) by RT-PCR. The nucleotide sequences of the...
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1-O-palmitoyl and 2-O-palmitoyl groups were labeled with \(^{14}\)C and nonradioactive PE. The products were separated by TLC, and the distribution of radioactivity on the thin-layer plate was analyzed (Fig. 4). In this assay, partially purified NAT from rat brain was used as a positive control (lane 2), and the COS-7 cell homogenate transfected with the insert-free vector was used as a negative control (lane 3). The results show that a radioactive band that co-migrated with authentic N-[\(^{14}\)C]palmitoyl-PE was formed with RLP-1 (lane 5) and RLP-3 (lane 6) as well as rat brain NAT, but not with LRAT (lane 4). This suggests that recombinant RLP-1 and RLP-3 possess the transacylation activity to form NAPE. Because the activity of RLP-3 was less than half that of RLP-1 and because RLP-3 generated a larger amount of a radioactive compound corresponding to free palmitic acid, we focused on RLP-1 in the following experiments.

To exclude a possible effect by the FLAG tag, we next overexpressed FLAG tag-free RLP-1 protein in COS-7 cells and used this protein in the following enzyme assays. As shown in Fig. 5A (lane 2), the cell homogenate revealed transacylation activity (235 ± 12 pmol/min/mg of protein). When the homogenate was subjected to ultracentrifugation, the enzyme activity was found in the cytosolic fraction (487 ± 12 pmol/min/mg of protein). In this assay, partially purified NAT from rat brain was used as a positive control (lane 5) as well as rat brain NAT, but not with LRAT (lane 4). This suggests that recombinant RLP-1 and RLP-3 possess the transacylation activity to form NAPE. The activity of RLP-3 was less than half that of RLP-1 and because RLP-3 generated a larger amount of a radioactive compound corresponding to free palmitic acid, we focused on RLP-1 in the following experiments.

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Identification of RLP-1 Products—To identify the major radioactive product as N-[\(^{14}\)C]palmitoyl-PE, this radioactive compound was extracted from silica gel and treated with recombinant NAPE-PLD (an enzyme that specifically hydrolyzes NAPEs to NAEs and phosphatidic acid) (46). By this treatment, the RLP-1 product was shown to be a radioactive band corresponding to free palmitic acid (Fig. 5B, lane 2). The NAPE-PLD product was treated with recombinant fatty acid amidase hydrolyase (an enzyme that hydrolyzes NAEs to free fatty acids and ethanolamine) (47, 48), a band corresponding to \(^{14}\)C-palmitic acid was observed (lanes 3 and 6). Thus, the RLP-1 product was shown to be N-palmitoyl-PE in which N-palmitoyl chain was labeled with \(^{14}\)C.
These bands co-migrated with authentic lyso-PC, PE, palmitic acid, and non-polar bands were also observed. All radioactive products, produced in the cytosol from the control cells (data not shown) and were not produced or were only slightly produced, were allowed to react with 40 μM 1,2-[14C]dipalmitoyl-PC and 75 μM 1,2-dioleoyl-PE in the standard reaction mixture containing 5 mM CaCl2, at pH 9.0. The products were separated by TLC with solvent system A. The positions of authentic compounds are indicated by arrows. NPPE, N-[14C]palmitoyl-PE; C16:0, [14C]palmitic acid.

We detected several radioactive bands in addition to the major product (N-palmitoyl-PE) and the remaining PC (Fig. 5A). To identify these bands, we separated them by TLC with longer plates in two different solvent systems (Fig. 5, C and D). These bands co-migrated with authentic lyso-PC, PE, palmitic acid, and N-palmitoyllyso-PE in both solvent systems. Faint non-polar bands were also observed. All radioactive products, including N-palmitoyl-PE, were abolished by heat treatment (data not shown) and were not produced or were only slightly produced in the cytosol from the control cells (lane 1), thus suggesting that the formation of these radioactive compounds is related to catalysis by RLP-1. Because the PC used as a substrate for the assay was radiolabeled at both the 1-O-palmitoyl and 2-O-palmitoyl groups, it was reasonable that radioactive lyso-PC was produced together with N-[14C]palmitoyl-PE as a result of the transfer of the radioactive 1-O-palmitoyl or 2-O-palmitoyl chain of PC to the amino group of PE. Moreover, when nonradioactive PE was removed from the reaction mixture, the formation of radioactive N-palmitoyl-PE and N-palmitoyllyso-PE was remarkably reduced (data not shown), strongly suggesting that PE serves as an acyl acceptor.

Verification of the Position of the Acyl Group Abstracted from PC—Because the PC had 14C in both the 1-O-palmitoyl and 2-O-palmitoyl groups, it remained unclear whether the sn-1- or sn-2-acyl group of PC was selectively abstracted. Therefore, we next tested 1-[14C]palmitoyl-2-palmitoyl-PC (Fig. 6A) and 1-palmitoyl-2-[14C]palmitoyl-PC (Fig. 6B) separately. The positional specificity of the radioactively labeled group in these PCs was verified by treatment of rat group IB secretory phospholipase A2 (40), which released [14C]palmitic acid from 1-palmitoyl-2-[14C]palmitoyl-PC, but only slightly produced radioactive lyso-PC from the same substrate (Fig. 6B, lane 4). With 1-[14C]palmitoyl-2-palmitoyl-PC, we observed a small amount (<5%) of [14C]palmitic acid released by group IB secretory phospholipase A2 (Fig. 6A, lane 4). When partially purified NAT from rat brain was allowed to react with these PCs, NAPE was produced only from 1-[14C]palmitoyl-2-palmitoyl-PC, whereas [14C]lyso-PC was formed with 1-palmitoyl-2-[14C]palmitoyl-PC. These results confirmed the selectivity of brain NAT toward the sn-1-acyl group (Fig. 6, A and B, lanes 1). Under the same assay conditions, however, cytosolic RLP-1 produced NAPE from both radioactive PCs (Fig. 6, A and B, lanes 3). The specific activity to form [14C]NAPE from each PC was 99 ± 10 (1-[14C]palmitoyl-2-palmitoyl-PC) and 244 ± 17 (1-palmitoyl-2-[14C]palmitoyl-PC) pmol/min/mg of protein. Thus, RLP-1 does not show obvious selectivity with respect to the sn-1 and sn-2 positions of PC.

Because unsaturated fatty acid chain is abundant at the sn-2 position of glycerophospholipids (49) and because N-oleoyl-PE and N-arachidonoyl-PE are known to be the precursors of N-oleoylphosphatidylcholine and anandamide, respectively (1, 12), it was interesting to investigate whether the oleoyl or arachidonoyl moiety of 2-oleoyl-PC or 2-arachidonoyl-PC serves as an acyl donor of RLP-1. When 1-palmitoyl-2-[14C]oleoyl-PC and 1-palmitoyl-2-[14C]arachidonoyl-PC were examined, the production of N-[14C]oleoyl-PE and N-[14C]arachidonoyl-PE was observed with RLP-1, but not with rat brain NAT (Fig. 6, C and D). Specific digestion by NAPE-PLD and fatty acid amide hydrolase confirmed that the N-acyl chain in the N-oleoyl-PE and N-arachidonoyl-PE produced was radiolabeled (data not shown). The specific activity was 162 ± 15 (1-palmitoyl-2-[14C]oleoyl-PC) and 84 ± 8 (1-palmitoyl-2-[14C]arachidonoyl-PC) pmol/min/mg of protein. We also tested [14C]labeled palmitic acid and palmitoyl-CoA as acyl donors, but both compounds were essen-
NAT was active at pH 6.0–9.5, and the optimal pH was slightly lower than the reported optimal pH of 7.0–10.0. The enzyme was active at pH 6.0–9.5, with the optimal pH being slightly lower than the reported optimal pH of 7.0–10.0. As for pH dependence, brain NAT was found to be a different enzyme protein from the known NAT. We also noted that brain NAT was partially inactive with RLP-1 as well as rat brain NAT (data not shown).

Characterization of the Catalytic Activity of RLP-1—The predominant presence in the cytosol and the ability to transfer both the sn-1- and sn-2-acyl groups of PC suggested that RLP-1 is a different enzyme protein from the known NAT. We also compared the catalytic properties of RLP-1 with those of partially purified NAT from rat brain. As for pH dependence, brain NAT was active at pH 6.0–9.5, and the optimal pH was ~8.0 (Fig. 7B). This result was in agreement with previous results (16, 20–23). On the other hand, RLP-1 was active at pH 7.0–10.0, and the optimal pH was ~9.0 (Fig. 7A). The stimulatory effect of Ca\(^{2+}\) on brain NAT was reported previously, and Ca\(^{2+}\) could be replaced with Sr\(^{2+}\) (20, 24). As shown in Fig. 8B, Ca\(^{2+}\) and Sr\(^{2+}\) at 5 mM stimulated brain NAT by 16- and 6-fold, respectively, and 5 mM EDTA almost completely inhibited the enzyme. Mg\(^{2+}\) hardly affected the activity. In contrast, Ca\(^{2+}\) only slightly stimulated RLP-1, and Sr\(^{2+}\) and Mg\(^{2+}\) showed no effect (Fig. 8A). In the presence of EDTA, the specific activity slightly decreased. (+)-Dithiothreitol (2 mM) acted as a stimulator for both enzymes (data not shown).

 Purification and Characterization of Recombinant RLP-1—To rule out the possibility that endogenous components of COS-7 cells affected the above-mentioned properties of recombinant RLP-1, we tried to purify FLAG-tagged RLP-1 from the cytosol of COS-7 cells using anti-FLAG M2 affinity gel. Through this purification procedure, the specific transacylation activity to form NAPE increased up to ~25 nmol/min/mg of protein. As analyzed by SDS-PAGE, a major protein band was seen at ~33 kDa, corresponding to the molecular mass of FLAG-RLP-1 (Fig. 9, lane 2). This band was also immunostained with anti-FLAG antibody. When the purified enzyme was allowed to react with 1,2-[1\(^{14}\)C]dipalmitoyl-PC and non-radioactive PE, the enzyme formed not only N-palmitoyl-PE, but also the products detected with the cytosol (PE, N-palmitoyllyso-PE, palmitic acid, lyso-PC, and non-polar bands). The purified enzyme also converted 1-[1\(^{14}\)C]palmitoyl-2-palmitoyl-PC, 1-palmitoyl-2-[1\(^{14}\)C]palmitoyl-PC, and 1-palmitoyl-2-[1\(^{14}\)C]oleoyl-PC, and 1-palmitoyl-2-[1\(^{14}\)C]arachidonoyl-PC to [1\(^{14}\)C]NAPEs, confirming its ability to transfer both the sn-1- and sn-2-acyl groups of PC to PE. It appeared that the enzyme preferred the sn-2-acyl chain to the sn-1-acyl chain. The activity in the presence of 5 mM Ca\(^{2+}\) was only 1.3-fold higher than that in the presence of 5 mM EDTA. These catalytic properties of purified RLP-1 were essentially identical to those of the cytosolic enzyme.

Organ Distribution of RLP-1—On the basis of the above-mentioned results, we thought that, by using the cytosolic fraction in the presence of 5 mM EDTA, the transacylation activity of RLP-1 in a tissue could be measured even if Ca\(^{2+}\)-dependent, membrane-bound NAT coexists. When the cytosolic fractions of various rat organs were tested under such conditions (Fig. 10A), the testis revealed by far the highest activity, with a specific activity of 20.6 ± 0.7 pmol/min/mg of protein. Much lower
activities were observed in several other organs such as the stomach, ileum, cecum, and colon. In addition, RT-PCR analysis showed by far the highest level of RLP-1 mRNA in the testis. In other organs, the mRNA was not seen after 30 cycles of PCR, but was detectable after 40 cycles (Fig. 10B). Although we could not rule out the possibility that other enzymes such as RLP-3 also contribute to the cytosolic transacylation activity, these results strongly suggest the functional expression of RLP-1 in rat testis.

Previous studies showed the presence of Ca\(^{2+}\)-dependent, membrane-bound NAT in rat testis (17, 24, 30). Hence, we were interested in seeing how Ca\(^{2+}\)-dependent NAT and RLP-1 share the transacylation activity in rat testis (Fig. 11A). In the presence of 5 mM Ca\(^{2+}\), the specific activity in the cytosolic fraction was comparable with that in the membrane fraction of the same tissue (27–30 pmol/min/mg of protein). However, the activity of the membrane-bound enzyme was considerably reduced by the removal of Ca\(^{2+}\) or by the addition of EDTA, whereas the change in the cytosolic activity was much smaller. In contrast, under the same assay conditions, the activity in the brains of 2-day-old rats was recovered mostly in the membrane fraction, and the membrane-bound enzyme was Ca\(^{2+}\)-dependent (Fig. 11B). As mentioned above, the transacylation activity in the cytosolic fraction of COS-7 cells overexpressing RLP-1 was 4.1-fold higher than that in the membrane fraction. These activities were not inhibited by EDTA and only slightly increased by Ca\(^{2+}\) (Fig. 11C). These results suggest that transacylation in the testis can be catalyzed by both RLP-1 and Ca\(^{2+}\)-dependent NAT, whereas that in the brain is attributable mostly to Ca\(^{2+}\)-dependent, membrane-bound NAT.

**DISCUSSION**

In animal tissues, NAEs, including the endocannabinoid anandamide, are principally generated from PE through the transacylation-phosphodiesterase pathway, which is composed of two consecutive enzyme-catalyzed reactions (1, 2). The first step is the transfer of a fatty acyl chain of a glycerophospholipid molecule to the amino group of PE by NAT (Fig. 1). The NAPE generated is then hydrolyzed to NAE and phosphatidic acid by NAPE-PLD. Recently, we succeeded in the cDNA cloning and functional expression of NAPE-PLD (13) and, through the use of the highly purified recombinant enzyme, suggested that NAPE-PLD is constitutively active (14). Therefore, the rate of NAE formation appears to be determined mostly by the availability of NAPE as discussed previously (2, 9). Because NAT is stimulated by Ca\(^{2+}\) (16–24), it is believed that the intracellular free Ca\(^{2+}\) concentration regulates the generation of NAPE (2, 9). Thus, NAT is generally considered to be an important enzyme functioning as the rate-limiting step in NAE biosynthesis (2, 9). However, its cDNA has not been cloned.

So far, NAT has been studied with crude or partially purified enzyme preparations from the brains (16, 21, 24) and testes (17) of rats and the brains (23) and hearts (20, 22) of dogs. The enzyme catalyzes the transfer of an O-acyl chain from the sn-1
position of various glycerophospholipids, including PC, PE, lyso-PC, and cardiolipin (16–24), to the amino group of PE and lyso-PE (16–24) and exhibits a broad specificity regarding the transferred fatty acyl species (24). The enzyme is membrane-bound (22, 24), and the stimulatory effect of Ca\textsuperscript{2+} can be replaced with Sr\textsuperscript{2+}, Mn\textsuperscript{2+}, and Ba\textsuperscript{2+} (20, 24). The addition of EDTA or EGTA potently inhibits NAT (16, 17, 21–24). The optimal pH for the enzyme reaction was reported to be in the range of 7–10 with different preparations or under different assay conditions (16, 20–23). The activity of NAT in rat brain considerably decreases during development (21, 29). Therefore, in this study, we used the brains of 2-day-old rats to partially purify NAT. The partially purified enzyme showed a specific activity of 213 pmol/min/mg of protein with an optimal pH of ~8.0 (Fig. 7B).

We noticed a similarity between the reactions catalyzed by LRAT and NAT, and in this study, we examined a possible PE N-acylation activity in the LRAT homologous proteins. Recently, on the basis of the sequence homology to LRAT, Anantharaman and Aravind (50) also predicted that members of this family function as acyltransferases related to lipid metabolism. We found that RLP-1, one of the LRAT homologous proteins in rat, transfers a fatty acyl chain from PC to the amino group of PE, resulting in the formation of NAPE. This was demonstrated by the production of radioactive NAPE from O-\textsuperscript{[14C]}-acyl-PC (an acyl donor) and nonradioactive PE (an acyl acceptor) by recombinant RLP-1 (Figs. 4 and 5). In addition, specific digestion of the resultant NAPE by NAPE-PLD and fatty acid amidohydrolase confirmed that its N-acyl chain is radiolabeled (Fig. 5B). To our best knowledge, this is the first report on a mammalian gene product catalyzing PE N-acylation. Free palmitic acid and palmitoyl-CoA did not serve as acyl donors, suggesting that transacylation occurred through an acyl-enzyme complex as proposed previously for NAT (9). We also observed the production of an NAPE-like compound by RLP-3. Further characterization of RLP-3 as a possible isoenzyme of RLP-1 will be required.

However, several experimental results revealed that RLP-1 is a different enzyme from the known NAT. First, the activity of recombinant RLP-1 was detected mainly in the cytosolic fraction, with lower activity in the membrane fraction (Figs. 5A and 11C). This result was in sharp contrast to the membrane association of NAT reported previously (22, 24) and confirmed in this study (Fig. 11B). Next, RLP-1 was almost independent of Ca\textsuperscript{2+}, and the activity was only slightly reduced even in the presence of 5 mM EDTA (Figs. 8A and 11C). On the other hand, we demonstrated that partially purified NAT from rat brain was remarkably stimulated by Ca\textsuperscript{2+} under our assay conditions (Figs. 8B and 11B). More important, RLP-1 did not show selectivity in terms of the sn-1 and sn-2 positions of PC as an acyl donor, unlike brain NAT, which exclusively transferred the sn-1-acyl group of PC (Fig. 6). Furthermore, as examined by RT-PCR, RLP-1 mRNA was expressed predominantly in the testis, with much lower expression levels in other rat tissues, including the brain (Fig. 10B). The cytosolic N-acylation activity was also by far the highest in the testis (Fig. 10A). This distribution was different from the reported distribution of rat NAT, with high activity in the brain and testis (24).

Thus, our results strongly suggest that RLP-1 is a novel PE N-acyltransferase enzyme distinguishable from the known NAT.

Although we focused on the transacylation activity of RLP-1 to synthesize NAPE, our results suggest that the enzyme is also involved...
in the formation of PE, N-palmitoyllyso-PE, and palmitic acid (Fig. 5, C and D). Such multifunction of RLP-1 was confirmed with the purified enzyme preparation. Because PE was reported previously to serve not only as an acyl acceptor, but also as an acyl donor of NAT (22), it was likely that RLP-1 also utilizes PE as an acyl donor. In fact, when radioactive PC was replaced with 1-palmitoyl-2-[14C]linoleoyl-PE, we observed production of NAPE and lyso-PE by RLP-1 (data not shown). These observations suggest that part of nonradioactive PE was used as an acyl donor together with radioactive PC and that the resultant lyso-PE was reacylated to radioactive PE by receiving a radioactive O-acyl chain from PC (Fig. 5, C and D). The formation of lyso-NAPE may be explained by two pathways. First, the NAPE generated is hydrolyzed to lyso-NAPE and a non-esterified fatty acid. Second, after O- [14C]acyl-PE is produced as described above, lyso-NAPE may be produced by intramolecular transfer of an O-[14C]acyl chain to the amino group. As for the formation of free palmitic acid, RLP-1 itself may also have a phospholipase A1/A2 activity, viz. it may use water as an acyl acceptor. Taken together, this study suggests that RLP-1 catalyzes not only N-acylation, but also O-acylation and hydrolysis.

It has been noted that RLP-1 is expressed predominantly in the testis among various rat tissues. Previously, Sugiuira et al. (17) examined the biosynthetic pathway of anandamide and other NAEs in rat testis and suggested that the N-acyl groups of NAPEs are derived from the sn-1 position of glycerophospholipids. Furthermore, Kondo et al. (30) reported that the contents of NAPE and NAPE in rat testis are dramatically increased by CdCl₂-induced inflammation. The authors revealed that the specific NAT activity in the testis microsomal fraction was not different between CdCl₂-administered and control rats, and the activation of NAT by an elevation of the intracellular free Ca²⁺ level was suggested to be a trigger for the formation of NAPE and NAPE (30). In this study, we confirmed the presence of Ca²⁺-dependent, membrane-bound NAT in rat testis, but also detected a cytosolic Ca²⁺-independent activity probably derived from RLP-1 (Fig. 11A). Considering the lack of a Ca²⁺ requirement and no specificity for the sn-1 position, RLP-1 appears to be less responsible for the formation of NAPE and NAPE (30).

Anandamide was discovered as an endocannabinoid and has recently received much attention because of its role in the regulation of male and female fertility in mammals (51, 52). The tissue level of anandamide is usually much lower than those of saturated and monounsaturated NAEs (2, 9–11). This fact has been explained as follows. 1) The arachidonic acid chain is much more abundant at the sn-2 position than at the sn-1 position of glycerophospholipids, and 2) NAT exclusively transfers the sn-1-acyl group to form NAPE (16–24). On the other hand, RLP-1 exhibited an ability to transfer the sn-2-acyl chain, and we actually observed the production of N-arachidonoyl-PE (anandamide precursor) from 1-palmitoyl-2-arachidonoyl-PC by RLP-1 (Fig. 6D). Moreover, the same reaction was seen with the cytosol of rat testis (data not shown). Therefore, although it is currently unclear why both NAT and RLP-1 coexist in the testis, it may be possible that RLP-1 constitutes an effective pathway for the formation of anandamide and other unsaturated NAEs. In addition, the low sensitivity to Ca²⁺ suggests that RLP-1 generates NAPEs and NAEs through a regulatory mechanism different from that of Ca²⁺-dependent NAT. Immuno-histochemical analysis to identify RLP-1-expressing cells will contribute to a better understanding of the role of RLP-1 in the testis. In regard to the predominant expression of RLP-1 in the testis, it should be noted that this organ abundantly expresses a complete set of the endocannabinoid system, including NAT, NAPE-PLD, fatty acid amidase hydrolase, and the CB1 cannabinoid receptor (13, 17, 24, 30, 52), suggesting the physiological importance of this system in the testis.

According to the data base, RLP-1, RLP-2, and RLP-3 show high similarity to human HRLP5, A-C1 and H-REV107, respectively. H-REV107 has been reported to be a class II tumor suppressor because it shows a growth-inhibiting activity in H-ras-transformed cell lines (42, 43). Similarly, A-C1, found as an H-REV107-related protein, significantly inhibits proliferation of H-ras-transformed NIH3T3 cells (44). These results suggest that members of the H-REV107-related protein family are involved in the control of cell proliferation and act as negative regulators of the oncogenic Ras signal. Therefore, RLP-1 as an H-REV107-related protein may play a role in the regulation of cell growth and differentiation in the testis. Overexpression of RLP-1 in H-ras-transformed cell lines may give a clue to its biological role. If such a biological activity is observed with RLP-1, it will be very interesting to investigate the relation of the biological activity to the catalytic activity shown in this study.

In conclusion, we have demonstrated that RLP-1 is capable of functioning as a Ca²⁺-independent PE N-acyltransferase, clearly distinguishable from the known NAT. Moreover, RLP-1 is expressed predominantly in the testis, suggesting the physiological importance of RLP-1 in this organ.

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