Identification of a Novel Human Lysophosphatidic Acid Acyltransferase, LPAAAT-theta, Which Activates mTOR Pathway

Wenwen Tang, Jian Yuan, Xinya Chen, Xiuting Gu, Kuntian Luo, Jie Li, Bo Wan, Yingli Wang and Long Yu*

State Key Laboratory of Genetic Engineering, School of Life Science, Fudan University, Shanghai, 200433, People’s Republic of China

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Lysophosphatidic acid acyltransferase (LPAAAT) is an intrinsic membrane protein that catalyzes the synthesis of phosphatidic acid (PA) from lysophosphatidic acid (LPA). It is well known that LPAAAT is involved in lipid biosynthesis, while its role in tumor progression has been of emerging interest in the last few years. To date, seven members of the LPAAAT gene family have been found in human. Here we report a novel LPAAAT member, designated as LPAAAT-theta, which was 2728 base pairs in length and contained an open reading frame (ORF) encoding 434 amino acids. The LPAAAT-theta gene consisted of 12 exons and 11 introns, and mapped to chromosome 4q21.23.

LPAAAT-theta was ubiquitously expressed in 18 human tissues by RT-PCR analysis. Subcellular localization of LPAAAT-theta-EGFP fusion protein revealed that LPAAAT-theta was distributed primarily in the endoplasmic reticulum (ER) of COS-7 cells. Furthermore, we found that the overexpression of LPAAAT-theta can induce mTOR-dependent p70S6K phosphorylation on Thr389 and 4EBP1 phosphorylation on Ser65 in HEK293T cells.

Keywords: 4EBP1, Endoplasmic reticulum, Lysophosphatidic acid acyltransferase, mTOR, p70S6K

Introduction

Lysophosphatidic acid acyltransferase (LPAAAT), also known as 1-acyl-sn-glycerol-3-phosphate acyltransferase (AGPAT, EC 2.3.1.51), is the enzyme that catalyzes the transfer of the fatty acid from an acyl donor to the sn-2 position of lysophosphatidic acid (LPA) to yield phosphatidic acid (PA) in the lipid metabolism (Kent, 1995). LPA or 1-acyl-sn-glycerol-3-phosphate consists of a glycerol backbone with a fatty acyl chain at the sn-1, a hydroxyl group at the sn-2, and a phosphate group at the sn-3 position. In the endoplasmic reticulum (ER) membrane, LPA is synthesized from glycerol-3-phosphate by glycerol-3-phosphate acyltransferase. LPA is then further acylated in the ER by LPAAAT to yield PA, the precursor of all glycerolipids (Aguado and Campbell, 1998).

LPA and PA are two phospholipids involved in signal transduction as well as in lipid biosynthesis in cells. They both play an important role in a variety of cellular signaling responses. LPA is well known as a lipid growth factor which is involved in the regulation of numerous cellular responses through the activation of specific G-protein-coupled receptors and it was originally considered as a membrane component and metabolic intermediate in lipid biosynthesis (Li et al., 2003). LPA is present in several biological fluids (serum, plasma, and aqueous humor) and is produced in various types of cells including platelets, fibroblasts, adipocytes and cancers. PA is known as a lipid second messenger that participates in a variety of intracellular signaling events and regulates a growing list of signaling proteins, including several protein kinases and phosphatases (English et al., 1996). Recently, PA has been recognized as a mediator of certain cell signaling functions associated with oncogenesis. These include ras/raf/Erk and Akt/mTOR (Bonham et al., 2003).

The mammalian target of rapamycin (mTOR; also named FRAP or RAFT1) belongs to the family of phosphatidylinositols kinase like kinases (PIKK) (Keith and Schreiber, 1995). The mTOR regulates both cell growth and cell cycle progression through its ability to integrate signals from nutrients (amino acids and energy) and growth factors (Shamji et al., 2003). So far, the best known downstream targets of mTOR are two protein families that control protein translation, the eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BPs) and the ribosomal protein S6 kinases (S6Ks). Through activation of...
S6, S6K enhances translation of mRNAs with repressive 5'-TOP tracts; many of these mRNAs encode components of the translational machinery. p70S6K (S6K1), one of the best studied S6Ks, is a serine-threonine kinase activated by mTOR. There are at least three phosphorylation sites on p70S6K, and mTOR is proposed to phosphorylate Thr389 in the hydrophobic motif of p70S6K in a rapamycin-sensitive manner. Similar to S6K1, 4EBP1 (also known as PHAS-I), a repressor of the translation initiation factor eIF4E and thus an inhibitor of protein biosynthesis, is regulated via mTOR-dependent signals. Hyper-phosphorylation of 4EBP1 disrupts its binding eIF4E, activating cap-dependent translation. Phosphorylation of 4EBP1 occurring on multiple sites in an ordered manner. Phosphorylation by mTOR on Thr37 and Thr46 of human 4EBP1 may prime it for subsequent phosphorylation at sites including Ser65 and Thr70. S6K1 and 4EBP1 function in parallel pathways that bifurcate downstream of mTOR (Fingar and Blenis, 2004). By virtue of its ability to sense environmental conditions, mTOR functions as a rheostat to regulate the rate of cell growth and cell proliferation by, at least in part, regulating protein biosynthesis. It is thus not surprising that mTOR and its downstream targets have emerged as novel targets for cancer therapeutics (Vivanco and Sawyers, 2002; Huang and Houghton, 2003). It is thus not surprising that mTOR and its downstream targets have emerged as novel targets for cancer therapeutics.

Materials and Methods

Cloning and sequencing of LPAAT-theta cDNA
One pair of primers LPAAT-theta-F, LPAAT-theta-R (Table 1) was designed to amplify LPAAT-theta from the liver cDNA library. PCR was performed using the PCR kit (Shenyang Biocolor Co.) at 94°C 5 min, 94°C (60 s), 57°C (60 s) and 72°C (90 s) for a total of 35 cycles, with a final extension at 72°C for 10 min in a PTC-200 DNA Engine (MJ Research, Waterdown). The PCR product was then subcloned into the pMD-18-T vector (Takara) and was sequenced. The generated sequences have been deposited to GenBank Data Library under Accession No. DQ345208.

Tissue distribution of LPAAT-theta. Human MTC (multiple tissue cDNA) panels (Clontech) including bone marrow, stomach, bladder, lung, placenta, pancreas, heart, spleen, liver, thymus, testis, intestine, uterus, ovary, brain, skeleton, muscle and prostate were served as templates to study the distribution of human LPAAT-theta mRNA using the primers LPAAT-theta-RF-F and LPAAT-theta-RF-R (Table 1). Thirty cycles of amplification (30 s at 94°C, 30 s at 54°C and 30 s at 72°C) were performed using Taq DNA polymerase (Shenyang Biocolor Co.). β-Macroglobulin (β-MG) forward (β-MG-F) and reverse (β-MG-R) primers (Table 1) were used to amplify a 290bp amplicon of C-terminal of β-MG, which was served as the internal control. Amplification products above were separated by DNA electrophoresis in 2% (w/v) agarose gel.

Plasmid construction. All inserted fragments were obtained by PCR amplification using the primers listed in Table 1. First, full-length LPAAT-theta was subcloned into the eukaryotic expression vector pCMV-Myc at EcoRI and SalI sites using primers Myc-LPAAT-theta-F and Myc-LPAAT-theta-R. The full-length LPAAT-theta was also cloned into the pEGFPN1 vector at EcoRI and XhoI sites to generate C-terminal enhanced GFP-tagged fusion protein. After confirmation by sequencing, all eukaryotic expression plasmids were extracted and purified for transfection using Endofree Plasmid Maxi Kit (Qiagen).

Table 1. Neucleotide sequence of oligonucleotides used for cloning of LPAAT-theta and the construction of expression vectors

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>LPAAT-theta-F</td>
<td>5'-GAGTTGGGCTGCCTGATCAGCTAC-3'</td>
</tr>
<tr>
<td>LPAAT-theta-R</td>
<td>5'-TAAGGCTGTCATCGTCTTGAG-3'</td>
</tr>
<tr>
<td>RF-PCR primers</td>
<td></td>
</tr>
<tr>
<td>LPAAT-theta-RF-F</td>
<td>5'-GCTACCTCTGTCCTGGAATTGAC-3'</td>
</tr>
<tr>
<td>LPAAT-theta-RF-R</td>
<td>5'-CTGGCATAAAGGGAGAGACTAC-3'</td>
</tr>
<tr>
<td>β-MG-F</td>
<td>5'-ATGAGATATCGCTGGGTGAAAC-3'</td>
</tr>
<tr>
<td>β-MG-R</td>
<td>5'-TGTTAAGAAGCGCTTCAAGATAC-3'</td>
</tr>
<tr>
<td>Gene expression primers</td>
<td></td>
</tr>
<tr>
<td>Myc-LPAAT-theta-F</td>
<td>5'-AATACTTCATGAGAGAGCGACGACGTTG-3'</td>
</tr>
<tr>
<td>Myc-LPAAT-theta-R</td>
<td>5'-CTGCTGCTGCAAGGAGATCCATCAG-3'</td>
</tr>
<tr>
<td>GFP-LPAAT-theta-F</td>
<td>5'-AAATCCTGAGATGAGGAGCGAGAGAGACG-3'</td>
</tr>
<tr>
<td>GFP-LPAAT-theta-R</td>
<td>5'-CTTCAGTTCCGCTGATGATACGATCC-3'</td>
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Cell culture, transfection and western blotting. HEK293T cells were cultured in DMEM (Gibco-BRL) supplemented with 10% FBS (Fetal Bovine Serum), and kept in the CO\textsubscript{2} incubator (37°C, 5% CO\textsubscript{2}). The cultured HEK293T cells were transfected with expression vectors using Lipofectamine\textsuperscript{TM} (Invitrogen) according to the manufacturer’s protocol. The cells were harvested 48 h after transfection and washed twice with ice cold PBS (phosphate-buffered saline) and then lysed on ice for 20 min in lysis buffer (Cell Signaling Technology; supplemented with cocktail tablets and 0.1 mM PMSF) with gentle shaking. The solution was then centrifuged at 12,000×g for 30 min at 4°C to remove the debris; and the supernatant was collected.

Protein samples separated by SDS-PAGE were electro-transferred onto nitrocellulose membrane.

**Fig. 1.** Nucleotide and deduced amino acid sequences of human \textit{LPAA\textsubscript{th}}. The nucleotide of 2728 bp cDNA is shown in the top lines and its deduced amino acid sequence is shown below. The ORF extends from nucleotide 304 to 1608, and encodes a protein of 434 amino acids. The poly adenylation signal (AAATAAA) and a stop codon (TAA) upstream of the ORF at the same reading frame are boxed. The sequence shaded in black is the PlsC (phosphate acyltransferase) domain. Asterisk represents the stop codon.
with primary antibody diluted with PBS containing 1% (w/v) BSA. The membrane was washed with PBST then incubated with HRP-conjugated secondary antibody at room temperature for 1 h. The membrane was washed again with PBST and then developed with ECL system (Santa Cruz).

Analysis of LPAAT-theta subcellular localization. To subcellularly localize the LPAAT-theta protein, we transiently expressed the EGFP-LPAAT-theta vectors in COS-7 cells. The transfection experiments were carried out in 6-well plates with a coverslip in each well and the transfected cells were grown for 24-36 h. The cells were washed with PBS twice for 5 min each, then fixed with cold fixing solution (4% paraformaldehyde in PBS), followed by 0.1% Triton X-100-PBS treatment. After fixation, the cells were stained with fluorescence markers specific for organelles, such as Concanavalin A/Texas Red for endoplasmic reticulum (ER), and

Fig. 2. Characterization of LPAAT-theta gene. (A) The amino acid alignment of the enzyme domains of the eight human LPAATs: LPAAT-alpha (GenBank No. U56417), LPAAT-beta (GenBank No. U56418), LPAAT-gamma (GenBank No. AF156774), LPAAT-delta (GenBank No. AF156776), LPAAT-epsilon (GenBank No. AF375789), LPAAT-zeta (GenBank No. AF406612), LPAAT-eta (GenBank No. AW734233), LPAAT-theta (GenBank No. DQ345298). Identity is indicated by black shading and the similarity is indicated by gray shading. Dashes and spaces introduced to optimize the alignment; dots omitted amino acid residues outside of the domain. The sequences were aligned by Clustal X software (Thompson et al., 1997) and viewed by GENEDOC software. (B) Unrooted phylogenetic tree of the eight members was constructed by comparing the whole sequences using the ClustalW software.
MitoTracker Red CMXRos for mitochondrial (Molecular Probes). Then the cells were washed five times with PBS. Images of the cells were acquired using a microscope equipped with a LEICA DC 500 camera and LEICA DMRA2 fluorescent optics (LEICA).

Results and Discussion

**LPAT-theta is a novel member of the lysophosphatidic acid acyltransferase family.** The LPAT genes play important roles in glycerol-phospholipid biosynthesis. To isolate more potential members of the human LPAT family, we searched the human EST database in GenBank (http://www.ncbi.nlm.nih.gov/blast), using the nucleotide acid sequence of PlsC (phosphate acyltransferase) domain in human LPAT-zeta as a query. After checking the retrieved ESTs, we assembled them into a contig using Vector NTI suite program (InfroMax, Inc.). This contig contains an open reading frame (ORF) of 1305 nucleotides. To verify the contig, PCR primers (LPAT-theta-clone-F, LPAT-theta-clone-R) (Table 1) were designed to perform PCR in a human liver cDNA library. PCR products were subcloned into pMD18-T vector (TaKaRa) and were sequenced. The sequencing result verified the contig sequence, and this sequence was subsequently submitted to GenBank with the GenBank Accession No. DQ345298.

The cDNA sequence has an ORF extending from nucleotide 304 to 1608, and it encodes a putative protein of 434 amino acids (Fig. 1). The molecular weight of the protein was predicted to be 48.7 kDa and the calculated pI was 9.0. After searching the SMART database (http://smart.embl.helidelberg.de/), we found that the protein contains a single PlsC domain (Fig. 1), the characteristic of the LPAT family, which suggested that this protein is a novel member of LPAT family. Therefore, we named the protein as LPAT-theta (Lysophosphatidic acid acyltransferase theta), which was approved by HUGO Nomenclature Committee (the HUGO nomenclature committee suggested the gene symbol as AGPAT 8). The multiple sequence alignments indicate that,
within the acyltransferase domain, LPAT-theta shares 16%-86% identical amino acid residues with the other seven members of human LPAT family, LPAT-alpha (18.1%), LPAT-beta (19.0%), LPAT-gamma (16.1%), LPAT-delta (20.8%), LPAT-epsilon (18.3%), LPAT-zeta (86.6%) and LPAT-eta (23.2%) (Fig. 2A). The alignments showed us that LPAT-theta had the highest similarity to LPAT-zeta. We can see the relationship among the LPAT family more clearly from the phylogenetic tree (Fig. 2B). All the members of LPAT family, including LPAT-theta here, contain two similar conserved motifs: HxST/xxD and FPEGT (Fig. 2A), in which His, Asp, Glu and Gly residues are essential for acyltransferase catalytic activity (Lewin et al., 1999). As we know, all the other human LPAT members have multiple hydrophobic regions, which may serve as transmembrane regions and may be related to their biological functions. Thus, we analyzed the hydrophobicity of the amino acid sequence of LPAT-theta (http://fasta.bioch.virginia.edu/fasta_www/grease.htm) and found that LPAT-theta also contained four transmembrane regions like other members of LPAT family (Fig. 2C). We also compared the whole amino acid sequence of our human LPAT-theta with those from several other sources (mouse, rat, dog, chick, xenopus and zebra fish). The homology comparison result showed that human LPAT-theta was 93.4% identity to mouse LPAT-theta, 87.7% identity to rat LPAT-theta, 83.0% identity to dog LPAT-theta, 77.4% identity to chick LPAT-theta, 74.1% identity to xenopus LPAT-theta and 72.0% identity to zebra fish LPAT-theta (Fig 2D), which suggested that LPAT-theta is one of the conserved proteins during evolution.

**Chromosomal localization and genomic organization of LPAT-theta.** We searched the UCSC genomic database (http://genome.ucsc.edu) and mapped the LPAT-theta gene to q21.23. The STS markers (RH171183, RH92405) and the known gene BC039573 situated adjacent to the location (Fig. 3). Our search also showed that the LPAT-theta gene has twelve exons and eleven introns and all sequences at the exon-intron junctions comply with the AG-G T consensus sequence (Table 2) (Shapiro and Sempley, 1987).

**Tissue distribution of LPAT-theta.** The expression patterns
of the members in LPAAT family are variable. **LPAAT-alpha** is ubiquitous and expressed in all tissues tested, while **LPAAT-beta** has a more distinct and differential tissue distribution, principally in the liver and the heart and to a less extent in hormone responsive tissues such as the prostate, ovaries, and testis (Eberhardt et al., 1997; Kume and Shimizu, 1997; Leung, 2001). Here, we study the distribution of LPAAT-theta by using the human MTC (multiple-tissue cDNA) panels (Clontech) and RT-PCR. As shown in Fig. 4, the **LPAAT-theta** gene is almost expressed in all the tissues detected, with lower expression levels in the liver, kidney, brain, prostate, thymus and placenta and very faint positive bands shown in pancreas.

**LPAAT-theta localized in the endoplasmic reticulum in COS-7 cells.** The subcellular localization of an enzyme is crucial for it to exert the physiological function. The reaction that LPA is acylated by LPAAT to yield PA mainly occurs in the endoplasmic reticulum (ER), so the members of LPAAT family were often thought to be located primarily in the ER. On the other hand, in addition to the key role in glycerophospholipids biosynthesis, LPAATs are also involved in the biosynthesis of triacylglycerol (TG) in eukaryotes. The TG biosynthesis also occurs mainly in the ER and all enzymes involved in the pathway are localized there (Agarwal and Garg, 2003). Among the members of LPAAT family, LPAAT-alpha, LPAAT-beta and LPAAT-eta have been verified to be localized in the ER (Eberhardt et al., 1997; Leung, 2001; Ye et al., 2005). Here, to accurately localize LPAAT-theta, we transiently expressed LPAAT-theta-EGFP in COS-7 cells. After 24 h of transfection, the cells were treated in the way we described in the materials and methods. The results are consistent with the subcellular localization of LPAAT-alpha, LPAAT-beta and LPAAT-eta. The LPAAT-theta-EGFP protein was co-localized with the Con-A stained ER, as showed by the yellow color in the merged image (Fig. 5C). On the contrary, there was no co-localization between LPAAT-theta-EGFP protein and the Mito Tracker Red CMXRos stained mitochondrial (Fig. 5F).

**LPAAT-theta induced p70S6K and 4EBP1 phosphorylation in HEK293T cells.** The lipid PA has mitogenic properties due, at least in part, to its capacity to modulate the mTOR.
pathway. Stimulation with serum or factors such as IL-2 induces an increase in cellular PA levels (Flores et al., 1996; Fang et al., 2001), which is required for activation of the mTOR targets p70S6K and 4EBP1 (Fang et al., 2001). Therefore, the PA generator enzymes are consequently potential regulators of mTOR. Here we detected the protein levels p70S6K and 4EBP1, as well as the levels of p70S6K’s phosphorylation on Thr389 and 4EBP1’s phosphorylation on Ser65 when LPAAT-theta was overexpressed in HEK293T cells. As shown in Fig. 6A, expression of LPAAT-theta induced a dramatic increase in p70S6K phosphorylation on Thr389 and 4EBP1 phosphorylation on Ser65 when LPAAT-theta was overexpressed in HEK293T cells. As shown in Fig. 6A, expression of LPAAT-theta induced a dramatic increase in p70S6K phosphorylation on Thr389 and 4EBP1 phosphorylation on Ser65. LPAAT-theta also increased the phosphorylation of endogenous p70S6K and 4EBP1 in transfected HEK293T cells. We also found that LPAAT-theta-induced p70S6K and 4EBP1 phosphorylation are mTOR-mediated, because they were both abolished by rapamycin (mTOR inhibitor). Further research indicated that LPAAT-theta stimulates p70S6K and 4EBP1 phosphorylation in a dose-dependent manner, as we observed more intense p70S6K and 4EBP1 phosphorylation at higher LPAAT-theta concentrations (Fig. 6B).

There are three different enzymes generating PA: phospholipase D (PLD), diacylglycerol kinase (DGK) and LPAAT. PLD is regarded as the main contributor of PA to mTOR (Fang et al., 2001; Fang et al., 2003). Recently, DGK has also been reported as a mediator of mTOR signaling (Avila-Flores et al., 2005). Here we demonstrate the contribution of LPAAT-theta to mTOR-dependent p70S6K and 4EBP1 phosphorylation, suggesting a role for this enzyme in the regulation of mTOR-dependent pathways.

In summary, we have identified LPAAT-theta, the eighth member of the LPAAT family. LPAAT-theta was ubiquitously expressed in 18 human tissues, LPAAT-theta was located primarily in the ER. Overexpression of LPAAT-theta in cultured cells can increase mTOR-mediated phosphorylation of p70S6K on Thr389 and 4EBP1 on Ser65 in a dose-
dependent manner. The present work is the first to provide direct evidence of a LPAAT member positive role on mTOR signaling. Because the mTOR pathway is critical for regulating normal and tumor growth, investigating the exact mechanisms that control the activation and possible interrelation of the different PA generators enzymes will be of valuable interest to conceive effective cancer therapeutic strategies.

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References


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