results showed that the cysteine at the end of motif 3 is highly conserved among many enzymes of many species. We termed these enzymes "motif 3-cysteine" acyltransferases in this work. The establishment of this classification allowed us to investigate the evolution of LPCAT1. We compared the "motif 3-cysteine" enzymes to determine when LPCAT1 first appeared. The phylogenetic tree suggested that the evolution of LPCAT1 occurred before the appearance of teleosts. It is said that the ancestor of the lung also appeared before the evolution of teleosts as a "respiratory pharynx", which became the air bladder in teleosts and the lung in other vertebrates (26). Thus, the evolution of LPCAT1 appears to coincide with that of the lung. Since both LPCAT1 and LPCAT2 possess lyso-PAF AT activity, the ancestor enzyme of LPCAT1 might be a PAF biosynthetic enzyme. Additionally, LPCAT1 might have evolved as the biosynthetic enzyme of pulmonary surfactant phospholipids. The high expression level of hLPCAT1 mRNA in the human lung underscores its involvement in pulmonary function.

The properties of hLPCAT1 that we have described here implicate it in the biosynthesis of pulmonary surfactant lipids. First, it synthesizes DPPC and DSPG *in vitro*, the major lipid components of pulmonary surfactant. Second, the evolution of LPCAT1 appears to coincide with the evolution of the lung. Third, hLPCAT1 is highly expressed in the human lung. In addition to these findings, hLPCAT1 mRNA (which was called AYTL2 in the report) was shown to be upregulated during *in vitro* differentiation of alveolar type II cells (30). Based on the above observations by us and others, we propose that hLPCAT1 is the biosynthetic enzyme of pulmonary surfactant lipids. Further studies are needed to elucidate the roles of hLPCAT1 in surfactant production *in vivo*. Given the fact that mutations in numerous genes involved in pulmonary surfactant function lead to various pulmonary diseases (for example ATP-binding cassette A3 and surfactant protein B) (3, 31), alterations in hLPCAT1 function may also be responsible for some lung dysfunctions.

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Footnotes

The nomenclature of the lysophospholipid acyltransferases in this study is based on the proposal of Shindou and Shimizu (6).

Figure Legends

Figure 1 Cloning and expression of hLPCAT1

A: The expression of hLPCAT1 was examined by RT-PCR using cDNA from A549, HEK293 and HeLa cells as templates. As a negative control, RT-PCR without template (no cDNA) was performed. B: The expression of hLPCAT1 in microsomes from vector- or FLAG-hLPCAT1-transfected CHO-K1 cells was detected by Western blot using anti-FLAG antibody (upper panel, 7 μg of protein per lane) or anti-LPCAT1 antiserum (lower panel, 1 μg of protein per lane). C: The endogenous expression of hLPCAT1 in A549, HEK293 and HeLa cells was determined by Western blot (10 μg of protein per lane) using anti-LPCAT1 antiserum. 1 μg of microsomes from FLAG-hLPCAT1 overexpressing CHO-K1 cells was loaded for comparison. D: Microsomes from vector-, hLPCAT1-wild type (WT)- or hLPCAT1-N213A-transfected HeLa cells were treated (+) or not (-) with N-glycosidase F and subjected to Western blot analysis. E: Microsomes from vector-, hPAFR-WT- or hPAFR-N169A-transfected HeLa cells were treated (+) or not (-) with N-glycosidase F and subjected to Western blot analysis. A, C: Same experiments were performed two times independently with similar results. D, E: Same experiments were performed three times independently with similar results.

Figure 2 Enzymatic activity of hLPCAT1

A: LPCAT (using palmitoyl-CoA), B: LPGAT (using palmitoyl-CoA) and C: lyso-PAF AT (using acetyl-CoA) activities were measured using microsomes from vector- or hLPCAT1-transfected HeLa cells. D: LPCAT activity was measured in the absence or presence of PC in the reaction mixture. n. s.: not significant. A-D: Values are means ± SD. Same experiments were performed three times independently with similar results. A-C: Unpaired t-test. D: Two-way ANOVA, Bonferroni.

Figure 3 NEM sensitivity of hLPCAT1

A: LPCAT activity of hLPCAT1 was measured after preincubation with 0-40 mM NEM. Values are means ± SD. Same experiments were performed three times independently with similar results. * $p < 0.001$ versus LPCAT1 with 0 M NEM, two-way ANOVA, Bonferroni. B: Several acyltransferases were compared to identify a conserved cysteine that may be important for the activity (arrowhead).

Figure 4 Analysis of Cys^{211} mutants

A: Microsomes from vector-, hLPCAT1- or the indicated hLPCAT1 mutant-transfected HeLa cells were subjected to Western blot (5 μg of protein per lane) using anti-FLAG antibody. B: LPCAT and C: LPGAT activities of microsomes from vector-, hLPCAT1-WT- or hLPCAT1 mutants-transfected HeLa cells were measured. B, C: Values are means ± SD. A-C: Same experiments were performed three times independently with similar results.

Figure 5 Phylogenetic tree of "motif 3-cysteine" acyltransferases

Multiple alignment of putative lysophospholipid acyltransferases containing a cysteine at the end of motif 3 was performed. Based on the result, a phylogenetic tree was drawn. Arrow: the time of bifurcation of LPCAT1 and other proteins.

Figure 6 Tissue distribution of hLPCAT1 mRNA

A: Quantitative PCR was performed using cDNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas as templates. Values are illustrated as the relative expression level of hLPCAT1 divided by that of glyceraldehyde-3-phosphate dehydrogenase. The value for human heart was set as 1. B: Quantitative PCR was performed using cDNA from human spleen, thymus, prostate, testis, ovary, small intestine, colon and leukocytes as templates. Values are illustrated as the relative expression level of hLPCAT1 divided by that of glyceraldehyde-3-phosphate dehydrogenase. The value for human thymus was set as 1.

Table 1. Primer design for constructing mutants

hLPCAT1 mutant	forward primer	reverse primer
C211S	AGAAGGAACTTTTACAAACAGGACCTGC	TCCTGTTTGTAAAAGTTCCTTCTGGAAAAATC
C211R	AGAAGGAACTCGTACAAACAGGACCTGC	TCCTGTTTGTACGAGTTCCTTCTGGAAAAATC
C211F	AGAAGGAACTTCTACAAACAGGACCTGC	TCCTGTTTGTAGAAGTTCCTTCTGGAAAAATC
N213A	AACTTGTACAGCCAGGACCTGCCTAATTAC	GGCAGGTCCTGGCTGTACAAGTTCCTTCTG
PAFR mutant	forward primer	reverse primer
N169A	CAGTGCTGGCTCAGGCGCCGTCACTCGCTGCTTTG	CAAAGCAGCGAGTGACGGCGCCTGAGCCAGCACTG

Figure 1

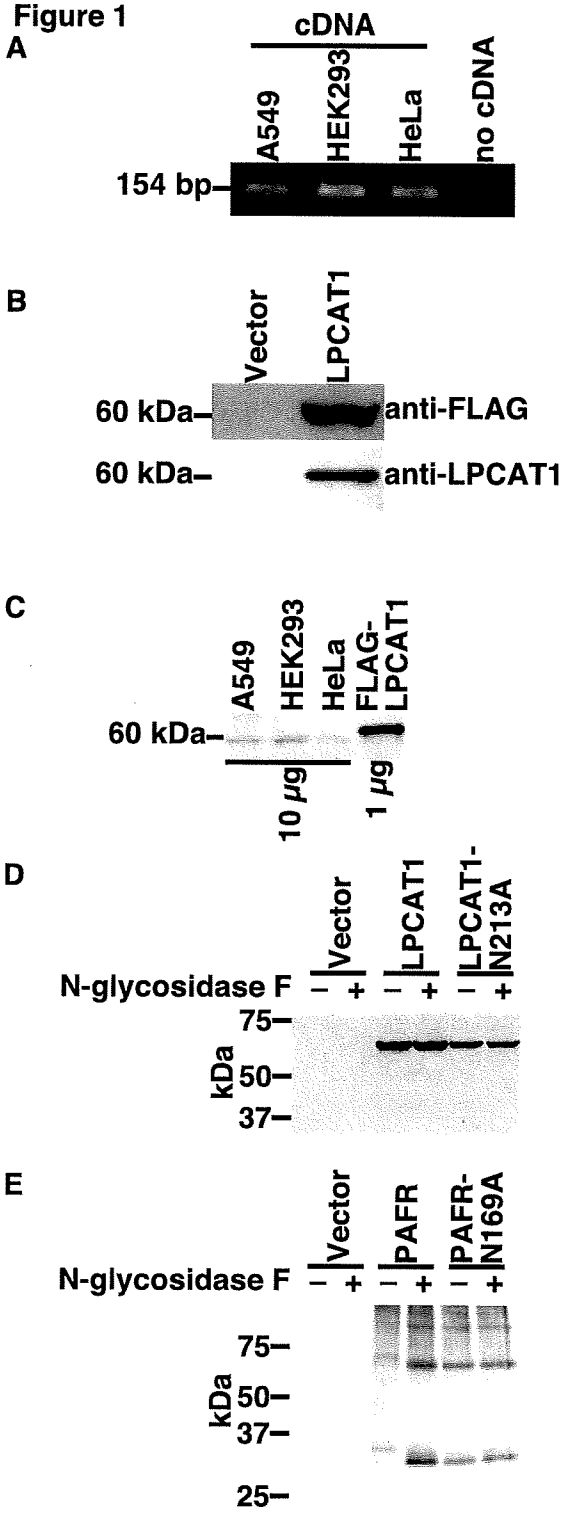

A
cDNA
A549
HEK293
HeLa
no cDNA
154 bp
B
Vector
LPCAT1
60 kDa
anti-FLAG
60 kDa
anti-LPCAT1
C
A549
HEK293
HeLa
FLAG-LPCAT1
60 kDa
10 μg
1 μg
D
Vector
LPCAT1
LPCAT1-N213A
N-glycosidase F
75
kDa
50
37
E
Vector
PAFR
PAFR-N169A
N-glycosidase F
75
kDa
50
37
25

Figure 2

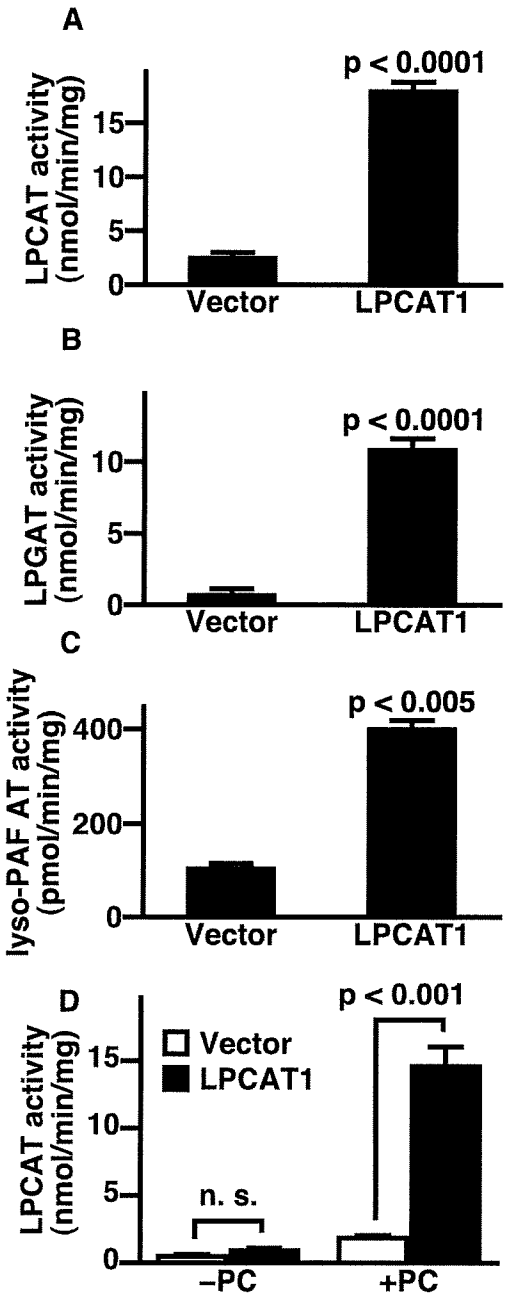
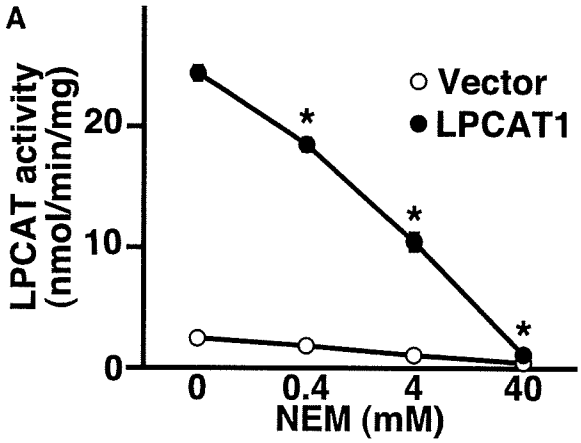

A
p < 0.0001
LPCAT activity (nmol/min/mg)
15
10
5
0
Vector
LPCAT1
B
p < 0.0001
LPGAT activity (nmol/min/mg)
10
5
0
Vector
LPCAT1
C
p < 0.005
lyso-PAF AT activity (pmol/min/mg)
400
200
0
Vector
LPCAT1
D
p < 0.001
Vector
LPCAT1
LPCAT activity (nmol/min/mg)
15
10
5
0
n. s.
–PC
+PC

Figure 3

A



B

	motif 3	NEM sensitivity
LPAAT1	^{175}VFPEGTR^{181}	unknown
LPGAT1	^{181}LFPEGGF^{187}	unknown
GPAT1	^{312}IFLEGTR^{318}	resistant
GPAT2	^{287}IFLEEPP^{293}	sensitive
GPAT3	^{300}IFPEGTC^{306}	sensitive
GPAT4	^{319}IFPEGTC^{325}	sensitive
LPCAT1	^{205}IFPEGTC^{211}	sensitive*
LPCAT2	^{217}VFPEGTC^{223}	unknown
LPEAT2	^{200}FFPEGTC^{206}	unknown

▲ *this study

Figure 4

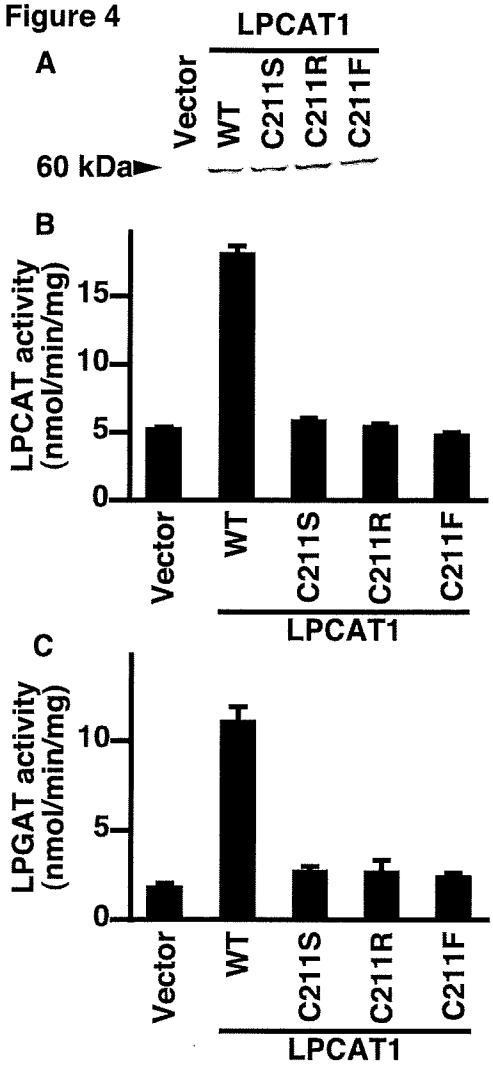
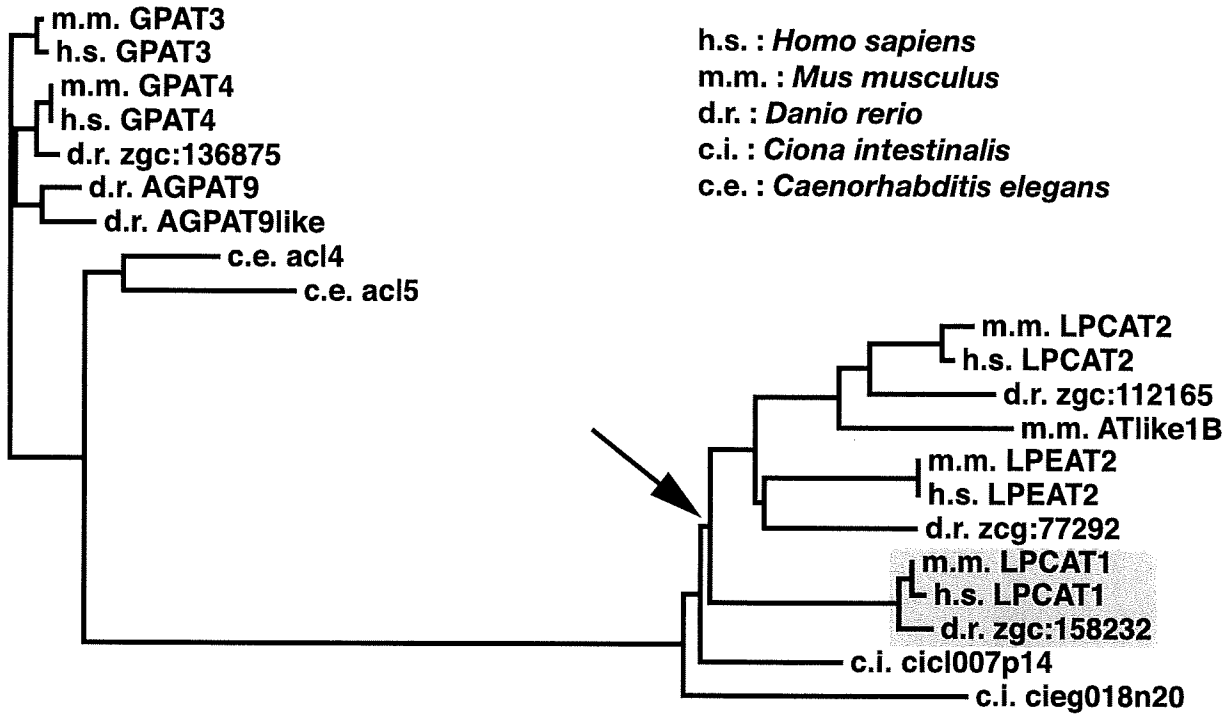

A
LPCAT1
Vector
WT
C211S
C211R
C211F
60 kDa
B
LPCAT activity (nmol/min/mg)
15
10
5
0
Vector
WT
C211S
C211R
C211F
LPCAT1
C
LPGAT activity (nmol/min/mg)
10
5
0
Vector
WT
C211S
C211R
C211F
LPCAT1

Figure 5


m.m. GPAT3
h.s. GPAT3
m.m. GPAT4
h.s. GPAT4
d.r. zgc:136875
d.r. AGPAT9
d.r. AGPAT9like
c.e. acl4
c.e. acl5
h.s. : Homo sapiens
m.m. : Mus musculus
d.r. : Danio rerio
c.i. : Ciona intestinalis
c.e. : Caenorhabditis elegans
m.m. LPCAT2
h.s. LPCAT2
d.r. zgc:112165
m.m. ATlike1B
m.m. LPEAT2
h.s. LPEAT2
d.r. zcg:77292
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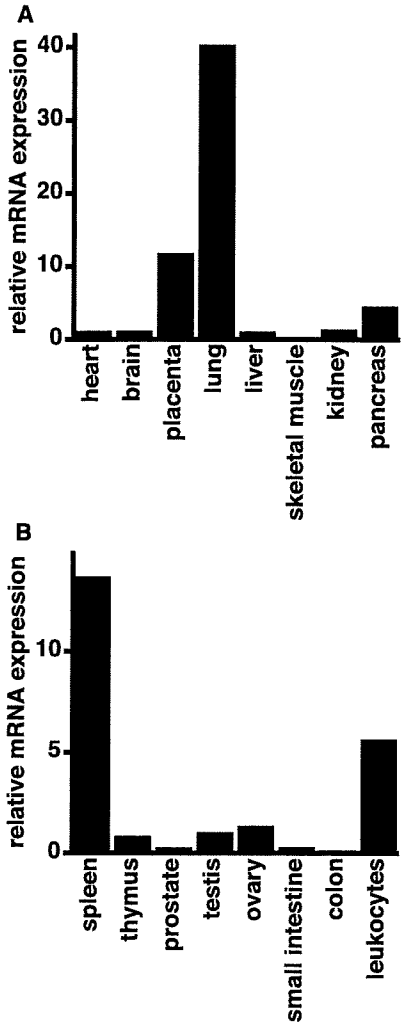
c.i. cicl007p14
c.i. cieg018n20

Figure 6


A
relative mRNA expression
40
30
20
10
0
heart
brain
placenta
lung
liver
skeletal muscle
kidney
pancreas
B
relative mRNA expression
10
5
0
spleen
thymus
prostate
testis
ovary
small intestine
colon
leukocytes

Identification of membrane O-acyltransferase family motifs

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