

# *Caenorhabditis elegans* *mboa-7*, a Member of the MBOAT Family, Is Required for Selective Incorporation of Polyunsaturated Fatty Acids into Phosphatidylinositol

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Phosphatidylinositol (PI) is a component of membrane phospholipids, and it functions both as a signaling molecule and as a compartment-specific localization signal in the form of polyphosphoinositides. Arachidonic acid (AA) is the predominant fatty acid in the *sn*-2 position of PI in mammals. LysoPI acyltransferase (LPIAT) is thought to catalyze formation of AA-containing PI; however, the gene that encodes this enzyme has not yet been identified. In this study, we established a screening system to identify genes required for use of exogenous polyunsaturated fatty acids (PUFAs) in *Caenorhabditis elegans*. In *C. elegans*, eicosapentaenoic acid (EPA) instead of AA is the predominant fatty acid in PI. We showed that an uncharacterized gene, which we named *mboa-7*, is required for incorporation of PUFAs into PI. Incorporation of exogenous PUFA into PI of the living worms and LPIAT activity in the microsomes were greatly reduced in *mboa-7* mutants. Furthermore, the membrane fractions of transgenic worms expressing recombinant MBOA-7 and its human homologue exhibited remarkably increased LPIAT activity. *mboa-7* encodes a member of the membrane-bound *O*-acyltransferase family, suggesting that *mboa-7* is LPIAT. Finally, *mboa-7* mutants had significantly lower EPA levels in PI, and they exhibited larval arrest and egg-laying defects.

## INTRODUCTION

Various kinds of fatty acids are distributed in membrane phospholipids in mammalian cells and tissues (Lands and Crawford, 1976; Holub and Kuksis, 1978; MacDonald and Sprecher, 1991). The fatty acyl residues of individual phospholipids seem to be under strict metabolic regulation. In general, saturated fatty acids are esterified at the *sn*-1 position, whereas polyunsaturated fatty acids (PUFAs), such as arachidonic acid (AA), are commonly found at the *sn*-2 position. Three-fourths or more of the phosphatidylinositol (PI) fraction in rat liver and brain constitutes the 1-stearoyl-2-arachidonoyl species (Holub and Kuksis, 1971; Baker and

Thompson, 1972). In contrast, the total pool of precursor phosphatidic acid (PA) in rat liver and brain has a fatty acid composition that does not resemble that of PI, showing a low AA content (Possmayer *et al.*, 1969; Akesson *et al.*, 1970; Baker and Thompson, 1972). Selectivity could be expressed during *de novo* synthesis at the level of formation of cytidine 5'-diphosphate (CDP)-diacylglycerol from PA and cytidine 5'-triphosphate or in the use of CDP-diacylglycerol in the final reaction. In fact, CDP-diacylglycerol synthase, which prefers 1-stearoyl-2-arachidonoyl PA as a substrate *in vitro*, has been cloned, although expression is restricted to testis, retina, and brain (Saito *et al.*, 1997).

An alternative mechanism for species selection has been proposed on the basis of turnover studies in rat brain *in vivo* (Baker and Thompson, 1972). [<sup>3</sup>H]AA and [<sup>14</sup>C]glycerol injected intracerebrally were incorporated almost exclusively into brain phospholipids. Comparison of PI and PA radioactivity suggest that the initial flux of AA into PI was independent of *de novo* synthesis from PA. The rapid turnover of the *sn*-2 acyl chains of glycerophospholipids was originally described by Lands (Lands, 1958; Lands, 1960; Lands and Merkl, 1963; Merkl and Lands, 1963), who proposed that acyl moieties of membrane phospholipids are rapidly metabolized by the action of phospholipase A<sub>2</sub>s and subsequently by lysophospholipid acyltransferases (Lands' cycle). To date, at least two lysophosphatidylcholine acyltransferases (LPCAT) were cloned from mammals: LPCAT1 catalyzes the incorporation of saturated fatty acids into lyso-

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Abbreviations used: AA, arachidonic acid; CoA, coenzyme A; EPA, eicosapentaenoic acid; GC, gas chromatography; GFP, green fluorescent protein; NGM, nematode growth medium; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; siRNA, small interfering RNA; WT, wild-type; X:Yn-Z, fatty acid chain of X carbon atoms and Y methylene-interrupted *cis* bonds (Z indicates the position of the terminal double bond relative to the methyl end of the molecules).

phosphatidylcholine (lysoPC) (Chen *et al.*, 2006; Nakanishi *et al.*, 2006), and LPCAT2 catalyzes the incorporation of acetic acid and AA into lysoPC (Shindou *et al.*, 2007). These enzymes have conserved motifs in common with the enzymes involved in the de novo phospholipid synthesis pathway (Kennedy pathway) such as acylglycerolphosphate acyltransferase and lysophosphatidic acid acyltransferase (LPAAT). Very recently, a novel lysophospholipid acyltransferase was identified from yeast that catalyzes the transfer of unsaturated fatty acids into most major lysophospholipids (Benghezal *et al.*, 2007; Jain *et al.*, 2007; Riekhof *et al.*, 2007; Tamaki *et al.*, 2007). In contrast to LPCAT1 and LPCAT2, this enzyme harbors a membrane-bound *O*-acyltransferase (MBOAT) motif present in a variety of acyltransferases of bacteria and mammals (Hofmann, 2000). Recent work on the lipid remodeling of glycosylphosphatidylinositol (GPI) anchors in yeast showed that Gup1p is required for the addition of a C26 fatty acid in the *sn*-2 position of GPI anchors (Bosson *et al.*, 2006). Gup1p is also a member of MBOAT family.

High levels of lysophosphatidylinositol acyltransferase (LPIAT) and LPCAT have been detected in the microsomal fraction of various mammalian tissues and cells (Keenan and Hokin, 1962; Keenan and Hokin, 1964; Inoue *et al.*, 1984). These experiments led to speculation that deacylation–reacylation reactions are important in establishing fatty acid pairing, and particularly in regulating the major molecular species of PI (Baker and Thompson, 1973; Holub, 1976). LPCAT and LPIAT show different substrate specificities for acyl donors, and they can be separated chromatographically (Sanjanwala *et al.*, 1989; Yamashita *et al.*, 2003), indicating that LPIAT and LPCAT are distinct enzymes. However, the gene(s) involved in LPIAT reaction have not been reported.

*C. elegans*, a free-living nematode, is a good model for studying the physiological functions of unsaturated fatty acids. Unlike mammals, *C. elegans* possesses all the required fatty acid biosynthetic enzymes, and it has no dietary requirements for essential fatty acids (Napier and Michaelson, 2001; Wallis *et al.*, 2002). Moreover, a series of mutants have been isolated that are unable to synthesize specific PUFAs due to mutations in genes encoding fatty acid desaturases (*fat* genes, Watts and Browse, 2002). *C. elegans* membrane fatty acids can be manipulated by dietary supplementation with fatty acids (Watts *et al.*, 2003; Watts and Browse, 2006). In contrast to mammals in which AA is a major PUFA in membrane phospholipids and eicosapentaenoic acid (EPA) is a minor component, *C. elegans* possesses abundant EPA but not AA (Satouchi *et al.*, 1993; Wallis *et al.*, 2002; Watts and Browse, 2002). The major PUFA attached to PI in wild-type worms is EPA and not AA. However, AA becomes an abundant PUFA in PI in *fat-1* mutants in which no n-3 PUFAs, including EPA, are produced (our unpublished data).

We used *C. elegans fat* mutants to establish a screening system to identify genes required for the incorporation of exogenous PUFAs into endogenous phospholipids. The *fat-4 fat-1* double mutants lack both n-3 and  $\Delta 5$  fatty acid desaturation activities; therefore, they fail to produce AA (20:4n-6) and n-3 PUFAs such as EPA (20:5n-3) (Kahn-Kirby *et al.*, 2004). The *fat-4 fat-1* mutants exhibit slow growth at low temperatures, and the growth defects are rescued by dietary supplementation with AA or EPA. Because it has generally been assumed that PUFAs such as AA and EPA are incorporated into phospholipids via a deacylation–reacylation remodeling pathway rather than a de novo synthesis pathway, it was reasonable to hypothesize that RNA interference (RNAi)-mediated knockdown of genes involved in the incorporation of exogenous PUFAs into phospholipids via the

remodeling pathway would cause growth retardation even in the presence of PUFAs in the medium. On the basis of this assumption, we performed comprehensive RNAi analysis with *fat-4 fat-1* mutants. In the present work, we showed that a previously uncharacterized gene encoding a 453-amino acid protein belonging to the MBOAT family is required for the incorporation of PUFA into PI.

## MATERIALS AND METHODS

### Materials

[1-<sup>14</sup>C]Stearic acid, [1-<sup>14</sup>C]oleic acid, [1-<sup>14</sup>C]linoleic acid, [1-<sup>14</sup>C]linolenic acid, [1-<sup>14</sup>C]eicosatrienoic acid, [1-<sup>14</sup>C]eicosatetraenoic acid, [1-<sup>14</sup>C]arachidonic acid, [1-<sup>14</sup>C]eicosapentaenoic acid, and [1-<sup>14</sup>C]arachidonoyl-coenzyme A (CoA) were purchased from American Radiolabeled Chemicals (St. Louis, MO). PI and lysoPI from porcine liver were purchased from Serdary Research Laboratories (London, ON, Canada). Lyso-phosphatidylserine (PS) from porcine brain, lysoPE from porcine liver, lysoPC from egg yolk, and *sn*-1-oleoyl-lysoPA were purchased from Avanti Polar Lipids (Alabaster, AL). AA and EPA were purchased from Cayman Chemical (Ann Arbor, MI). 1,2-Dipalmitoyl PI was purchased from Cayman Chemical. *Rhizopus delemere* lipase was purchased from Seikagaku (Tokyo, Japan). Phospholipase A<sub>2</sub> from honey bee venom was purchased from Sigma-Aldrich (St. Louis, MO).

### General Methods and Strains

Maintenance and genetic manipulation of *C. elegans* were carried out as described previously (Brenner, 1974). The *C. elegans* wild-type strain was Bristol N2, and *Escherichia coli* OP50, a bacterium that does not synthesize or require PUFAs (Tanaka *et al.*, 1996), was used as the sole food source. The following mutations were used: *fat-1(wa9)*, *fat-3(wa22)*, *fat-4(wa14)* (Watts and Browse, 2002), *eri-1(mg366)* (Kennedy *et al.*, 2004), and *mboa-7(gk399)* mutation was backcrossed at five times before further analysis.

### Large-Scale RNAi Screening for Genes Involved in Incorporation of Exogenous AA into Phospholipids

Feeding RNAi was performed as described previously (Kamath *et al.*, 2001). In total, 5720 bacterial RNAi feeding strains from the Ahringer library (Ashrafi *et al.*, 2003) were tested with wild-type, *fat-4 fat-1*, and *fat-3 fat-1* mutants under 25  $\mu$ M AA-supplemented conditions at 15°C. We selected RNAi clones that caused growth defects only in *fat-4 fat-1* and/or *fat-3 fat-1* mutants even under AA-supplemented conditions. We used AA instead of EPA as an exogenous PUFA source due to its lower cost. Because *fat-4 fat-1* mutants lacking endogenous AA and EPA do not exhibit embryonic or larval lethality, phospholipids having these fatty acyl chains seemed not to be essential for survival of *C. elegans*. Therefore, we excluded genes required for survival in wild-type animals. For dietary supplementation of plates with AA or EPA, an 80 mM stock solution of fatty acids in ethanol was added to nematode growth medium (NGM) at a final concentration of 25  $\mu$ M. Plates were poured, covered, and allowed to dry in the dark at room temperature overnight. All screenings were carried out at 15°C in the RNAi-hypersensitive *eri-1(mg366)* background (Kennedy *et al.*, 2004).

### Cloning of *C. elegans mboa-7* and a Human Orthologue

Full-length *mboa-7* cDNA was amplified by polymerase chain reaction (PCR) from a *C. elegans* cDNA library with primers *mboa-7-F*, 5'-CAG GTC TGC AGA TGG AAA ATA TCC TTG GCT T-3' and *mboa-7-R*, 5'-CTT CCG ATT TTT GAG CTT TTT TCG GC-3'. The PCR-amplified *mboa-7* cDNA was cloned into pPD95.67 at the PstI and SmaI sites. Human *mboa-7* (*h-mboa-7*) and mouse LPIAT (*m-mboa-7*) cDNAs were amplified from HeLa cell and mouse liver cDNA libraries, respectively, and cloned into pCAGGS-MCS vector (Niwa *et al.*, 1991) at the EcoRI and XhoI sites. The following primers were used for cloning of *h-mboa-7* and *m-mboa-7*: *h-mboa-7-F1*, 5'-CAG CTG AAT TCA TGT CGC CTG AAG AAT GGA C-3'; *h-mboa-7-R1*, 5'-GTG ACC TCG AGC TCC TCC CGG AGC TTC TCC G-3'; *m-mboa-7-F*, 5'-ACG GTT GAA TTC ATG ACA CCC GAA GAA TGG AC-3'; and *m-mboa-7-R*, 5'-CAG CAG CTC GAG CTC TTC CCG GAG CTT TTC-3'. *mboa-7*, *h-mboa-7*, and *m-mboa-7* sequences have been submitted to the GenBank databases under accession numbers EU016382, EU016381, and EU016380, respectively.

### Preparation of Transgenic Worms

DNA injection into the *C. elegans* germ line was carried out as described previously (Mello *et al.*, 1991). The array *xE1[mboa-7::GFP]* contained the plasmids pKE1 (*Pmboa-7::mboa-7::GFP*) and pRF4 [*rol-6(su1006)*]. The arrays *xE2[mboa-7<sup>H350A</sup>::GFP]* and *xE3[mboa-7<sup>H350A</sup>::GFP]* contained the plasmids pKE2 (*Pmboa-7::mboa-7<sup>H350A</sup>::GFP*) and pRF4. The array *xE4[h-mboa-7::GFP]* contained the plasmids pKE3 (*Pmboa-7::h-mboa-7::GFP*) and pFXneges-1EGFP (*Pges-1::EGFP*). pKE1, pKE2, and pKE3 were prepared as follows.

**pKE1.** A 6594-base pair genomic fragment containing 4000 base pairs upstream of the initiation codon and part of the coding sequence truncated at the 3' end was amplified by PCR from wild-type worms with primers *mboa-7* genome-F, 5'-ACT GAC TGC AGT AAA ACC TTC TTA AAC C-3', and *mboa-7* genome-R, 5'-CTT CCG ATT TTT GAG CTT TTT TCG GC-3'. The fragment was cloned in frame with the green fluorescent protein (GFP) gene into the pPD95.67 vector (a kind gift from Dr. Andrew Fire, Stanford University School of Medicine) at the PstI and SmaI sites.

**pKE2.** The two fragments containing a point mutation to change His 350 to Ala were amplified by PCR from the plasmid pKE1 with primers *mboa-7<sup>H350A</sup>*-F1, 5'-TAA AAT CAT GGG ATC CCA CAC TGG AAG TGA AGT CT-3' and *mboa-7<sup>H350A</sup>*-R1, 5'-CAT ATG TTC CCG CCC AGA CAG CA-3', and *mboa-7<sup>H350A</sup>*-F2, 5'-TGC TGT CTG GGC CGG AAC ATA TG-3' and *mboa-7<sup>H350A</sup>*-R2, 5'-ATC TGG GTA TCT CGA GAA GCA TTG AAC ACC ATA AC-3' (underlined nucleotides represent the His-to-Ala mutation). The two overlapping fragments were fused by PCR using primers *mboa-7<sup>H350A</sup>*-F1 and *mboa-7<sup>H350A</sup>*-R2 and integrated into the BamHI and XhoI sites of the pKE1 vector using an In-Fusion Dry-Down PCR cloning kit (Clontech, Mountain View, CA).

**pKE3.** Full-length *h-mboa-7* cDNA was amplified by PCR from *h-mboa-7* pCAGGS-MCS vector (see above) with primers *h-mboa-7*-F1, 5'-CCT GGT CTA GAT GTC GCC AGA AGA ATG GAC CTA CCT CG-3' and *h-mboa-7*-R1, 5'-GCT CGG GAT CAT CCT CCC GGA GCT TCT CCG GGG C-3'. The fragment was cloned into the pPD95.67 vector at the XbaI and BamHI sites, yielding a vector pKE4. Next, 4000 base pairs upstream of the initiation codon of *mboa-7* was amplified from the pKE1 vector with primers *Pmboa-7*-F, 5'-ACT GAC TGC AGT AAA ACC TTC TTA AAC C-3' and *Pmboa-7*-R, 5'-TTC CAT CTA GAT ACC TGA AAC AAA AAA CGA ATA C-3'. The fragment was cloned into the pKE4 vector at the PstI and XbaI sites.

### Phenotypic Analysis of *mboa-7* Mutants

Adult wild-type and *mboa-7(gk399)* worms were allowed to lay eggs for 2–3 h at 20°C, and the progeny were scored for embryonic lethality and larval arrest. Unhatched eggs were examined 24 h after being laid, and hatched but arrested larvae were examined 48 h after being laid. For growth rate analysis, first larval stage worms were synchronized after bleach treatment of gravid hermaphrodites, and they were incubated on AA- or EPA-supplemented NGM plates at 15°C. Growth rate was scored by counting adult worms under a stereomicroscope. The average number of unladen fertilized eggs that accumulated inside of adult animals was quantified as described previously (Koelle and Horvitz, 1996).

### Lipid Analysis

Lipids of synchronized young adult worms were extracted by the method of Bligh and Dyer (1959). For fatty acid analysis, phospholipids were separated by one-dimensional thin-layer chromatography (TLC) on silica gel 60 plates (Merck Biosciences, Darmstadt, Germany) in chloroform:ethanol:water:triethylamine (30:35:7:35, vol/vol). Phospholipids were identified by comigration with known standards. The area of silica gel corresponding to each phospholipid was scraped off the plates and treated with dehydrated methanol:acetyl chloride (10:1) to extract the fatty acid methyl esters. The fatty acid methyl ester derivatives were analyzed with a GC 353B gas chromatograph equipped with a flame ionization detector (GL Sciences, Tokyo, Japan) and a TC-FFAP capillary column (60 m × 0.25 mm internal diameter, 0.25 μm; GL Sciences). The oven temperature was programmed to increase from 170°C to 230°C at 5°C/min followed by a hold of 15 min. The injector and detector temperatures were both set at 250°C. Helium was used as carrier gas at 1 ml/min. Individual fatty acids were identified by comparing the retention times with those of known fatty acid standards. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis was performed on a Quatro microtandem quadrupole mass spectrometer (Waters-Micromass, Manchester, United Kingdom) equipped with an ESI interface. PI species were detected by negative ionization, and the mobile phase consisted of acetonitrile:methanol:water (6:7:2, vol/vol/vol) containing 0.1% ammonium formate, pH 6.4. Dry argon was used as the collision gas ( $2.4 \times 10^{-3}$  mbar; 1 bar ≈  $10^5$  Pa). Collision gas-induced fragmentation of PI species generated a common dehydrated inositol phosphate fragment, with  $m/z = 241$ , and parent ion scans of this  $m/z = 241$  fragment provided diagnostic determination of PI. The data were acquired and processed with MassLynx NT 4.0 software (Waters-Micromass). PI molecular species were determined by analysis of fatty acyl fragments generated by collision gas-induced fragmentation under negative ionization. Quantitation of individual PI species was obtained based on the ion current response of the PI species relative to that of the internal standard 1,2-dipalmitoyl PI (16:0/16:0 PI). It is noted that due to different ionization efficiency, ion counts obtained for different PI species do not allow us to estimate the relative abundance of the different PI species with regard to each other.

### Preparation of *sn-2-acyl LysoPI*

*sn-2-acyl lysoPI* was prepared as follows. PI (3–5 μmol) from porcine liver dissolved in 1 ml of diethyl ether was incubated with 100 μl of 100 mM CaCl<sub>2</sub>,

600 μl of 50 mM Tris-malate, pH 5.7, and 400 μl of enzyme solution containing 16 mg of *Rhizopus delemere* lipase for 1 h at 40°C while stirring vigorously. After the incubation, the reaction was terminated by adding 1 ml of methanol. Remaining PI and fatty acid were removed by three extractions with 4 ml of diethyl ether:petroleum ether (1:1, vol/vol). 2-Acyl lysoPI remaining in the lower layer was extracted by the method of Bligh and Dyer (1959).

### Acyltransferase Assay

Synchronized young adult worms (1 g, wet weight) were suspended in 4 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 0.15 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.25 M sucrose (homogenizing buffer), and they were sonicated three times on ice for 30 s. The *C. elegans* homogenate was centrifuged at  $11,000 \times g$  for 30 min at 4°C. The resulting supernatant was further centrifuged at  $105,000 \times g$  for 60 min. The pellet was suspended in homogenizing buffer (without EDTA, dithiothreitol, and PMSF) and immediately used for the enzyme assay described below. HeLa cells were harvested, washed with ice-cold phosphate-buffered saline, and sonicated three times on ice for 3 s in homogenizing buffer. The membrane fraction of HeLa cells was prepared as described above.

In vitro fatty acid incorporation assay was performed as described previously (Tanaka *et al.*, 1999), with slight modifications. Each assay contained 32 nmol of lysophospholipid, 320 μg of protein of the membrane fraction of *C. elegans*, and the desired 20 nmol of fatty acid in a total volume of 0.8 ml of assay buffer (0.15 M KCl, 5 mM MgCl<sub>2</sub>, 0.25 M sucrose, 7.5 mM ATP, 0.4 mM CoA, and 50 mM potassium phosphate buffer, pH 6.8). Fatty acids were added as a fatty acid–albumin complex (Chen and Nilsson, 1993), and the specific activity was 10 mCi/mmol. The reaction was incubated at 20°C for 5 min, and then it was stopped by mixing with 2 ml of methanol. The lipids were extracted and separated by TLC as described above. The area of silica gel for each phospholipid was scraped off the plate, and radioactivity was measured.

[<sup>14</sup>C]Arachidonoyl-CoA:lysophospholipid acyltransferase activity was measured in a similar manner except that 10 nmol of [<sup>14</sup>C]arachidonoyl-CoA instead of <sup>14</sup>C-free fatty acid and increasing concentrations of various lysophospholipids were used. In this assay, MgCl<sub>2</sub>, ATP, and CoA were omitted from the assay buffer. Acyltransferase activity of the HeLa cell microsomes was measured similarly, except that the incubation temperature was 37°C, and 80 μg of microsomal protein was used.

### Cell Culture and Transfection

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. The small interfering RNAs (siRNAs) for human *mboa-7* were transfected into HeLa cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The *h-mboa-7*-specific siRNA sequence spans nucleotide positions 1082–1106 (5'-AUG GUC AGG AAG CUC AGG UAG UAG C-3').

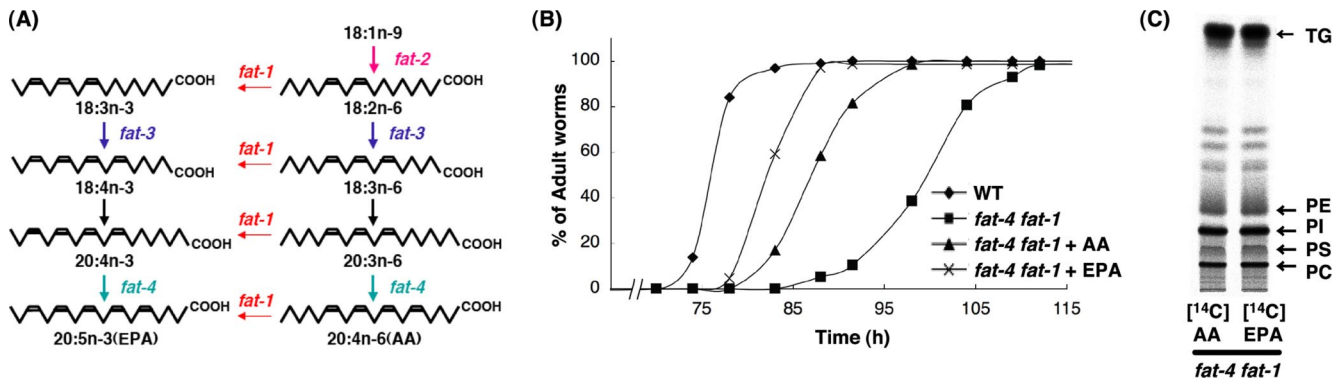
### Quantitative Real-Time Reverse Transcription (RT)-PCR

Total RNA from HeLa cells was extracted with ISOGEN (Nippongene, Toyama, Japan) and reverse transcribed with the SuperScript First-strand Synthesis System for RT-PCR (Invitrogen). Oligonucleotide primers for PCR were designed with Primer Express software (Applied Biosystems, Foster City, CA). The sequences of the oligonucleotides used in PCR reaction were *h-mboa-7*-F2, 5'-GCC CTC CCT GAT GGA GAC A-3' and *h-mboa-7*-R2, 5'-GTA GGT GCG GTA GCG GAA GA; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-F-3' and 5'-GCC AAG GTC ATC CAT GAC AAC T; GAPDH-R-3' and 5'-GAG GGG CCA TCC ACA GTC TT-3'. GAPDH was amplified as an internal control, and expression of experimental transcript each sample was normalized to that of GAPDH.

### In Vivo Incorporation of Exogenous Fatty Acids into *C. elegans* and HeLa Cells

For incorporation of exogenous fatty acids into *C. elegans*, synchronized first-stage larvae (800–1200 animals) were cultured with 1 μCi of each <sup>14</sup>C-fatty acids for 54 h on NGM plates at 20°C until they reached the young adult stage. Lipids of young adult worms were extracted by the method of Bligh and Dyer (1959), and they were separated by one-dimensional TLC on silica gel 60 plates (Merck Biosciences) in chloroform:ethanol:water:triethylamine (30:35:7:35, vol/vol). Incorporation of <sup>14</sup>C-fatty acid into individual phospholipids was expressed as the percentage of radioactivity incorporated into total lipids.

HeLa cells ( $1.5 \times 10^5$ ) were plated in six-well dishes and incubated for 24 h. siRNAs for *h-mboa-7* were then transfected into HeLa cells with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. The final concentration of siRNA was 40 nM. After incubation for 48 h, the cells were washed and incubated in DMEM containing 0.1% bovine serum albumin for 24 h. Then, 100 nmol per dish of [<sup>14</sup>C]AA (0.1 μCi) or [<sup>14</sup>C]EPA (0.1 μCi) was added to the medium as an albumin complex (Chen and Nilsson, 1993). After 1-h incubation, the cell lipids were extracted and separated by TLC as de-



**Figure 1.** (A) *C. elegans* PUFA synthetic pathway from bacterial fatty acids. Lipid structures are abbreviated as in 20:4n-6, which has 20 carbons and four double bonds, the first occurring at the n-6 position. *fat-1*, n-3 desaturase; *fat-2*,  $\Delta 12$  desaturase; *fat-3*,  $\Delta 6$  desaturase; *fat-4*,  $\Delta 5$  desaturase. (B) Rescue of the growth defects of *fat-4 fat-1* mutants by dietary supplementation with AA or EPA. Synchronized populations of first-stage larvae were propagated on *E. coli* OP50 supplemented with AA or EPA, and they were allowed to grow at 15°C. At least 150 worms were scored for each assay and scored as adult if they form vulva. Representative plots of multiple experiments are shown. WT, wild-type. (C) One-dimensional thin-layer chromatographic separation of total lipids from *fat-4 fat-1* mutants radiolabeled with  $[^{14}\text{C}]$ AA (left) and  $[^{14}\text{C}]$ EPA (right).

scribed above. Incorporation of  $^{14}\text{C}$ -fatty acid into individual phospholipids was expressed as the percentage of radioactivity incorporated into total lipids.

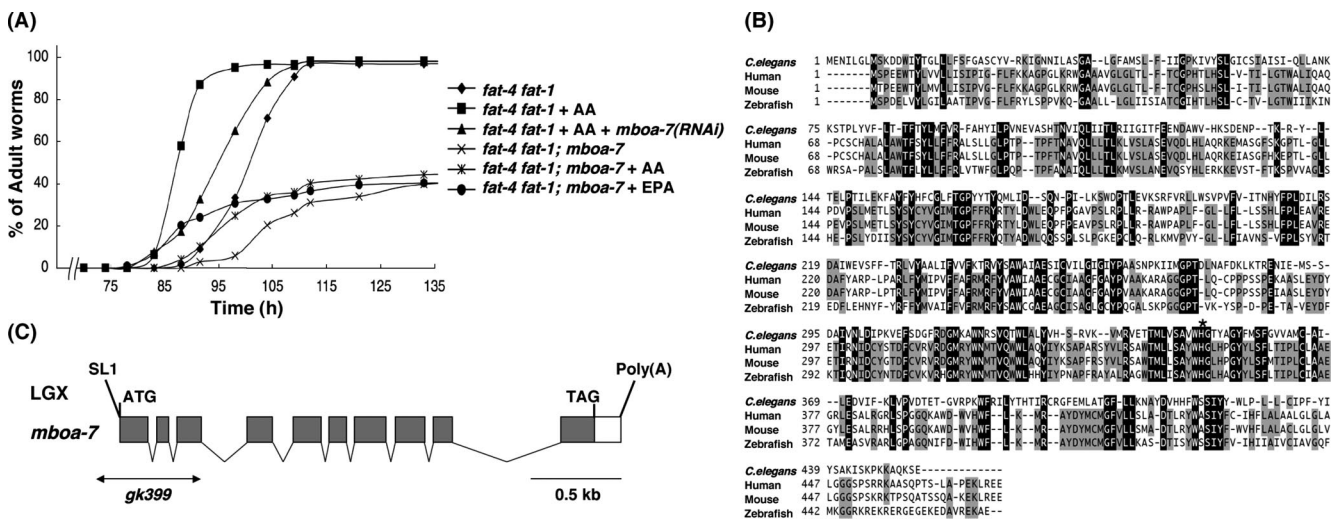
## RESULTS

### Identification of Genes Involved in the Incorporation of Exogenous AA into PI

*fat-4 fat-1* mutants, which do not produce AA(20:4n-6) and n-3 PUFAs such as EPA(20:5n-3), were viable and fertile (Figure 1A; Watts and Browne, 2002; Kahn-Kirby *et al.*, 2004), but they exhibited slow growth at 15°C (Figure 1B). Dietary supplementation with 25  $\mu\text{M}$  AA or EPA led to incorporation of these PUFAs into the phospholipid fractions of *fat-4*

*fat-1* mutants (Figure 1C), and it rescued the growth defects of this strain (Figure 1B).

To identify genes that are involved in the incorporation of exogenous PUFAs into phospholipids in *C. elegans*, we performed a large-scale RNAi screen by using *fat-4 fat-1* mutants under AA-supplemented conditions (see *Materials and Methods*). In this screen, we found that knockdown of a previously uncharacterized gene, F14F3.3, caused weak growth defects despite supplementation with AA (Figure 2A). F14F3.3 encodes a protein with structural similarity to those of MBOATs, which transfer acyl groups to proteins, sterols, or diacylglycerol (Figure 2B; Hofmann, 2000). Our



**Figure 2.** (A) Knockdown and knockout of *mboa-7* inhibit growth rescue by dietary supplementation with AA or EPA. Growth of each strain was scored as described in Figure 1B, except that *E. coli* HT115 was used as a food source. All experiments were performed at 15°C. (B) Multiple sequence alignment of *C. elegans* *mboa-7* and homologous sequences in human (BB1/LENG4; Fukunaga-Johnson *et al.*, 1996; Wende *et al.*, 2000), mouse, and zebrafish. Residues identical in all four sequences are shaded in black, and residues identical in three proteins are shaded in gray. The numbers on the left indicate amino acid positions. The histidine residue indicated by asterisk is the predicted active site of the MBOAT motif in *mboa-7* (Hofmann, 2000; Bosson *et al.*, 2006). Accession numbers for the sequences used were as follows: *C. elegans*, EU016382; human, EU016381; mouse, EU016380; and zebrafish, NP\_609029. (C) Genomic structure of *mboa-7* (F14F3.3). The *mboa-7* gene is located on chromosome X. Gray boxes indicate coding exons, and white boxes indicate 5' and 3' untranslated sequences. The positions of the ATG initiation codon, stop codon (TAG), the trans-spliced leader SL1, and the poly(A) tail are shown. The extent of the deletion in *mboa-7(gk399)* is indicated by a horizontal line.

**Table 1.** *C. elegans* MBOAT family<sup>a</sup>

| Gene          | Sequence name | Human homologue |
|---------------|---------------|-----------------|
| <i>mboa-1</i> | B0395.2       | ACAT-1, ACAT-2  |
| <i>mboa-2</i> | H19N07.4      | DGAT1           |
| <i>mboa-3</i> | C54G7.2       | MBOAT1, MBOAT2  |
| <i>mboa-4</i> | C08F8.4       | MBOAT1, MBOAT2  |
| <i>mboa-5</i> | ZK550.1       | MBOAT1, MBOAT2  |
| <i>mboa-6</i> | R155.1        | MBOAT5          |
| <i>mboa-7</i> | F14F3.3       | BB1/LENG4       |
| <i>mom-1</i>  | T07H6.2       | Porcupine       |
| <i>hhat-1</i> | ZC101.3       | HHAT            |
| <i>hhat-2</i> | Y57G11C.17    | HHAT            |

<sup>a</sup> BB1, genes overexpressed by human bladder and breast cancer cell lines (Fukunaga-Johnson *et al.*, 1996); LENG4, leukocyte receptor cluster member 4 (Wende *et al.*, 2000); and HHAT, Hedgehog acyltransferase.

genome database search revealed that *C. elegans* genome possesses 10 MBOAT family members (Table 1; WormBase [http://www.wormbase.org/]), three of which are homologous to the enzymes transferring acyl groups to specific proteins such as Wnt and Hedgehog (Hofmann, 2000). We named the other seven members membrane-bound *O*-acyltransferase-1-7 (*mboa-1-7*); *mboa-1* and *mboa-2* are homologous to acyl-CoA:cholesterol acyltransferase and diacylglycerol *O*-acyltransferase 1, respectively; and *mboa-3*, *mboa-4*, and *mboa-5* show significant homology to human MBOAT1, MBOAT2. *mboa-6* corresponds to human MBOAT5. *C. elegans* F14F3.3 identified in this study encodes homologous sequence to human BB1 (Fukunaga-Johnson *et al.*, 1996), which also belongs to MBOAT family, and it was named *mboa-7*. We considered *mboa-7* a candidate gene for phospholipid fatty acid remodeling, and we examined the growth rate of *mboa-7* deletion mutants [*mboa-7(gk399)*; Figure 2C]. In the *fat-4 fat-1* mutant background, *mboa-7* mutants exhibited severe growth defects even under conditions of AA or EPA supplementation (Figure 2A), suggesting that *mboa-7* is required for use of exogenous PUFAs in *C. elegans*.

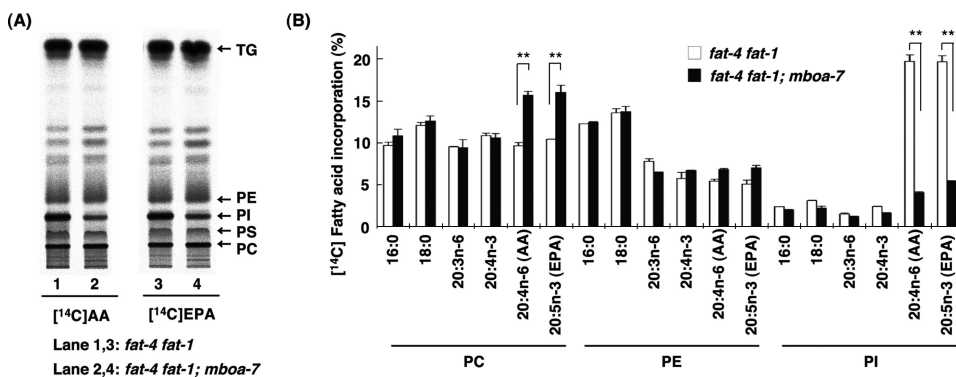
Next, we examined the incorporation of exogenous [<sup>14</sup>C]AA and [<sup>14</sup>C]EPA into the lipid fraction of *mboa-7* mutants in the *fat-4 fat-1* mutant background in which endogenous AA and EPA are absent. In *mboa-7(+/+)* animals, both [<sup>14</sup>C]AA and [<sup>14</sup>C]EPA were incorporated into all the major membrane phospholipids, especially PI and phos-

phatidylcholine (PC) (Figure 3, A and B). In contrast, in *mboa-7* mutants, incorporation of [<sup>14</sup>C]AA and [<sup>14</sup>C]EPA into PI was reduced, and uptake of radioactive AA and EPA into the PC fraction was increased. Incorporation of other fatty acids into PI was not affected significantly (Figure 3B, 16:0, 18:0, 20:3n-6, and 20:4n-3). These data indicated that *mboa-7* is involved in the selective incorporation of AA and EPA into PI.

### *mboa-7* Is Required in LPIAT Activity

It is generally thought that incorporation of PUFAs into cellular phospholipids requires acyl-CoA:lysophospholipid acyltransferase activity (MacDonald and Sprecher, 1991). To examine the possibility that the *mboa-7* gene product is associated with lysophospholipid acyltransferase activity, we measured lysophospholipid acyltransferase activity by using membrane fractions of wild-type and *mboa-7* mutants. First, we used free <sup>14</sup>C-fatty acids as acyl donors (see *Materials and Methods*). In this system, free <sup>14</sup>C-fatty acids are converted to [<sup>14</sup>C]acyl-CoAs by the endogenous acyl-CoA synthase, and then they are incorporated into lysophospholipids by acyltransferases during the incubation. As shown in Figure 4, A and B, significant incorporation of [<sup>14</sup>C]EPA into lysoPC, lysoPI, lysoPS, and lysoPE were detected in the membrane fraction of wild-type worms, whereas incorporation of [<sup>14</sup>C]EPA into lysoPI was greatly reduced in the membrane fraction of *mboa-7* mutants. We then analyzed the acyl donor selectivity using lysoPI as an acyl acceptor. The wild-type membrane fraction showed a preference for [<sup>14</sup>C]AA and [<sup>14</sup>C]EPA as acyl donors for lysoPI similar to the situation in mammalian cells (Figure 4C; Baker and Thompson, 1973; Holub, 1976). However, incorporation of [<sup>14</sup>C]AA and [<sup>14</sup>C]EPA into lysoPI was significantly reduced in the membrane fraction of *mboa-7* mutants. Incorporation of [<sup>14</sup>C]oleic acid (18:1n-9) into lysoPI was also reduced, but the extent was not so significant compared with [<sup>14</sup>C]AA and [<sup>14</sup>C]EPA. Incorporation of [<sup>14</sup>C]EPA into lysoPC, lysoPE, and lysoPS (Figure 4, A and B), and incorporation of other <sup>14</sup>C-free fatty acids such as 18:2n-6, 18:3n-3, and 20:4n-3 into lysoPI (Figure 4C) were not affected in the membrane fraction of *mboa-7* mutants, suggesting that acyl-CoA synthesis from free fatty acid and CoA seemed to be normal in the membranes of *mboa-7* mutants.

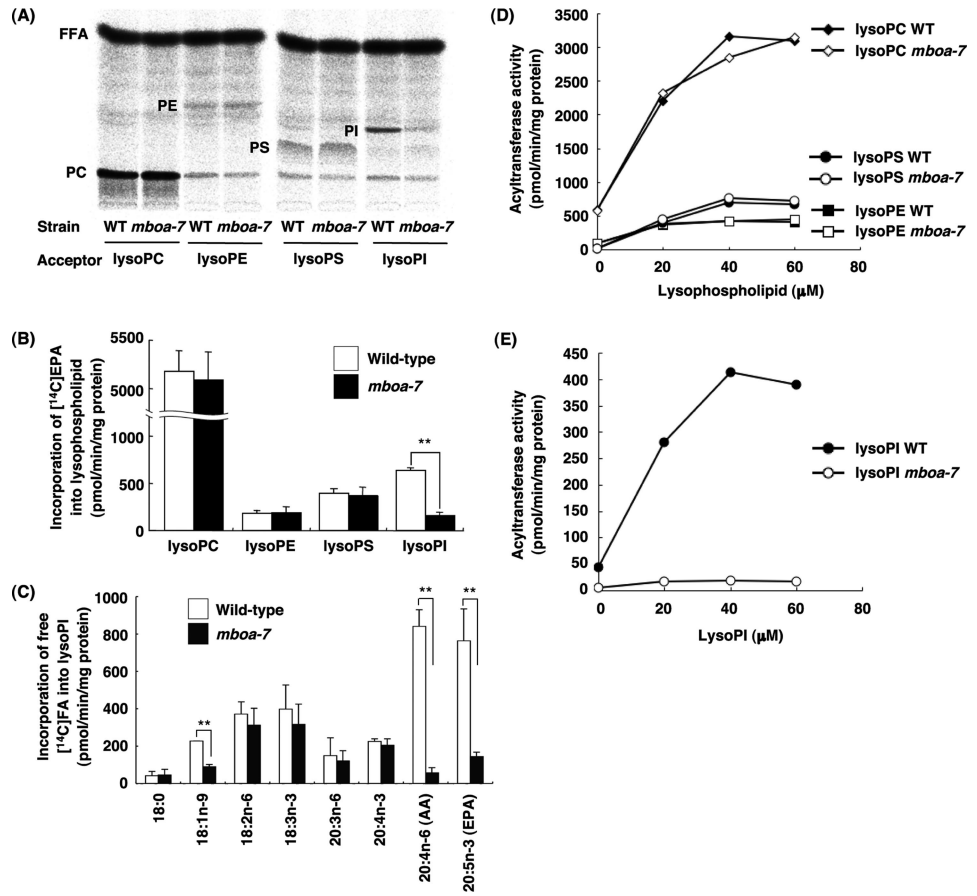
Then, we examined incorporation of [<sup>14</sup>C]arachidonoyl-CoA into various lysophospholipids (Figure 4, D and E). Incorporation of [<sup>14</sup>C]arachidonoyl-CoA was elevated with increasing concentrations of lysophospholipids, and it reached a plateau in the membrane of wild-type worms. Incorporations of [<sup>14</sup>C]arachidonoyl-CoA into lysoPC, ly-



**Figure 3.** Incorporation of exogenous [<sup>14</sup>C]AA and [<sup>14</sup>C]EPA into phospholipids of *mboa-7* mutants. (A) One-dimensional thin layer chromatographic separation of total lipids from *fat-4 fat-1* and *fat-4 fat-1; mboa-7* mutants radiolabeled with exogenous [<sup>14</sup>C]AA (left) and [<sup>14</sup>C]EPA (right). (B) Incorporation of various radiolabeled fatty acids into phospholipids of *fat-4 fat-1* and *fat-4 fat-1; mboa-7* mutants. The amount of incorporation was expressed as the percentage of radioactivity incorporated into total lipids. All experiments were carried out at 20°C.

Note that the radiolabeled fatty acids are not metabolized to AA or EPA *in vivo* due to the lack of  $\Delta 5$  and *n-3* fatty acid desaturation activities. Each bar represents the mean  $\pm$  SEM of at least three independent experiments. \*\**p* < 0.01.

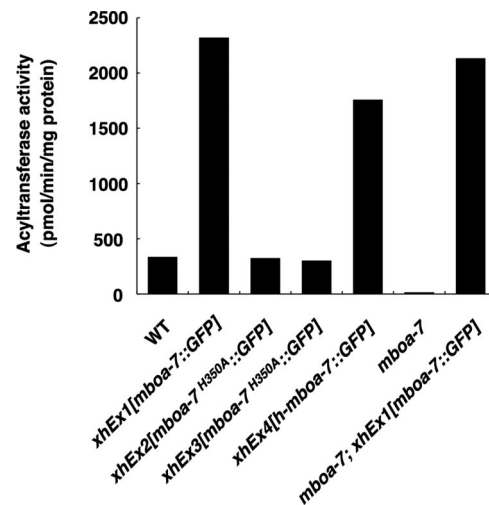
**Figure 4.** The membrane fraction of *mboa-7* mutants lacks LPIAT activity. (A and B) In vitro fatty acid incorporation assay using the membrane fractions of wild-type and *mboa-7* mutants and different lysophospholipids as acyl acceptors. [ $^{14}$ C] EPA was used as an acyl donor. (A) One-dimensional thin layer chromatographic separation of total lipids of the reaction mixture after incubation. FFA, free fatty acid. (B) Each bar represents the mean  $\pm$  SEM of at least three independent experiments. \*\* $p < 0.01$ . (C) In vitro fatty acid incorporation into lysoPI with different radiolabeled fatty acids as acyl donors. Each bar represents the mean  $\pm$  SEM of at least three independent experiments. \*\* $p < 0.01$ . (D) Acyl-CoA:lysophospholipid acyltransferase activity in the membrane fractions of wild-type and *mboa-7* mutants. [ $^{14}$ C]Arachidonoyl-CoA (12.5  $\mu$ M) and the indicated concentration of lysoPC, lysoPE, or lysoPS were used. (E) [ $^{14}$ C]Arachidonoyl-CoA:lysoPI acyltransferase activity in the membrane fractions of wild-type and *mboa-7* mutants. [ $^{14}$ C]Arachidonoyl-CoA (12.5  $\mu$ M) and the indicated concentration of lysoPI were used.



soPE, and lysoPS were not affected in the membranes of *mboa-7* mutants; however, incorporation of [ $^{14}$ C]arachidonoyl-CoA into lysoPI was specifically reduced at any lysoPI concentration. These results indicate that the *mboa-7* is required for LPIAT activity with a preference for AA and EPA as the acyl donor.

To reveal that the *mboa-7* gene product (MBOA-7) shows LPIAT activity, we generated transgenic worms expressing GFP-tagged MBOA-7 (*xhEx1[mboa-7::GFP]*; see *Materials and Methods*), and we examined the enzyme activity of MBOA-7::GFP. Expression of MBOA-7::GFP fully rescued the phenotypes of *mboa-7(gk399)* mutants in the *mboa-7* mutant background (see below), indicating that MBOA-7::GFP is functional in vivo. Consistent with the results described above, the membrane fraction of MBOA-7::GFP-expressing worms showed remarkably increased LPIAT activity with [ $^{14}$ C]arachidonoyl-CoA as an acyl donor (Figure 5, *xhEx1[mboa-7::GFP]*). Next, we mutated histidine 350 of MBOA-7 (histidine to alanine; H350A), the predicted active residue of the membrane-bound O-acyltransferase family (Figure 2B; Hofmann, 2000; Bosson *et al.*, 2006), and generated the transgenic worms expressing the MBOA-7<sup>H350A</sup>::GFP (*xhEx2[mboa-7<sup>H350A</sup>::GFP]*, and *xhEx3[mboa-7<sup>H350A</sup>::GFP]*). As shown in Figure 5, the membrane fractions of these two MBOA-7<sup>H350A</sup>::GFP transgenic lines did not show increased LPIAT activity compared with those of wild-type worms. These data strongly suggest that *mboa-7* gene encodes LPIAT.

All the acyltransferase assays described above were performed using *sn*-1-acyl-2-lysoPI (*sn*-2-lysoPI) as an acyl acceptor. Next, we examined whether MBOA-7 incorporates



**Figure 5.** Recombinant MBOA-7 and h-MBOA-7 (a human homologue of MBOA-7) expressed in *C. elegans* show remarkably increased LPIAT activity. [ $^{14}$ C]Arachidonoyl-CoA:lysoPI acyltransferase activity in the membrane fractions of the indicated strains were measured. 40  $\mu$ M lysoPI and 12.5  $\mu$ M [ $^{14}$ C]arachidonoyl-CoA were used as an acyl donor and an acyl acceptor, respectively. The membrane fraction of MBOA-7::GFP-expressing worms showed remarkably increased LPIAT activity both in wild-type and *mboa-7* mutants (*xhEx1[mboa-7::GFP]* and *mboa-7; xhEx1[mboa-7::GFP]*, respectively). Human MBOA-7::GFP also catalyzed LPIAT activity (*xhEx4[h-mboa-7::GFP]*). MBOA-7<sup>H350A</sup>::GFP, which is mutated at the predicted active site of the membrane-bound O-acyltransferase motif of MBOA-7, had no LPIAT activity (*xhEx2[mboa-7<sup>H350A</sup>::GFP]* and *xhEx3[mboa-7<sup>H350A</sup>::GFP]*).

PUFA into the *sn-1* position of lysoPI. For this study, the substrate *sn-2*-acyl-1-lysoPI (*sn-1*-lysoPI) was prepared by phospholipase A<sub>1</sub> digestion of PI (see *Materials and Methods*), and it was immediately used for acyltransferase assay. The wild-type membrane fraction showed significant LPIAT activity by using *sn-1*-lysoPI as a substrate (black plus gray bars in the Supplemental Figure S1A), and the enzyme activity was reduced to ~65% in the membranes of *mboa-7* mutants. Because *sn-1*-lysoPI is known to easily isomerize to form *sn-2*-lysoPI (see Supplemental Figure S1B; Rouser *et al.*, 1966), it was possible that <sup>14</sup>C-acyl donor was incorporated into *sn-2*-lysoPI and *sn-1*-lysoPI. To check the positional specificity, [<sup>14</sup>C]PI produced after the incubation was treated again with bee venom phospholipase A<sub>2</sub> (for details, see the legend to Supplemental Figure S1). By measuring the radioactivity of <sup>14</sup>C-free fatty acid and [<sup>14</sup>C]lysoPI produced after the phospholipase A<sub>2</sub> treatment, we can estimate the incorporation of <sup>14</sup>C-acyl donor into *sn-1*-lysoPI (*sn-1*-LPIAT activity, black bar) and *sn-2*-lysoPI (*sn-2*-LPIAT activity, gray bar) (Supplemental Figure S1B). The *sn-2*-LPIAT activity was observed in the microsomes of wild-type worms, whereas the activity was almost undetectable in those of *mboa-7* mutants, indicating that *sn-2*-LPIAT activity in wild-type worms was catalyzed by MBOA-7 and that significant isomerization of *sn-1*-lysoPI occurred probably during the incubation. In contrast to *sn-2* LPIAT activity, similar levels of *sn-1* LPIAT activity (black bar) were detected in wild-type and *mboa-7* mutants. These results indicate that MBOA-7 prefers *sn-2*-lysoPI as an acyl acceptor rather than *sn-1*-lysoPI and that *sn-1*-LPIAT other than MBOA-7 exists in *C. elegans*.

#### PI Molecular Species in *mboa-7* Mutants

To address possible involvement of MBOA-7 in regulating the distribution of PI molecular species, we next analyzed phospholipid fatty acid composition of wild-type and *mboa-7* mutants by gas chromatography (GC) (Table 2). First, we measured PC, PE, and PI content of wild-type and *mboa-7* mutants, and we found that phospholipid contents were not affected in the *mboa-7* mutants (PC, 33.0% in wild-type vs. 34.6% in *mboa-7* mutants; PE, 39.3% in wild-type vs. 43.7% in *mboa-7* mutants; and PI, 1.9% in wild-type vs. 2.0% in *mboa-7* mutants). GC analysis of PC, PE, and PI revealed

that the *mboa-7* mutation significantly reduced the EPA content of PI but not PC or PE. In *mboa-7* mutants, the amount of EPA in PI was reduced to 8.9% of total PI fatty acids compared with 26.2% in wild-type animals. Concomitantly, palmitic acid (16:0) and oleic acid (18:1n-9) in PI increased in *mboa-7* mutants (17.5% in wild-type vs. 26.4% in *mboa-7* mutants and 7.5% in wild-type vs. 15.2% in *mboa-7* mutants, respectively). Reduction of EPA-containing PI molecular species was also confirmed by ESI-MS/MS analysis (Supplemental Figure S2, A and B). Consistent with the GC analysis, the amounts of EPA-containing PI molecular species such as 18:0/20:5, 18:1/20:5, 18:0(alkyl)/20:5, and 16:0/20:5 were reduced in *mboa-7* mutants. Instead, other PI molecular species such as 18:1/18:1, 18:0(alkyl)/18:1, 16:0/18:1, and 16:1/18:1 increased in *mboa-7* mutants. Collectively, these results indicate that *mboa-7* is a major determinant of PI molecular species in *C. elegans*.

#### Human Homologue of *C. elegans mboa-7*

*C. elegans mboa-7* is homologous to human BB1, which also belongs to the MBOAT family (Figure 2B; Fukunaga-Johnson *et al.*, 1996; Hofmann, 2000). BB1 was reported to be up-regulated in metastatic breast and bladder carcinomas (Fukunaga-Johnson *et al.*, 1996), but the function of this gene has not yet been elucidated. To confirm that human BB1/*h-mboa-7* is involved in incorporation of AA into PI, we performed knockdown experiments by using gene-specific siRNAs in HeLa cells. In cells transfected with *h-mboa-7* siRNA duplex, *h-mboa-7* mRNA levels were reduced to ~30% of those in control siRNA-transfected cells at 72 h after the transfection (Supplemental Figure S3A). [<sup>14</sup>C]AA added to the culture was incorporated into PC, PE, and PI of control HeLa cells time-dependently for at least 3 h (data not shown). Incorporation of [<sup>14</sup>C]AA into cellular PI was specifically reduced from 13.1% of the total radioactivity in control cells to 6.7% in the siRNA-transfected cells (Supplemental Figure S3B). In contrast, incorporation of [<sup>14</sup>C]AA into PC was increased in siRNA-transfected cells. Incorporation of [<sup>14</sup>C]EPA into PI was also reduced similar to that of [<sup>14</sup>C]AA in siRNA-transfected cells, although uptake of [<sup>14</sup>C]EPA into PI was substantially lower than that of [<sup>14</sup>C]AA in control cells (4.1 vs. 13.1%) (data not shown).

**Table 2.** Fatty acid composition of wild-type and *mboa-7* mutants

|         | Wild-type  |            |            | <i>mboa-7</i> |            |            |
|---------|------------|------------|------------|---------------|------------|------------|
|         | PC         | PE         | PI         | PC            | PE         | PI         |
| 16:0    | 1.3 ± 0.1  | 3.4 ± 0.3  | 17.5 ± 0.3 | 1.3 ± 0.1     | 3.4 ± 0.1  | 26.4 ± 2.6 |
| 18:0    | 2.5 ± 0.2  | 8.9 ± 0.2  | 21.1 ± 3.0 | 2.6 ± 0.2     | 7.0 ± 0.2  | 18.7 ± 3.2 |
| 18:1n-9 | 1.9 ± 0.1  | 1.6 ± 0.1  | 7.5 ± 1.7  | 1.3 ± 0.0     | 1.4 ± 0.0  | 15.2 ± 2.0 |
| 18:1n-7 | 26.9 ± 1.7 | 31.8 ± 2.1 | 12.0 ± 3.5 | 32.6 ± 0.8    | 37.7 ± 0.2 | 12.7 ± 2.6 |
| 18:2n-6 | 7.7 ± 0.2  | 7.4 ± 0.0  | 2.8 ± 0.9  | 4.2 ± 0.1     | 5.6 ± 0.0  | 5.1 ± 0.5  |
| 18:3n-6 | 2.5 ± 0.2  | 1.5 ± 0.2  | N.D.       | 2.1 ± 0.0     | 1.6 ± 0.1  | N.D.       |
| 18:3n-3 | 0.8 ± 0.1  | 0.3 ± 0.0  | N.D.       | 0.3 ± 0.0     | 0.2 ± 0.0  | N.D.       |
| 20:3n-6 | 4.4 ± 0.1  | 2.5 ± 0.0  | N.D.       | 4.6 ± 0.1     | 3.1 ± 0.1  | N.D.       |
| 20:4n-6 | 3.2 ± 0.1  | 1.5 ± 0.1  | N.D.       | 3.9 ± 0.2     | 1.8 ± 0.1  | N.D.       |
| 20:4n-3 | 6.4 ± 0.0  | 2.7 ± 0.0  | N.D.       | 5.7 ± 0.0     | 2.8 ± 0.0  | N.D.       |
| 20:5n-3 | 32.9 ± 0.5 | 15.3 ± 1.3 | 26.2 ± 2.3 | 33.0 ± 0.6    | 13.0 ± 0.7 | 8.9 ± 0.6  |
| Others  | 9.5 ± 1.1  | 23.0 ± 0.9 | 12.9 ± 1.1 | 8.4 ± 0.3     | 22.4 ± 0.5 | 12.9 ± 7.5 |

Data are mol% and represent the average ± SEM of three independent experiments. Others are the total mol% of 16:1n-7, 17:0, 17:iso, 17:Δ, 18:0DMA, and 19:Δ.

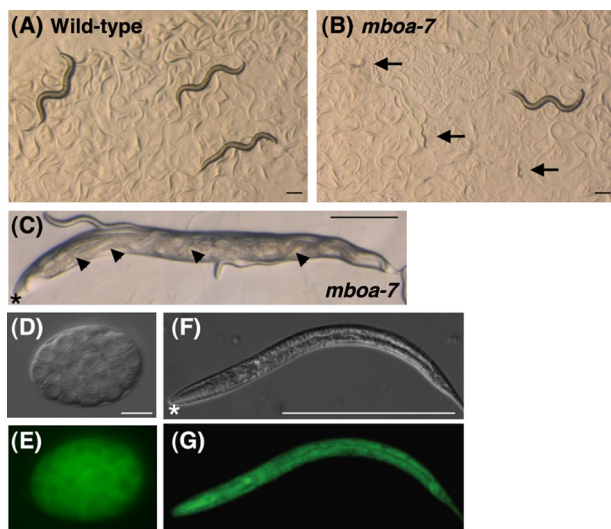
<sup>a</sup> N.D., not detectable.

Acyltransferase activity was also measured in microsomal fractions from siRNA-transfected HeLa cells by using [<sup>14</sup>C]arachidonoyl-CoA. As shown in Supplemental Figure S3C, incorporation of [<sup>14</sup>C]arachidonoyl moiety into lysoPI was specifically reduced in the membrane fraction of the siRNA-transfected cells, whereas incorporation of [<sup>14</sup>C]arachidonoyl moiety into lysoPC and lysoPS was unaffected. Incorporation of [<sup>14</sup>C]arachidonoyl moiety into lysoPE was very low in the membrane of control HeLa cells under the present assay conditions. These results indicate that human BB1/*h-mboa-7* is also involved in incorporation of AA into PI.

To investigate whether human BB1/*h-mboa-7* possesses LPIAT activity, we measured LPIAT activity by using membrane fractions of transgenic worms expressing *h-mboa-7::GFP* (*xhEx4[h-mboa-7::GFP]*; see *Materials and Methods*). The membrane fractions of the human MBOA-7::GFP-expressing worms showed increased LPIAT activity (Figure 5), suggesting that human BB1, a homologue of *C. elegans mboa-7*, is human LPIAT.

### Phenotypic Analysis of *mboa-7* Mutants

Finally, to elucidate the physiological functions of LPIAT, we analyzed the phenotypes of *mboa-7* mutants and examined the expression pattern of *mboa-7*. Mutation of *mboa-7* caused larval arrest at an early developmental stage with incomplete penetrance (Figure 6B; Table 3). Under normal conditions (20°C), ~22% of *mboa-7* mutants were arrested at an early larval stage (Table 3), and the penetrance of the defects increased under high temperature conditions (48% at 25°C). We also found that 14% of *mboa-7* mutants accumu-



**Figure 6.** Phenotypes of *mboa-7* mutants. (A, B) For comparison of growth, wild-type and *mboa-7* embryos were placed onto culture plates, incubated at 20°C, and photographed after 72 h of growth. Arrows indicate *mboa-7* mutants that have arrested development at an early larval stage. Bar, 200  $\mu$ m. (C) Some of the *mboa-7* mutants show bags of worms phenotype where embryos hatch within the mother, leaving a cuticle sack containing multiple wriggling larvae (arrowheads). Asterisks indicate the anterior of the mother worm. Bar, 200  $\mu$ m. (C–G) Expression of MBOA-7::GFP in an early embryo (D and E) and a second-stage larva (F and G). Nomarski micrographs (D and F) and corresponding MBOA-7::GFP expression (E and G). MBOA-7::GFP was ubiquitously expressed throughout development. Asterisks indicate the anterior of the larva (F and G). Bar, 10  $\mu$ m (D and E) and 200  $\mu$ m (F and G).

**Table 3.** Summary of *mboa-7* mutant defects

| Strain  | Larval arrest (%) | No. of eggs in uterus | % of worms with >20 eggs in uterus |
|---|-------------------|-----------------------|------------------------------------|
| Wild-type                                     | 0.2 $\pm$ 0.2     | 14.0 $\pm$ 0.6        | 2.5 (1/60)                         |
| <i>mboa-7</i>                                 | 21.7 $\pm$ 1.7    | 21.9 $\pm$ 0.7        | 70.0 (21/30)                       |
| <i>mboa-7;xhEx1</i><br>[ <i>mboa-7::GFP</i> ] | 2.1 $\pm$ 1.6     | 10.8 $\pm$ 0.5        | 0 (0/30)                           |

Phenotypes of *mboa-7* were analyzed as described in *Materials and Methods*. Data represents the mean  $\pm$  SEM of at least three independent experiments. Larval arrest refers to the percentage of larvae that have arrested development at an early larval stage ( $n > 500$  for each strain). MBOA-7::GFP is expressed under the control of its own promoter. All strains were raised at 20°C.

lated unlaidd eggs that hatched internally and formed “bags of worms” (Figure 6C), suggesting that adult *mboa-7* mutants display egg-laying defects (Trent *et al.*, 1983). Wild-type young adult animals had an average of 14 fertilized eggs in the uterus. In contrast, in *mboa-7* mutants, the average number of eggs in the uterus was 21.9, and two thirds of *mboa-7* mutants had >20 eggs in the uterus, whereas wild-type animals rarely possessed >20 eggs (Table 3). Vulval structure of *mboa-7* mutants seemed normal as judged by differential interference contrast microscopy. These data suggest that LPIAT activity is required for early larval development and egg-laying behavior in *C. elegans*.

We next examined expression of MBOA-7::GFP driven by the *mboa-7* promoter. MBOA-7::GFP fully rescued the larval arrest and egg-laying defects of *mboa-7* mutants described above (Table 3). The MBOA-7::GFP fusion protein was expressed ubiquitously throughout development from early embryo to larval and adult stages (Figure 6, D–G). Strong expression was observed in pharyngeal muscles, body wall muscles, vulval cells, distal tip cells, intestinal cells, and spermatheca at adult stage (data not shown).

### DISCUSSION

In the present study, we used a comprehensive RNAi screen of *C. elegans* to clone the previously uncharacterized gene named *mboa-7* involved in the PI remodeling pathway originally reported in 1962 (Keenan and Hokin, 1962). Incorporation of exogenous EPA into PI of living worms and LPIAT activity in microsomes were greatly reduced in *mboa-7* mutants. *mboa-7* encodes a member of the MBOAT family, including sterol acyltransferase and the lysophospholipid acyltransferase recently identified from yeast, suggesting that *mboa-7* encodes LPIAT. Moreover, *mboa-7* mutants showed significant reductions in the EPA content of PI but not other phospholipids and they exhibited defects in early larval development and egg-laying behavior. These data show that *mboa-7* is a major determinant of PI molecular species and that specific PI molecular species are critical for certain biological functions.

Previous studies revealed that LPIAT purified from rat liver microsomes (Yamashita *et al.*, 2003) or bovine heart microsomes (Sanjanwala *et al.*, 1989) shows absolute specificity for lysoPI as the acyl acceptor and no activity with lysoPC, lysoPE, and lysoPS. Consistent with these observations, the incorporation of exogenous EPA or AA into PI in *mboa-7* mutants and the *in vitro* incorporation of AA or EPA



into LysoPI in the microsomal fraction of *mboa-7* mutants were specifically reduced compared with those in wild-type worms. Acyl donor specificity for LPIAT with lysoPI as a substrate has been reported in the microsomal fraction of various tissues and cells (Baker and Thompson, 1973; Holub, 1976; Kameyama *et al.*, 1983; Inoue *et al.*, 1984; Sanjanwala *et al.*, 1989), and it differs minimally depending on the source and the state of the enzyme. In general, acyl-CoAs with more than three double bonds are particularly good substrates. EPA (20:5n-3) and AA (20:4n-6) were incorporated into lysoPI in the microsomes of wild-type *C. elegans* and the incorporation of both fatty acids was greatly reduced in the microsomes of *mboa-7* mutant, indicating that the incorporation of these PUFAs into the PI fraction is mediated by *mboa-7*. These biochemical features may explain the enrichment of EPA in PI of wild-type worms and that of AA in PI of *fat-1* mutants in which AA is a dominant PUFA (see *Introduction*).

Furthermore, we found that incorporation of fatty acids such as 18:2n-6, 18:3n-3, and 20:4n-3 into lysoPI was unaffected in the *mboa-7* mutants, suggesting the existence of other lysophospholipid acyltransferases that have different acyl donor specificities. It is not clear at present whether another lysoPI-specific acyltransferase with different acyl donor specificity exists or if lysophospholipid acyltransferase with no head group specificity catalyzes the transfer of these fatty acids into PI exists. Very recently, a novel lysophospholipid acyltransferase was identified from yeast that catalyzes the transfer of unsaturated fatty acids into major lysophospholipids such as lysoPE, lysoPC, lysoPS, lysoPI, and lysoPA (Benghezal *et al.*, 2007; Jain *et al.*, 2007; Riekhof *et al.*, 2007; Tamaki *et al.*, 2007). Because other MBOAT family genes were found in *C. elegans*, one or more of these gene products may express the remaining LPIAT activity in *mboa-7* mutants.

The physiological significance of phospholipid fatty acid remodeling remains to be elucidated because the genes responsible for the reactions have not been identified and appropriate animal models deficient of such genes have not been analyzed. In the present study, we showed that *C. elegans mboa-7* mutants had significantly lower EPA levels in PI, demonstrating that *mboa-7* regulates PI molecular species, supporting the hypothesis proposed by Lands in the 1960s (Lands and Crawford, 1976; Lands, 2000). We also found that *mboa-7* mutants showed defects in early larval development and egg-laying behavior, indicative of the importance of specific PI molecular species in certain physiological functions. It has been assumed that specific PI molecular species are critical for normal phosphoinositide function (Hodgkin *et al.*, 1998; Arnhold *et al.*, 1999; Shirai *et al.*, 1999), which plays important roles in signal transduction, such as the phospholipase C-signaling pathway (Rebecchi and Pentylala, 2000; Rhee, 2001). Interestingly, mutants of *egl-8*, a phospholipase C isoform in *C. elegans*, exhibit egg-laying defects similar to that observed in our *mboa-7* mutants (Trent *et al.*, 1983; Lackner *et al.*, 1999). Both *egl-8* and *mboa-7* mutants have apparently normal vulvae, and they exhibit an induction of egg laying in response to exogenous serotonin (Bastiani *et al.*, 2003; our unpublished data). The *mboa-7* mutants will provide a useful tool for studying the physiological links between PI molecular species and PI signaling.

Although the EPA content of PI is significantly reduced in the *mboa-7* mutants, an appreciable amount of EPA is still present in PI. It is possible that other LPIAT may exist in *C. elegans* or that EPA can be incorporated into PI via different pathways. LPAAT may catalyze the incorporation of PUFA into the *sn-2* position of lysoPA during de novo phospho-

lipid synthesis. Both mammalian LPAAT $\alpha$  and LPAAT $\beta$  show relatively broad substrate specificity for acyl-CoAs and incorporate AA into lysoPA (Stamps *et al.*, 1997; Eberhardt *et al.*, 1997). Thus, it is possible that PI-containing AA can be synthesized once AA is incorporated into PA via the de novo pathway. Alternatively, other lysophospholipid acyltransferases, such as lysoPC acyltransferase (Sanjanwala *et al.*, 1988) and lysoPS acyltransferase (Holub, 1980), are also known to prefer PUFA for acylation at the *sn-2* position. Once phospholipids other than PI acquire PUFA via a remodeling system, these lipids can be converted by the action of phospholipase C or D into diacylglycerol or PA, respectively, both of which serve as a precursor of PI synthesis.

The *mboa-7* gene product belongs to the large and diverse MBOAT family (Hofmann, 2000). All member proteins have several membrane-spanning regions, typically between eight and 10, and they share a region of detectable sequence similarity. Biochemically characterized members of the family encode enzymes that transfer organic acids, typically fatty acids, onto hydroxyl groups of membrane-embedded targets. As mentioned above, several groups very recently cloned a yeast gene named *ALE1* (Riekhof *et al.*, 2007), *SLC4* (Benghezal *et al.*, 2007), or *LPT1* (Jain *et al.*, 2007; Tamaki *et al.*, 2007) that encodes an acyltransferase of the MBOAT family. Deletion of this gene in yeast results in strong reduction of the activities of various lysophospholipid acyltransferases such as LPEAT, LPCAT, LPSAT, LPIAT, and LPAAT, suggesting that this newly identified acyltransferase is involved in the metabolism of a variety of lysophospholipids in the cells. In this context, *mboa-7* seems to be involved only in the remodeling of PI according to the data presented in this work. In contrast, it is well known that in GPI-anchored proteins, fatty acyl chains at the *sn-2* position of the diacylglycerol moiety receive remodeling. In yeast, the gene called *GUPI* is proposed to catalyze the incorporation of a C26:0 fatty acid at the *sn-2* position of the diacylglycerol moiety in GPI-anchored proteins (Bosson *et al.*, 2006). *GUPI* is also a member of MBOAT family, but the human and *C. elegans* orthologues have not been cloned so far. According to our preliminary observations, *mboa-7* is not involved in the remodeling of GPI-anchored proteins. However, it will be interesting to compare these two molecules to elucidate the domain involved in the recognition of acyl-CoA molecules and the head group of lysophospholipids.

In conclusion, we found that MBOA-7, another member of the MBOAT family, catalyzes the incorporation of AA and EPA into lysoPI. Substrates of several members of the MBOAT family are still unclear, and we are now testing whether they possess lysophospholipid acyltransferase activity.

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