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cDNA cloning and characterization of human and mouse Ca^{2+}-independent phosphatidylethanolamine *N*-acyltransferases

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ABSTRACT

The formation of *N*-acylphosphatidylethanolamine by *N*-acylation of phosphatidylethanolamine (PE) is the initial step in the biosynthetic pathway of bioactive *N*-acylethanolamines, including the endocannabinoid anandamide and the anti-inflammatory substance *N*-palmitoylethanolamine. We recently cloned a rat enzyme capable of catalyzing this reaction, and referred to the enzyme as Ca^{2+}-independent *N*-acyltransferase (iNAT). Here we report cDNA cloning and characterization of human and mouse iNATs. We cloned iNAT-homologous cDNAs from human and mouse testes, and overexpressed them in COS-7 cells. The purified recombinant proteins abstracted an acyl group from both *sn*-1 and *sn*-2 positions of phosphatidylcholine, and catalyzed *N*-acylation of PE as well as phospholipase A_1/A_2-like hydrolysis. The iNAT activity was mainly detected in soluble rather than particulate fractions, and was only slightly increased by Ca^{2+}. These results demonstrated that the human and mouse homologues function as iNAT. As for the organ distribution of iNAT, human testis and pancreas and mouse testis exhibited by far the highest expression level, suggesting its physiological importance in the specific organs. Moreover, mutagenesis studies showed crucial roles of His-154 and Cys-241 of rat iNAT in the catalysis and a possible role of the N-terminal domain in membrane association or protein–protein interaction.

1. Introduction

N-Acylethanolamines (NAEs) are ethanolamides of long-chain fatty acids present in a variety of organisms. NAEs have received much attention because of various biological activities [1,2]. In particular, anandamide (*N*-arachidonoylethanolamine) has been characterized as an endogenous ligand of cannabinoid receptors [3] and vanilloid receptor [4] in mammalian tissues. Saturated and monounsaturated NAEs are inactive on cannabinoid receptors. However, *N*-palmitoylethanolamine was reported as an anti-inflammatory and analgesic substance [5,6], and *N*-oleoylethanolamine [7] and *N*-stearoylethanolamine [8] as anorexic mediators. Involvement of peroxisome proliferator-activated receptor-α (PPARα) and the G protein-coupled receptors GPR55 and GPR119 in these biological activities were suggested [9–11].

In animal tissues NAEs are principally biosynthesized from membrane glycerophospholipids by two steps of enzyme reactions [1,2,12–15]. The first reaction is the transfer of a fatty acyl group from the *sn*-1 position of a glycerophospholipid molecule to the primary amine of phosphatidylethanolamine (PE), resulting in the formation of *N*-acyl-PE (NAPE) [1,2,12–14]. This reaction is attributable to the catalysis by membrane-associated, Ca^{2+}-dependent *N*-acyltransferase (NAT) [1,2,12–14]. The second reaction is hydrolysis of NAPE to generate NAE and phosphatidic acid, and is catalyzed by NAPE-hydrolyzing phospholipase D (NAPE-PLD), a member of the metallo-β-lactamase family [16,17]. Recent studies revealed that the pathways composed of multiple enzymes are also responsible for the formation of NAE from NAPE [18–20].

H-rev107-like protein 5 (HRLP5) or HRAS-like suppressor family, member 5 (HRASLS5) is a gene of human (GenBank™ accession number, AJ416558) and mouse (NM_025731), which has been noted to be homologous to the tumor suppressor gene H-Rev107. However, its function remained unclear. We recently found that a rat gene, which appeared to be a homologue of HRLP5, encodes an enzyme capable of catalyzing *N*-acylzation of PE [21]. This enzyme was, however, detected mainly in cytosolic rather than membrane fraction, and was little stimulated by Ca^{2+}. Moreover, the enzyme transferred a fatty acyl group from not only *sn*-1 position but also *sn*-2 position of phosphatidylcholine (PC) to PE. The enzyme was, therefore, considered to be different from the known Ca^{2+}-dependent NAT, and we referred to this enzyme as Ca^{2+}-independent *N*-acyltransferase (iNAT) [21]. So far, the physiological significance of iNAT remains unclear. In addition, iNAT of mammals other than rat has not yet been examined.

In the present study, we isolated iNAT-homologous cDNAs from human and mouse, and characterized their recombinant proteins as

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iNAT enzymes. We further performed structural analysis of iNAT by mutagenesis technology.

2. Materials and methods

2.1. Materials

1,2-[1-^{14}C]Dipalmitoyl-PC (4.107 GBq/mmol) and [1-^{14}C]palmitic acid (2.06 GBq/mmol) were purchased from PerkinElmer Life Science (Boston, MA); 1-palmitoyl-2-[1-^{14}C]palmitoyl-PC (2.22 GBq/mmol), 1-palmitoyl-2-[1-^{14}C]arachidonoyl-PC (1.962 GBq/mmol), horseradish peroxidase-linked anti-mouse IgG, Hybond P, and an ECL Plus kit were from GE Healthcare Life Sciences (Piscataway, NJ); 1,2-dipalmitoyl-PC, 1,2-dioleoyl-PE, 1-palmitoyl-2-arachidonoyl-PC, fatty acid-free bovine serum albumin, anti-FLAG M2 monoclonal antibody, anti-FLAG M2 affinity gel, and FLAG peptide were from Sigma (St. Louis, MO); Dulbecco's modified Eagle's medium, Lipofectamine, fetal calf serum, pCR2.1-TOPO, pcDNA3.1(+), TRIzol, and Moloney murine leukemia virus reverse transcriptase were from Invitrogen (Carlsbad, CA); Nonidet P-40, dimethyl sulfoxide, and iodoacetic acid were from Nacalai Tesque (Kyoto, Japan); *Rhizopus delemar* lipase was from Seikagaku Corp. (Tokyo, Japan); (±)-dithiothreitol (DTT), EDTA, and 3 (2)-*t*-butyl-4-hydroxyanisole were from Wako Pure Chemical (Osaka, Japan); random hexamer, *Ex Taq* DNA polymerase, and Pyrobest DNA polymerase were from TaKaRa Bio (Ohtsu, Japan); KOD-Plus DNA polymerase was from TOYOBO (Osaka, Japan); human testis QUICK-Clone cDNA and human tissue cDNA panels were from Clontech (Mountain, CA); protein assay dye reagent concentrate was from Bio-Rad (Hercules, CA); and precoated Silica Gel 60 F_{254} aluminum sheets (20×20 cm, 0.2 mm thick) for TLC were from Merck (Darmstadt, Germany). 2-Palmitoyl-glycerophosphocholine was prepared from 1,2-dipalmitoyl-PC using *Rhizopus delemar* lipase, and was used to prepare 1-[1-^{14}C]palmitoyl-2-palmitoyl-PC from [1-^{14}C]palmitic acid [22]. The prepared 1-[1-^{14}C]palmitoyl-2-palmitoyl-PC was purified by TLC with a mixture of chloroform, methanol, and H_2O (65:25:4, v/v). Authentic radioactive compounds for TLC were prepared as described previously [21].

2.2. cDNA cloning

The cDNA containing the coding region of human or mouse iNAT was generated by PCR using the forward primers 5′-AAGCTTATGGGCCTGAGCCCGGGCGCC-3′ (human) and 5′-GAATTCATGATCCCGGGGCTAGGCGGA-3′ (mouse), and the reverse primers 5′-GAATTCTCAGGCAGTTATTGGTTTGGG-3′ (human) and 5′-GGTACCAGCAGTTATGGGTTTGGGTCTTAT-3′ (mouse). These primers were designed based on the cDNA sequences of human (AJ416558) and mouse (NM_025731). Human testis QUICK-Clone cDNA was used as a template for human iNAT. Mouse cDNAs were prepared from total RNA (5 μg) of adult mouse testis using Moloney murine leukemia virus reverse transcriptase and random hexamer. PCR was performed with the aid of Pyrobest DNA polymerase at a denaturing temperature of 94 °C for 30 s, followed by annealing at 58 °C (human) or 60 °C (mouse) for 30 s and extension at 72 °C for 1 min (human) or 2 min (mouse) (35 cycles). The resultant PCR products were subcloned into the pCR2.1-TOPO vector. The cDNA was then digested with HindIII (human) or KpnI (mouse) and EcoRI, and inserted into the eukaryotic expression vector pcDNA3.1(+). For the N-terminally FLAG-tagged human and mouse iNATs, the cDNAs were generated by PCR using the forward primers 5′-AAGCTTATGGATTACAAGGATGACGACGATAAGGCCTGAGCCCGGGCGCC-3′ (human) and 5′-GGTACCATGGATTACAAGGATGACGACGATAAGATCCCGGGGCTAGGCGG-3′ (mouse), and the above-mentioned reverse primer (human) and 5′-GAATTCTCAAGCAGTTATGGGTTTGGGTCT-3′ (mouse). PCR was performed as described above using the pCR2.1-TOPO vector harboring full-length cDNA for iNAT as a template. The PCR products were ligated into pcDNA3.1(+). cDNA cloning of rat iNAT and construction of N-terminally FLAG-tagged rat iNAT were performed as described previously [21]. All the constructs were sequenced in both directions with the aid of an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA).

2.3. Mutagenesis

Rat iNAT mutants were constructed by PCR method using the expression vector harboring rat iNAT as a template. For the point mutants H154L and C241S were prepared by the megaprimer PCR. The primers used for the first step of PCR were as follows: for H154L, 5′-AAGCTTATGGATTACAAGGATGACGACGATAAGATCCCGGGGCACCGCG-GACCC-3′ (forward, Primer 1) and 5′-TAGATGGCCCAAAGTTCATAGCC-3′ (reverse), and 5′-GGCTATGAACTTTGGGCCATCTA-3′ (forward) and 5′-GAATTCTCAAGCAGTTATGGGTTTGGGTCT-3′ (reverse, Primer 2); for C241S, Primer 1 (forward) and 5′-CGAAGTGCTCACTGTTCCCCTCG-3′ (reverse), and 5′-CGAGGGGAACAGTGAGCACTTCG-3′ (forward) and Primer 2 (reverse). PCR was performed by KOD-Plus DNA polymerase at a denaturing temperature of 94 °C for 20 s, followed by annealing at 56 °C for 20 s and extension at 68 °C for 1 min (25 cycles) in 5% (v/v) dimethyl sulfoxide. The cDNA fragments resultant from each PCR reaction were then combined by the megaprimer PCR with Primer 1 (forward) and Primer 2 (reverse) for both the point mutants. For the deletion mutants, the cDNAs were generated by PCR using the forward primers 5′-AAGCTTATGGATTACAAGGATGACGACGA-TAAGGGCCTGAGCCCAGCAGCCAGC-3′ (ΔN1), 5′-AAGCTTATGGATTA-CAAGGATGACGACGATAAGCTGGTGGTCCAGTTCTTGCCG-3′ (ΔN2), and 5′-AAGCTTATGGATTACAAGGATGACGACGATAAGGCAGCGGAGAG-CAATCAAAAA-3′ (ΔN3) and Primer 2 as the reverse primer (ΔN1, Δ2, and Δ3). PCR amplification was performed under the conditions as described above. The PCR products, which contained HindIII and EcoRI sites derived from the primers, were subcloned into the corresponding sites of pcDNA3.1 (+). All the constructs were sequenced in both directions to check the introduction of the desired mutations.

2.4. Overexpression of recombinant iNATs

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_2 and 95% air incubator. The cells were then treated with 8 μg of the expression vector harboring cDNA for iNAT 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 each for 3 s 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 soluble fraction, whereas the pellet was suspended in 20 mM Tris–HCl (pH 7.4) and used as particulate fraction. The protein concentration was determined by the method of Bradford [23] with bovine serum albumin as the standard.

2.5. Purification of recombinant iNAT

The soluble fraction was prepared from COS-7 cells overexpressing the FLAG-tagged iNAT grown in ten 100-mm dishes 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 soluble fraction was added 1 ml of anti-FLAG M2 affinity gel pre-equilibrated with buffer A, and the mixture was then incubated overnight with gently mixing to allow the FLAG-tagged iNAT to bind to the gel. The gel was then packed into a column and washed three times with each 12 ml of buffer A. The enzyme was eluted with buffer A containing 0.1 mg/ml of FLAG peptide, and every 0.5-ml fraction was collected.