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# THE STRUCTURE AND FUNCTIONS OF HUMAN LYSOPHOSPHATIDIC ACID ACYLTRANSFERASES

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## 1. ABSTRACT

Lysophosphatidic acid (LPA) and phosphatidic acid (PA) are two phospholipids involved in signal transduction and in lipid biosynthesis in cells. LPA acyltransferase (LPAAT), also known as 1-acyl *sn*-glycerol-3-phosphate acyltransferase (1-AGPAT) (EC 2.3.1.51), catalyzes the conversion of LPA to PA. Two human isoforms of LPAAT, designated as LPAAT-α (AGPAT1) and LPAAT-β (AGPAT2), have been extensively characterized. These two proteins contain extensive sequence similarities to microbial, plant and animal LPAAT sequences. LPAAT-α mRNA is uniformly expressed throughout most tissues with the highest level found in skeletal muscle; whereas LPAAT-β is differentially expressed, with the highest level found in heart and liver, and negligible level in brain and placenta. The LPAAT-α gene is located on chromosome 6p21.3, an area within the class III region of the major hiscompatibility complex (MHC) and the LPAAT-β gene is mapped to chromosome 9q34.3. Enhanced transcription of LPAAT-β is suggested for neoplasm of the female genital tract. Additionally, ectopic LPAAT expression in certain cytokine-responsive cell lines can effect amplification of cellular signaling processes, such as those leading to enhancement of synthesis of tumor necrosis factor-α and interleukin-6 from cells following stimulation with interleukin-1β; this suggests that the LPAAT genes represent candidates for affecting the development of certain cancers or inflammation-associated diseases.

## 2. INTRODUCTION

The initial step of phospholipid biosynthesis involves the acylation of glycerol-3-phosphate (G3P) at the *sn*-1 position by G3P acyltransferase (G3PAT) to form lysophosphatidic acid (LPA). The second step involves the acylation of LPA at the *sn*-2 position by LPA acyltransferase (LPAAT) to form phosphatidic acid (PA). Besides being the precursor molecule for all glycerophospholipid biosynthesis (1), PA has also been identified as a phospholipid signaling molecule that can be rapidly upregulated upon cellular activation and mitogenesis (2, 3). The LPAAT pathway of PA synthesis is also involved in the reciprocal remodeling of plasma membrane lipids through deacylation by phospholipase A2 (PLA2) and reacylation by acyltransferase to specific phospholipids in the *sn*-2 position (4). Two isoforms of the human homologs of LPAAT have been cloned and expressed by several research groups (5-8). This article reviews the current findings of the structure and cellular functions of these two LPAATs. As one of the isoforms, LPAAT-α, has been located within the class III region of the major histocompatiblity complex (MHC) (3), the potential roles of this enzyme in MHC-associated diseases and immunological functions will also be discussed.

## 3. CLONING AND SEQUENCE ANALYSIS OF HUMAN LPAAT cDNAS

Human cDNAs coding for LPAAT were mostly identified from the GenBank database of expressed sequence tags (dbEST) based on sequence homology (9) with the yeast (10) or certain plant (11) LPAAT protein sequences. Sequence comparison of these cDNA clones suggests the presence of at least two types of potential human LPAAT isoforms, designated here as LPAAT-α and LPAAT-β. The full-length cDNA for LPAATs were then

isolated from various cDNA libraries using the human EST

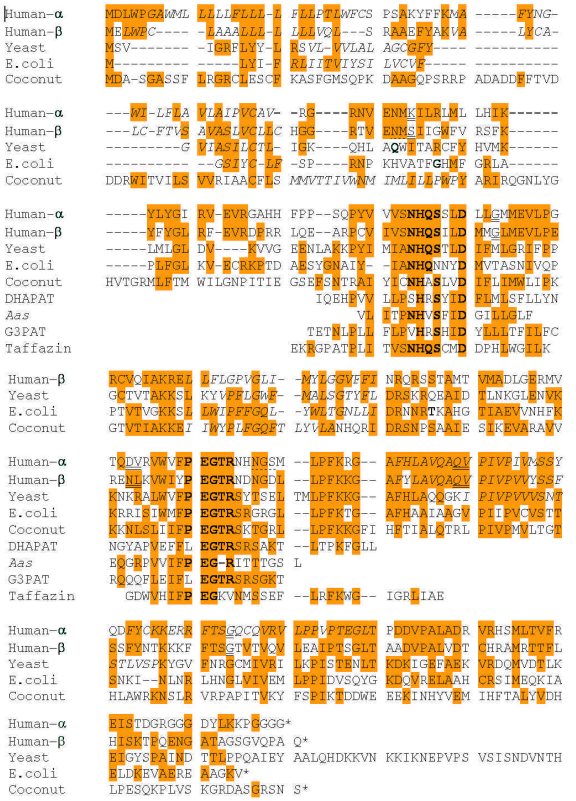


**Figure 1.** LPAAT amino acid sequence alignment from human α- and β-isoforms, yeast, *E. coli*, coconut, and other related sequences. Amino acids identical in at least two sequences are highlighted. The last ten amino acids of yeast LPAAT is not shown. Possible transmembrane domains are shown in italics. The amino acids at the junctions of exon/intron boundaries of LPAAT-α and –β are doubly underlined and the potential glycosylation site are underlined with dotted lines. For non-LPAAT sequences [DHAPAT, human dihydroxyacetonephosphate acyltransferase (31); *aas*, *E. coli* 2-acylglyceroPE acyltransferase (32); G3PAT, murine G3P acyltransferase (33); taffizin (15)], only regions around the conserved motif NHQSxxD…PEGTR (bold) are shown. The amino acids Q44 in yeast and T122 in E. coli that are important for acyl CoA substrate specificity, and G39 in E. coli that is essential to temperature sensitivity are also indicated in bold.

sequences as probes (5-8). Nucleotide sequence analysis of full-length LPAAT-α and LPAAT-β cDNA clones showed, respectively, open reading frames capable of encoding a 283 and a 278 amino acids polypeptides. The calculated molecular weights of LPAAT-α and LPAAT-β were 31.7 kDa and 30.9 kDa with predicted isoelectric points of 9.66 and 8.80, respectively.

As expected, sequence comparison of LPAAT-α and LPAAT-β with proteins in the GenBank database (9) showed that these two proteins were homologous to LPAATs identified in yeast, plants, and bacteria and to putative LPAAT homologs in *C. elegans* and *Drosophila*. An example of amino acids alignment of human LPAAT-α

and LPAAT-β with a yeast, a plant and a bacterial LPAAT is shown in figure 1. Multiple amino acid sequence alignments of the individual LPAATs show the two human α- and β-isoforms have a 34% match overall and the overall matches among human α- and β-isoforms and the other LPAATs range from 20% to 4% (5). Other human proteins that are homologous to LPAAT have recently been designated as LPAAT-γ (GenBank accession no. AF156774), LPAAT-δ (GenBank accession no. AF156776), and LPAAT-ε (GenBank accession no. AF375789). With the exception of two additional transmembrane domains near their C-termini, the rest of the hydrophobicity profiles of LPAAT-γ and LPAAT-δ are very similar to those of LPAAT-α and LPAAT-β. LPAAT-δ and LPAAT-ε have a 27% match within a stretch of 259 amino acids. Two cytosolic proteins, endophilin I (12) and one that can be ADP-ribosylated upon Brefeldin-A treatment (CtBP/BARS) (13), involved in, respectively, synaptic and golgi membrane vesicle formation have also been found to have LPAAT activity. These two proteins do not have any primary sequence homology with LPAAT α- and β described here. Change in LPAAT activity in these proteins may alter the ratio of LPA, a molecule with an inverted-cone shape, and PA, a cone shaped molecule, in the membrane and thus may affect the direction of the membrane curvature during fission. LPAAT-α and LPAAT-β are probably responsible for the bulk of LPAAT activity in most cells, as the catalytic activities of LPAAT-γ, -δ, -ε, endophilin I and BARS are relatively low (13), suggesting that these proteins may use other substrates yet to be identified.

## 4. CONSERVED MOTIFS IN LPAAT SEQUENCES

Sequence alignment of the human LPAAT-α and -β coding sequences with LPAAT coding sequences from other species (Figure 1) shows that the regions around the the amino acid sequences NHQSxxD and PEGTR corresponding to amino acids 103-109 and 177-181 of LPAAT-α are the most conserved among all LPAAT species. Localized sequence similarities between LPAATs and certain acyltransferases that do not use LPA as substrate (14), such as G3PAT, 2-acylglycerophosphatidylethanolamine acyltransferase (*aas*) and dihydroxyacetonephosphate acyltransferase (DHAPAT), can also be found within these two particular regions. Tafazzin, a protein encoding the sequence responsible for Barth syndrome, an X-linked inherited disease associated with the symptoms of cardiac and skeletal myopathy, short stature and neutropenia (15), was the only other protein identified that also contained sequence similarities within these two regions. These two short stretches of amino acids may represent a consensus sequence important for catalytic activity. Using the *E. coli* G3PAT as a model enzyme for this family of acyltransferases, site-specific mutagenesis experiments have shown that the invariant His and Asp residues within the motif N**H**QSxx**D** and the Glu and Gly residues within the motif P**EG**TR are essential for catalytic functions (14,16). Based on the proposed model for G3PAT catalysis (16), LPAAT may use this invariant His as a base to abstract a proton from the hydroxyl group at the *sn*-2 position of LPA to make it more nucleophilic to attack the thioester of acyl-CoA. The invariant Asp may act

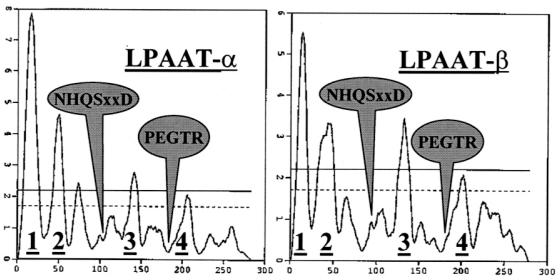


**Figure 2.** Hydrophobicity analysis (19) of proteins encoded by hLPAAT-α and hLPAAT-β. The x-axis shows the corresponding amino acid position in each LPAAT. Residues above the horizontal dotted lines are considered likely to be hydrophobic regions, whereas those above the horizontal solid lines are considered most likely to be in transmembrane regions. Predicted transmembrane domains are indicated by underlined numbers in each panel.

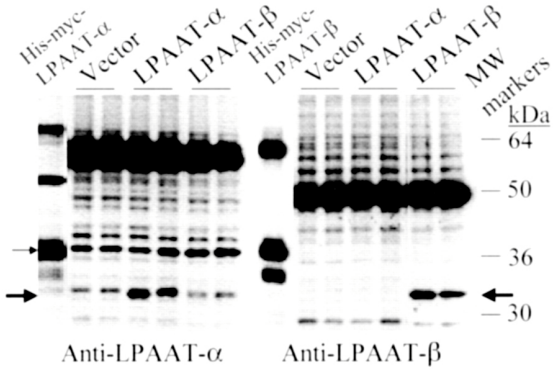


**Figure 3.** Western blot analysis of cell extracts overexpressing hLPAAT-α or hLPAAT-β after the proteins were resolved in SDS tris-glycine gels. The left panel was probed with a polyclonal antibody specific for hLPAAT-α and the right panel was probed with a polyclonal antibody specific for hLPAAT-β. The large arrows on the sides indicate the positions of the 32 kDa proteins of which the expression level were substantially increased in LPAAT-producing ECV304 cells compared to that in vector control cells. The small arrow indicates the 37 kDa His-myc-LPAAT fusion proteins expressed in Sf9 cells. The apparent MWs of the SeeBlue (Novex, San Diego, CA) pre-stained standards are shown on the right.

with the His in a charge relay system to enhance the nucleophilicity of the LPA hydroxyl group. The residues in P**EG**TR may partly involve in binding the substrate (14) LPA. Regions beyond the two conservative motifs have also been found to affect LPAAT activity. A mutation (Q to L) located in the 44$^{th}$ amino acid of yeast LPAAT has been implicated in altering its substrate specificity, which led to utilization of $C_{26}$-CoA and growth even without sphinogolipid biosynthesis (10). A temperature sensitive *E. coli* LPAAT mutation (17) has been mapped to the 39$^{th}$ amino acid with a change from Gly to Glu (18). Changing the Thr122 of E. coli LPAAT to Ala increases the substrate specificity of the enzyme for oleoyl (C18:1)- and linoleyl (C18:3)-CoA, whereas changing this Thr

to Leu increases the substrate specificity for lignoceroyl (C24:0)-CoA (18).

## 5. MEMBRANE TOPOLOGY OF LPAAT

The proteins encoded by LPAAT-α and LPAAT-β (Figure 2) have a similar hydrophobicity profile. Four transmembrane stretches are predicted based on sequence comparison using the dense alignment surface method (19), suggesting that the human LPAATs are integral membrane proteins. LPAAT-α-fusion protein containing a short epitope-tag at the carboxyl-terminus has been expressed in CHO cells (8). Immunohistochemical staining of the permeabilized cells with an epitope-specific antibody showed LPAAT was expressed predominantly in the endoplasmic reticulum (ER). The localization of LPAAT proteins to ER is consistent with the prediction based on the primary structure that LPAAT being a transmembrane protein. Based on the hydrophobicity profile, the two conserved motifs N**H**QSxx**D** and P**EG**TR essential for catalytic activity would be separated by the 3$^{rd}$ transmembrane domain (Figure 2). If this were indeed the case, these two motifs would be located on opposite side of the ER membrane with respect to each other, which is inconceivable as both motifs would be required to act in concert in close proximity to convert LPA and acyl-CoA to PA. Since the 4$^{th}$ transmembrane domain is even less hydrophobic than the 3$^{rd}$ one, a more plausible topographic structure would be only the 1$^{st}$ two highly hydrophobic transmembrane domains are utilized with the $NH_2$-terminus and the rest of the molecule after the 2$^{nd}$ transmembrane domain reside on the cytoplasmic side of the ER. This model is consistent with the finding that the synthesis of glycerolipid intermediates occurs on the cytoplasmic side of the ER (20). Western blot analysis of CHO cell extracts overexpressing the C-terminus-tagged hLPAAT-α revealed a single protein band of ~27 kDa (8). It was suggested that the reduction in size compared to the predicted hLPAAT-α size of 32 kDa was due to cleavage of the two transmembrane domains at the $NH_2$-terminus. On the contrary, we consistently observed the presence of a product with mobility in between the 30 kDa and 36 kDa markers in cell extracts overexpressing either hLPAAT-α or hLPAAT-β (Figure 3). Moreover, expression of either hLPAAT-α or -β fusion proteins containing an extra 50 amino acids encoding 6 histidines, a linker and a myc-epitope at $NH_2$-terminus have been found to be fully active and led to the detection of proteins of ~37 kDa in size in Sf9 cell extracts by Western blot analysis, suggesting the absence of any processing at the $NH_2$-terminal ends. It is possible that the discrepancy in the reported MW for recombinant LPAAT-α in SDS gels could be due to the anomalous migration of certain protein markers in electrophoresis. Expression constructs with the 1$^{st}$ two transmembrane domains deleted will be required to resolve some of these issues.

## 6. CHROMOSOMAL LOCALIZATION AND GENOMIC STRUCTURE OF LPAATS

Sequence analysis of a 300-kilobase pair segment of DNA within the MHC class III region (GenBank# U89336) shows that one of the genes embedded in this region corresponds to hLPAAT-α. Hence the hLPAAT-α gene is mapped to the chromosome band 6p21.3, corresponding to the human major histocompatibility complex (MHC) that spans 4 megabase pairs (8). Figure 4 shows the genomic structure of hLPAAT-α and the relative distance and orientation of two other genes found in the vicinity. The hLPAAT-α gene is composed of 7 exons and 6 introns spanning a distance of 7.9 kb. The coding region of hLPAAT-α extends from near the beginning of exon 2 to the 1$^{st}$ quarter of exon 7. A minor splice variant has been found in U937 cells where a canonical 5'-intron splice site (gtgagtagtg) located in intron B at 47 bp downstream from the end of exon 2 was used instead (7). If this protein is expressed, it would use a different reading frame in the area upstream of exon 3 and a different initiation AUG located slightly downstream of the regular AUG initiation codon (7).

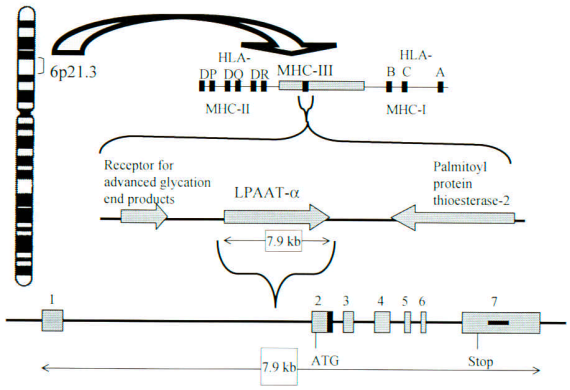


**Figure 4.** The genomic structure of hLPAAT-α and the relative distance and orientation of two other genes found in the vicinity within the MHC class III region. The internal bar in exon 7 refers to a region that may be spliced out in certain cDNA clones.

The location of the human LPAAT-β gene was mapped by fluorescence *in situ* hybridization (FISH) to band 9q34.3 near the q terminus of chromosome 9 (6). A comparison of the sequences of LPAAT-α and LPAAT-β at exon / intron boundaries (Table 1) shows they have similar coding exon sizes, suggesting the two genes have similar genomic organizations. With the exceptions at the 3'-end of intron A and at the 5'-end of intron B for LPAAT-β (6), all introns contain the 5'-gt …ag-3' consensus splice sites. Besides LPAAT-α and LPAAT-β, the chromosomal bands 6p21.3 (MHC class III region) and 9q34 contain other gene family members that are present in both areas such as *NOTCH-4*, *PBX-2*, and *TN-X* in 6p21.3 and *NOTCH-1*, *PBX-3*, and *TN-C* in 9q-34 (8), suggesting these two regions may come about from duplication of a primary common sequence.

## 7. DETECTION OF LPAAT MRNA IN HUMAN TISSUES

Northern blot analysis (Figure 5, left panels) shows that LPAAT-α is expressed in all human tissues

tested with the highest expression level found in skeletal muscle (5). The uniformity of LPAAT-α expression has also been found in additional tissues such as prostate, testis, ovary, small intestine, and colon (7) as well as in mouse tissues (21). A 2 kb and a 1.3 kb forms, possibly due to alternative utilization of polyadenylation signals at the 3'- utr, have been found in murine LPAAT-α mRNA (21), whereas only one major human LPAAT-α mRNA of 2.3 kb in size has been detected by Northern analysis (5,7). In contrast, LPAAT-β (Figure 5, right panels) demonstrates a distinct tissue distribution of mRNA expression. LPAAT-β is most highly expressed in liver and heart tissues. LPAAT-β is also expressed at moderate levels in pancreas, lung, skeletal muscle, kidney, spleen, and bone marrow; at low levels in thymus, and at undetectable levels in brain and placenta. This differential pattern of LPAAT-β expression has been confirmed independently (6) with the only discrepancy being that high level, instead of moderate level, of LPAAT-β has been detected in pancreas, possibly due to slight lot variations in commercial RNA blots (Clontech, Palo Alto, CA). In addition, moderate LPAAT-β expression has been found in prostate, testis, ovary, small intestine, and colon with the small intestine containing relatively higher amounts (6). Within the various brain sections, high expression has been found in the subthalamic nucleus and spinal cord; and least in the cerebellum, caudate nucleus, corpus callosum, and hippocampus (6). LPAAT-β can also be detected in myeloid cell lines THP-1, HL-60, and U937 with the mRNA levels remain the same with or without phorbal-ester treatment (6). The size difference between LPAAT-α and LPAAT-β mRNA is consistent with the sequence data, in which LPAAT-α has a longer 3'-UTR. The differential tissue expression pattern between LPAAT-α and LPAAT-β mRNA would suggest these two genes are regulated differently and are likely to have independent functions. Table 2 summarizes the differences and similarities between LPAAT-α and LPAAT-β.

PA has been implicated in mitogenesis of several cell lines (2). PA level has been found to be increased in either ras or fps transformed cell lines compared to the parental Rat2 fibroblast cell line (22). To test whether LPAAT expression may be enhanced in certain tumor cells, we examined the expression of LPAAT-α and LPAAT-β mRNA in human tumor panel blots (Invitrogen, Carlsbab, CA) that contained tumor RNAs isolated from various malignant tissues and RNAs from the normal tissues in the surgical margins (23). The same blots were also reprobed using cDNAs encoding phosphatidic acid phosphatase isoform PAP2-α, an enzyme that degrades, rather than generates, PA. Of a total of eight different tissues examined, LPAAT-β mRNA was found to be elevated in three tumors tissues (uterus, fallopian tube, and ovary), as compared to its expression in the corresponding normal tissues (Figure 6, upper panels), whereas no significant difference was found in LPAAT-α mRNA level between the various tumor tissues and the normal adjacent tissues. In two of the tumor tissues (fallopian tube and

**Table 1.** Exon / intron organization of human LPAAT-α and LPAAT-β genes

```
Exon /(bp, aa)                                  Intron / size (bp)
1α (309+,  0)...TACCCCTCTGAGgtgtgggcggga...A(4308)...catcccctgcagGTGGCCAGAATG
                                                                        Met
1β (   ?,  0)                                ...A.........ggggcctgagcgCGCGGGGGAGAA

2α (209,  66)...CGAGAACATGAAgtgaggggcaag...B( 226)...atgaccccacagGATCTTGCGTCT
                GluAsnMet                                          IluLeuArg
2β (248,  61)...GGAGAACATGAGgcaaggccgggg...B.........cctcccctgcagCATCATCGGCTG
                GluAsnMet                                          IluIluGly

3α (134,  44)...TCGATCTGCTTGgtgagaccccac...C( 336)...accctgcccaagGGATGATGGAGG
                 AspLeuLeu                                          MetMetGlu
3β (134,  44)...TGGACATGATGGgtaggccgggcc...C.........tctgtctcccagGCCTCATGGAGG
                 AspMetMet                                          LeuMetGlu

4α (176,  58)...CTCACCCAGGACgtgagtcatcct...D( 164)...tttctccccagGTGAGGGTCTGG
                LeuThrGlnAsp                                       ValArgValTrp
4β (176,  58)...GTCAGGGAGAACgtgagttagcaa...D.........ctctccctgcagCTCAAAGTGTGG
                ValArgGluAsn                                       LeuLysValTrp

5α (  96, 32)...GTGCAGGCCCAGgtgactactgct...E( 126)...acccttccccagGTTCCCATTGTC
                ValGlnAlaGln                                       ValProIluVal
5β (  96, 32)...GTCCAGGCACAGgtaggctgagcc...E.........ccctgcctgcagGTGCCCATCGTC
                ValGlnAlaGln                                       ValProIluVal

6α (  73, 24)...GCTTCACCTCGGgtgagggctttg...F( 517)...cccttgccccagGACAATGTCAGG
                 PheThrSer                                          GlnCysGln
6β (  73, 24)...TCTTCACTTCAGgtaccccacat...F.........cttcccccacagGAACAGTCACAG
                 PheThrSer                                          ThrValThr

7α (1234, 56)   CCCTCATCTCCAgtgactcagcct...G( 266)...cttccctcccagTGTAGCCTCCTG
7β (  784, 58)
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Exon (capital letters) and intron (lowercase letters) junction boundaries of the 7 exons and 6 introns are shown. Exons for LPAAT-α or LPAAT-β, respectively, are denoted by a number followed by the symbol α or β. The 1st two nucleotides at the 5'-end and the last two nucleotides at the 3'-end of each intron are shown in bold. The exact sizes of exons 1α aand 1β are not known since the transcription initiation sites were not determined. Splicing of intron G, located within the 3'-utr of exon 7α, was found in a small portion of LPAAT-α EST clones in GenBank.

**Table 2.** The Biochemical and Genetic Properties of human LPAAT-α and LPAAT-β

| | **LPAAT**-a | **LPAAT**-b |
|---|---|---|
| Chromosomal Location | 6p21.3 | 9q34.3 |
| Tissue Distribution | Uniformly expressed in all tissues | Differentially expressed in selective tissues |
| Tumor vs Matched Normal Tissues | Evenly expressed in both tissues | Overexpressed in selective tumor tissues |
| Protein Type | Integral membrane protein | |
| Membrane Topology | Catalytic domains on the cytoplasmic side of ER | |
| Glycosylation Site | Not utilized because not located on the luminal side of ER | |
| Conserved Motifs | **SNHQS**xL**D**-------(F/Y)**PEGTR**ND | |
| Molecular Weight | 31.7 kDa | 30.9 kDa |
| Isoelectric Point | 9.66 | 8.80 |
| Acyl Donor | Acyl CoA | |
| Acyl Acceptor | 1-acyl or 1-alkyl sn-glycerol-3-phosphate | |
| Size of mRNA | 2.3 kb | 1.7 kb |
| Cytokine Signaling | Enhanced when LPAAT is overexpressed in 2 cell lines | |

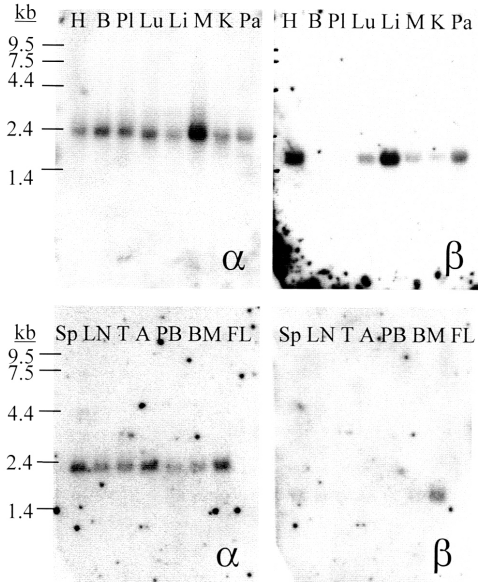


**Figure 5.** Differential expression of LPAAT mRNA in various human tissues. Nylon membranes containing 2 µg/lane poly-$A^+$ mRNA from various human tissues (Clontech, Palo Alto, CA) were hybridized sequentially with [$^{32}$P]LPAAT-α and -β cDNA fragments and exposed to X-ray films for 72 h and 16 h, respectively. The labels of the lanes are H, heart; B, brain; Pl, placenta; Lu, lung; Li, lung; M, skeletal muscle; K, kidney; Pa, pancreas; Sp, spleen; LN, lymph node; T, thymus; A, appendix; PB, peripheral blood lymphocytes; BM, bone marrow; FL, fetal liver. Reprinted (5) with permission from Mary Ann Liebert, Inc.

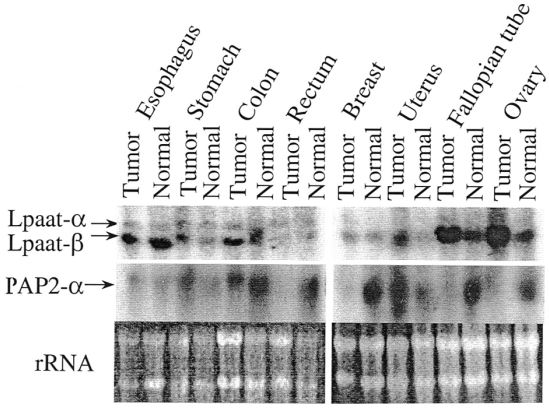


**Figure 6.** Northern Analysis of LPAAT-α and LPAAT-β mRNA in various human tumor tissues and donor-matched normal tissues. Nylon membranes containing 20 µg/lane total RNA from the tumor tissues and normal tissues (Invitrogen, Carlsbab, CA) were hybridized with a mixed probe containing equal amounts of [$^{32}$P]LPAAT-α and LPAAT-β cDNA fragments and then reprobed with a [$^{32}$P]PAP2-α1 cDNA fragment. The bottom panel depicts an ethidium bromide-stained gel. Reprinted (23) with permission from Mary Ann Liebert, Inc.

ovary) where LPAAT-β mRNA was elevated, PAP2-α mRNA expression was found to be suppressed, as it was also in tumors of the colon, rectum, and breast (Figure 6, middle panels). Using *in situ* hybridization with anti-sense LPAAT-β cDNA probe, the expression of LPAAT-β was found to be augmented in human tumor tissue in 10/11 ovarian, 14/20 breast, and 7/16 prostate biopsies as compared to normal adjacent tissues (34).

## 8. COMPLEMENTATION AND EXPRESSION OF LPAAT IN LPAAT-DEFICIENT *E. COLI*

*E. coli* strain JC201 contains a temperature-sensitive mutation in the *plsC* locus that encodes the LPAAT gene (17). This growth defect at temperature above $42^0$C can be restored by expression of either human LPAAT-α (5,7) or LPAAT-β (5). "Flip-flop" TLC analyses of the [$^{32}$P]phospholipid content of strains JC200, JC201 and JC201 expressing human LPAAT-α or LPAAT-β (5) show that (Figure 7) mutation of *plsC* in JC201 leads to a reduction in PA levels compared to that in wild-type JC200. *E.coli* JC201 cells transformed with either human LPAAT cDNA expression vector show an increase in PA, accompanied by a marked decrease in its precursor, LPA, compared to control cells. The predominant PA product generated by human LPAAT-α or -β has a faster mobility on TLC plates in the second direction employed in this chromatographic technique than does that of the major PA species endogenous in *E. coli*; this suggests that human LPAAT-α and LPAAT-β isoforms may prefer different substrates as compared to that used by *plsC*. In contrast to the results obtained by overnight labeling with [32P]PO4-3, 30 min labeling with [3H]oleic acid showed a marked decrease of label incorporation into PE and PG in JC201 cells containing the *plsC* mutation (7). This deficient oleic acid incorporation can be restored by expression of human LPAAT-α in JC201 cells (7), again suggesting that human LPAAT can complement the temperature-sensitive defect and the glycerolipid metabolism defect in *E. coli*. JC201.

## 9. EXPRESSION OF HUMAN LPAATS IN MAMMALIAN AND IN INSECT CELLS

Ectopic expression of LPAAT-α led to a 1.3- to 2.1-fold increase in LPAAT activity in COS7 (7) and CHO cells (8) when compared to the levels in vector controlcells. Since both COS7 and CHO cells contain considerable endogenous activity, LPAAT-α was expressed in insect Sf9 cells (8), which has negligible endogenous LPAAT activity, to assess its preference for acyl-CoA substrates. For LPAAT-α, the rank order for the utilization of acyl-CoAs was found to be C12:0 ~ C14:0 ~ C16:0 ~ C18:2 > C16:1 ~ C18:2 ~ C18:3 > C18:0 ~ C18:1 ~ C20:4 >> C20:0 ~ C24:0 (8), showing that LPAAT-α can utilize fatty acids of acyl chain lengths from 12 to 18 carbons with only a slight dependence on the degree of unsaturation, but does not use saturated long chain fatty acids (C20:0 or C24:0).

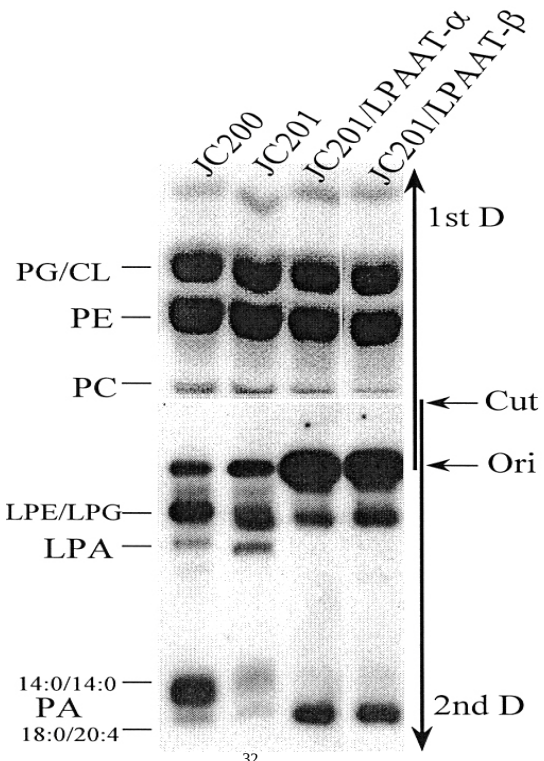


**Figure 7.** Analysis of [$^{32}$P]phospholipid contents from *E. coli* strains by ffTLC. The numbers above and below PA indicate the side chains of the PA standards used. Ori refers to the origins where the samples were applied. TLC was run in a basic solvent in the 1st direction to resolve neutral and cationic lipids, whereas PA, LPA and other anionic lipids stay near the origin. The plate was cut slightly above the Ori in the first direction, and the bottom half of the plate was rotated 180º and run in an acidic solvent to enable migration of the anionic lipids. The top part of the plate was exposed to X-ray film overnight; while the bottom part, for 6 days. Reprinted (5) with permission from Mary Ann Liebert, Inc.

As for LPAAT-β, transient expression in COS7 cells led to a more substantial (6- to 17-fold) increase in in LPAAT activity (6). The rank order for the utilization of acyl-CoAs was found to be C14:0 ~ C18:0 ~ C20:4 > C16:0 (6), indicating that LPAAT-β can utilize a variety of fatty acyl-CoAs. The efficient incorporation of arachidonyl-CoA (C20:4) into LPA to form PA by LPAAT-β and, to some extent, by LPAAT-α could be of biological significance, since arachidonic acid, the major prostagladin precursor, can be readily released from the PA formed by the action of phospholipase A2 (4). Other than the predicted acyl-acceptor LPA, the LPAAT enzymes do not use as substrates other glycerolipids with a free hydroxyl group at the *sn*-2 position, such as lysoPC, MAG or DAG (5,6); this suggests that the free phosphate domain of LPA is necessary for substrate recognition by LPAAT. The enzyme diacylglycerol acyltransferase has been found to have more similarities with acyl-CoA:cholesterol acyltransferase (24), and it is likely that the acyltransferases that utilize LPC (25) or MAG (26) as a substrate represent different enzymes from the LPAATs described here. More experiments using a variety of lipid substrates and acyl donors will be required to determine if there are subtle differences between the substrate specificities of LPAAT-α and LPAAT-β.

In contrast to *E. coli*, wherein changes in the steady state levels of LPA and PA can be readily detected upon LPAAT overexpression (Figure 7), significant differences in the steady state levels of these metabolites between the LPAAT transfected cells and the control cells have not been detected in mammalian cells despite the several-fold differences in *in vitro* LPAAT activity (5). It is possible that the PA generated via the LPAAT pathway in mammalian cells is rapidly metabolized by enzymes such as PLA2 to form LPA, PA-phosphatase to form DAG, or CDP-DAG synthase to form CDP-DAG. The partitioning of PA between DAG and CDP-DAG can be an important regulatory point in phospholipid signaling (1), as DAG is the lipid activator of protein kinase C and CDP-DAG is the precursor to phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL). PI, in turn, is the precursor for the synthesis of a series of well-studied lipid second messengers, such as phosphatidylinositol-4,5-bisphosphate ($PIP_2$), of which DAG and inositol-1,4,5-triphosphate ($IP_3$). A key enzyme involved in the synthesis of $PIP_2$, PI(4) 5-kinase α, can also be activated by PA (27).

## 10. LPAAT OVEREXPRESSION ENHANCES CYTOKINE SIGNALING RESPONSES

As a key enzyme involved in the second step of glycerophospholipid biosynthesis (17), LPAAT activity in cells can lead to the generation of PA. PA has been implicated in cytokine-induced inflammatory responses and the modulation of numerous protein kinases involved in signal transduction (2, 27, 28). LPAAT-α or LPAAT-β overexpression in two cytokine responsive cell lines, A549 and "ECV304" (the latter being recently revealed as a derivative of the bladder carcinoma T24 line and not of endothelial origin per communication from American Type Culture Collection), has led to increased synthesis of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF) upon stimulation (5). Figure 8 shows the production of IL-6 (panel A) and TNF (panel B) in A549 cells stably transfected with LPAAT-α expression vector increases by >10 fold and ~4 fold, respectively, relative to A549 cells transfected with control vector after 16 hrs of stimulation with IL-1β and murine TNF. Similarly, the production of IL-6 (panel C) and TNF (panel D) in ECV304 cells stably transfected with either LPAAT-α or LPAAT-β expression vector also increases by >10 fold relative to cells transfected with control vector after stimulation with IL-1β. However, there is little effect on the basal level of cytokine release (less than 10 pg/ml) in either cell line, suggesting that overexpression of LPAAT amplifies the cytokine signaling

response but not steady-state basal signals.

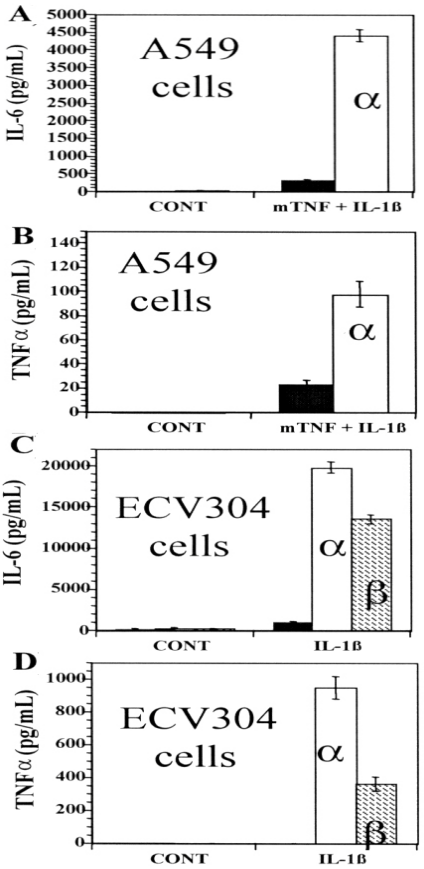


**Figure 8.** Enhancement of cytokines release in A549 and ECV304 cells transfected with LPAAT expression vectors. Cells were incubated 16 hrs in the absence (CONT) or the presence of both human IL-1β (1 ng / ml) + murine TNF (10 ng / ml) (panels A and B) or of IL-1β (1 ng / ml) alone (panels C and D). The solid, open, and hatched bars represent the concentration of IL-6 (panels A and C) or TNFα (panels B and D) measured in the culture medium of cells transfected, respectively, with control vector, with LPAAT-α, and with LPAAT-β. Each data point represents the concentration of IL-6 or TNFα measured by ELISA from triplicate samples (Mean± SD). Reprinted (5) with permission from Mary Ann Liebert, Inc.

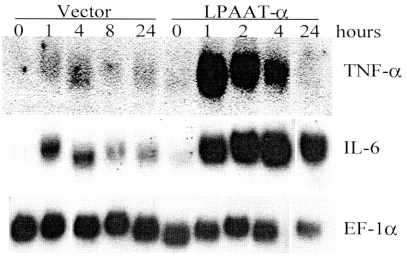


**Figure 9.** LPAAT overexpression enhances the IL-1β induced transcription of TNF and IL-6 mRNA. RNA was extracted from ECV304 cells transfected either with control vector or with LPAAT-α expression plasmid at various time points after IL-1β treatment. Northern blot analyses were performed using 32P-probes derived from TNF, IL-6 and EF-1α cDNA after X-ray film exposure for 72, 24, and 2 hr, respectively. Reprinted (5) with permission from Mary Ann Liebert, Inc.

To determine whether LPAAT expression was enhancing the IL1-β-dependent release of TNF and IL-6 through activation of the transcription of these cytokine mRNAs, Northern analysis of TNF and IL-6 mRNA levels (Figure 9) was performed in ECV304 cells transfected with either LPAAT expression vectors or control vector at various times after IL-1β stimulation (5). As compared to vector control cells, which show some induction of both TNF and IL-6 mRNAs after IL-1β stimulation, cells transfected with LPAAT-α expression vector showed an additional 8 to 12 fold increase in TNF mRNA level and a 4 to 6 fold increase in IL-6 mRNA level within the first few hours after IL-1β induction, with little variation in the mRNA level of a housekeeping gene, EF-1α.

## 11. LPAAT EXPRESSION AND MHC RESPONSE

TNF-α has been shown to induce MHC and adhesion molecule expression in certain cell types and is therefore implicated in the amplification of allograft or xenograft rejection (29, 30). As overexpression of LPAAT can enhance cellular responses to cytokine stimulation, leading to elevation of the transcription of IL-6 and TNF (Figure 9), and enhanced synthesis of IL-6 and TNF proteins (Figure 8), it is possible that overexpression of LPAAT in certain cells might enhance the *in vivo* cellular immune response to incompletely matched organ grafts. Development of specific inhibitors for LPAAT enzymes may be of interest to attenuate the MHC response and other immune functions.

## 12. ACKNOWLEDGMENTS

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