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Membrane topology of human AGPAT3 (LPAAT3)

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Abstract

Integral membrane lysophospholipid acyltransferases (AT) are involved in many reactions that produce phospholipids and triglycerides. Enzymes that utilize lysophosphatidic acid (LPA) as an acceptor substrate have been termed LPAATs, and several are members of the 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT) gene family. Amino acid sequence comparisons with other acyltransferases reveal that AGPATs contain four conserved motifs (I–IV), whose invariant residues appear to be important for catalysis and/or substrate recognition. Although the enzymatic activities of many AGPATs are known, for many members their structural organization within membranes and their exact biological functions are unclear. Recently, a new function for AGPATs was discovered when it was determined that human AGPAT3/LPAAT3 is involved in the structure and function of the Golgi complex. Here we have determined the topological orientation of human AGPAT3/LPAAT3. AGPAT3/LPAAT3 possesses two transmembrane domains, one of which separates motifs I and II, which are thought to form a functional unit that is critical for enzymatic activity. This is a surprising result but similar to a recent study on the topology of human LPAAT 1. The data is consistent with a structural arrangement in which motif I is located in the cytoplasm and motif II is in the endoplasmic reticulum and Golgi lumen, suggesting a different model for AGPAT3/LPAAT3's enzymatic mechanism.

Keywords

Golgi complex; 1-acylglycerol-3-phosphate O-acyltransferase; AGPAT3; lysophosphatidic acid acyltransferase; LPAAT3; membrane topology

1. Introduction

Integral membrane lysophospholipid acyltransferases (LPATs) comprise a large and diverse family of enzymes involved in the conversion of lysophospholipids into phospholipids, which are classified both by sequence similarities and substrate preferences [1]. For example, lysophosphatidic acid (LPA) acyltransferases (LPAATs) that are members of the 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT) gene family convert LPA to phosphatidic acid by transfer of an acyl chain from fatty acyl-CoA. There are ten AGPATs encoded in the human genome [1,2,3], but because sequence comparisons can only provide an approximate idea of enzymatic activity, Shimizu and colleagues have suggested a standard nomenclature for these enzymes based on their substrate preferences [1].

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Although the enzymatic activities of many LPATs have been experimentally determined or predicted from sequence comparisons, in many cases the exact biological function of these enzymes has not been elucidated. Several members of this family are clearly involved in *de novo* phospholipid synthesis, synthesis of triglycerides, and reacylation of pre-existing lysophospholipids via the Lands cycle [2,4]. However, in the latter case, the biological role of many reacylation reactions is unclear. Recently, we have found that remodeling of membrane lysophospholipid/phospholipid levels influences trafficking in the secretory and endocytic pathways in mammalian cells [5,6]. More specifically, we found that human AGPAT3/LPAAT3, an LPA acyltransferase, is localized to the endoplasmic reticulum (ER) and Golgi complex and functions to regulate both the structure and trafficking functions of the Golgi [7]. In addition, mouse AGPAT3/LPAAT3 has been shown to also have lysophosphatidylinositol acyltransferase activity, perhaps serving specific functions in the testis [8]. Thus, integral membrane LPATs have now been demonstrated to have an expanded range of biological roles.

Our understanding of the structure of integral membrane LPATs is limited to mutagenesis studies that have identified important motifs involved in catalysis, and, in a very limited number of cases, membrane topology. Members of the AGPAT family, and other acyltransferases, are characterized by the presence of four conserved acyltransferase sequence motifs (I–IV), although certain plant acyltransferases are missing motifs III and IV, suggesting that they are dispensable [9,10]. Motifs I and II are likely involved in some aspect of catalysis or substrate recognition, although the exact reaction mechanism is poorly characterized. Mutations in either motif I or II generally cause a significant loss of enzymatic activity, and have therefore been assumed to define a structural and functional unit.

The topological orientation of LPATs within intracellular membranes has not been extensively studied, but is important for understanding the function(s) of these enzymes, e.g., on which side of the membrane products are made and thus available for subsequent events. The topological orientation of AGPATs has only been partially determined for one family member, human AGPAT1/LPAAT1. Surprisingly, the results showed that motif I is separated from motif III (and probably motif II) by a transmembrane domain [11]. This result is not consistent with previous ideas that Motifs I and II/III act as a functional unit on the same side of the membrane during catalysis. Other potential transmembrane domains have not been mapped.

To further explore the functional organization of an AGPAT, we determined the topological orientation of human AGPAT3/LPAAT3. Using two independent methods, we confirmed that motif I and motifs II/III are located on opposite sides of the membrane. Moreover, in contrast to several predictive algorithms, our mapping studies show that AGPAT3/LPAAT3 likely has only two transmembrane domains, thus providing a framework for future structure-function studies on these important enzymes.

2. Materials and Methods

2.1 Cell Culture and Immunofluorescence

All cells were grown in MEM + 10% NuSerum (or FBS) in a 37°C chamber with 5% CO_2. Cells grown on coverslips were transfected with pEGFP N-1 AGPAT3/LPAAT3 and c-myc or HA epitope tags inserted into the sequence. Cells were then fixed in 3.7% formalin in PBS, washed in PBS and permeabalized with either 0.1% Triton X-100 in PBS or a digitonin solution (3 μg/ml digitonin, 0.3 M sucrose, 5 mM $MgCl_2$, 120 mM KCl, 0.14 mM $CaCl_2$, 2 mM EGTA, 25 mM HEPES pH to 7.6 with KOH). Cells were then incubated with diluted primary antibodies: 9B11 mouse monoclonal anti-c-myc (1:1000) (from Cell

Signaling, Beverly, MA), anti-protein disulfide isomerase (PDI) (1:1000) (from Affinity Bioreagents, Rockford, IL), or 12CA5 mouse monoclonal anti-HA (1:100) (from Covance, Princeton, NJ) followed by secondary antibody anti-mouse or anti-rabbit TRITC (1:100) (from Jackson ImmunoResearch Laboratories, West Grove, PA). Peptide antibody for AGPAT3/LPAAT3 was designed and generated by Pacific Immunology (Ramona, CA) and characterized as described [7]. Rabbit anti-GFP was kindly provided by Anthony Bretscher (Weill Institute of Cell and Molecular Biology, Cornell University). Cells were then viewed and imaged using the Zeiss Axioscope 2.

2.2 Cloning/Mutagenesis

AGPAT3/LPAAT3 (Accession No. BC011971) cDNA was obtained in pCMV-SPORT6 from the EST collection of the IMAGE Human library (Invitrogen, Carlsbad, California). Using PCR and the multiple cloning site of pEGFP N-1 (Clontech, Mountain View, CA), AGPAT3/LPAAT3 was inserted in frame with the EGFP C-terminus before the stop codon. To make the catalytic mutant of AGPAT3/LPAAT3, insert either c-myc or HA sequences, and mutate K373/K374/K375 to A373/A374/A375, Quick Change II (Stratagene) mutagenesis kit with mutagenic primers was used. All constructs were verified by double stranded DNA sequencing. DNA constructs were transfected into cells grown on coverslips using Fugene 6 transfection reagent (Roche, Nutley, NJ) and processed for immunofluorescence experiments within 24–48 h unless otherwise indicated.

2.3 Protease Protection Assay

Cells were grown on 300 cm^2 plates and then scarped to harvest and spun down in microcentrifuge tubes. The pellet was resuspended in 240 μl PBS and homogenized by passage though a 26 gauge syringe. Nuclei were pelleted from the homogenate by microcentrifugating at 600 × g. Microsomes, Golgi and other membranes were further pelleted by spinning the supernatant at 100,000 × g for 30 min. The pellet was resuspended in 240 μl PBS and to this was added 2 μg/ml trypsin and 0.1% Triton X-100 when appropriate and incubated for 15 min [12]. Samples were separated by SDS-PAGE and analyzed by Western blotting.

2.4 Sequence Analyses Programs

The following programs were used to predict transmembrane domains and AGPAT3/LPAAT3 topology: HMMTOP 2.0 (http://www.enzim.hu/hmmtop/html/document.html) [13]; PSORTII (http://psort.ims.u-tokyo.ac.jp/) [14]; TopPred (http://www.enzim.hu/hmmtop/html/document.html) [15]; SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html)[16]; TMPred (http://www.ch.embnet.org/software/TMPRED_form.html) [17]. Generation of the two-dimensional topology models in Fig. 6 was done with TOPO2 (http://www.sacs.ucsf.edu/TOPO2/) software [18].

3. Results and Discussion

3.1 AGPAT3/LPAAT3 Primary Sequence and Domains

Human AGPAT3/LPAAT3 is a 376-residue protein and highly conserved with other vertebrate homologs: human AGPAT3/LPAAT3 is 75% identical (+12.5% strongly similar) to the *D. rerio* ortholog (Fig. 1). The four conserved motifs (I–IV) thought to be important for enzymatic activity are shown in yellow shading. No N-linked glycosylation sites are found, but a C-terminal di- or –tri lysine sequence is present in all members (green shaded residues). In type I transmembrane proteins, this sequence can serve as an ER “retention” signal for COPI mediated retrieval from post ER compartments [19]. Motifs I–IV are placed

in relationship to putative transmembrane domains that are predicted by several membrane topology algorithms (Fig. 2A). Two of the programs place all four motifs on one side of the membrane, whereas others bisect motifs I and II.

To empirically determine sequences of AGPAT3/LPAAT3 that are exposed to the cytoplasm or to the lumen of the ER and Golgi complex, c-myc and HA epitopes and GFP were inserted into the wild type amino acid sequence of AGPAT3/LPAAT3. HA and c-myc are short epitopes and unlikely to disrupt membrane insertion or folding, and GFP is known to leave AGPAT3/LPAAT3 functional if fused to the C-terminus [7]. The epitope tags were placed so that we could definitively map domains that are separated by a transmembrane segment (Fig. 2A).

3.2 Identification of Transmembrane Regions Using Selective Membrane Permeability and Immunofluorescence

The first method we used to map transmembrane domains used selective membrane permeabilization and immunofluorescence [20,21]. Cells transfected with AGPAT3/LPAAT3 cDNA containing each of these sequence tags were selectively permeabalized with either digitonin or Triton X-100. At the concentrations used, digitonin permeabilizes only the plasma membrane and not the ER or Golgi, thus allowing antibodies to enter the cell and bind to sequences exposed to the cytoplasm. Triton X-100 solubilizes all membranes and thus exposes all sequences to antibodies whether they are luminal or cytoplasm. The results showed that regions around myc41, HA93, and GFP377 were all exposed to the cytoplasm because antibodies bound to those epitotopes when incubated with digitonin and did not need Triton X-100 (Fig. 2B). The regions around myc175, however, were not detected in digitonin permeabilized cells, but were in those permeabilized with Triton X-100. Thus, this region must be located in the lumen. Protein disulfide isomease (PDI) is a resident ER protein in the lumen, which was used as a control. These results predict two possible transmembrane passes in the sequence of AGPAT3/LPAAT3.

3.3 Identification of Transmembrane Regions Using Protease Protection Assay

The second method used to determine the topology of AGPAT3/LPAAT3 was an *in vitro* protease protection assay [12]. In this case, cells were transfected to express the various tagged versions of AGPAT3/LPAAT3, homogenized in sucrose containing buffer to maintain organelle integrity and topology, and then a post-nuclear supernatant containing ER and Golgi complex membranes was incubated with trypsin or trypsin with Triton X-100. The logic is that regions of the protein near an epitope tag that faces the cytoplasmic or outer surface will be subjected to digestion, resulting in a loss of detection by western blotting. If a strong band is detected, then the membrane protects that sequence from proteolysis in the absence of Triton X-100. The results showed that constructs with myc4, myc41, HA93, and GFP377 all experienced a significant loss in signal when treated with trypsin, even in the absence of Triton X-100 (Fig. 2C). In contrast, the myc175 construct exhibited a loss in signal only when Triton X-100 was included with trypsin.

3.4 Putative C-terminal Tri-lysine ER retrieval motif

Our previous studies found that human AGPAT3/LPAAT3 is found in both the ER and Golgi complex, using both C-terminal GFP fusion constructs and antibodies against that endogenous protein (Fig. 3A–C). The location of the C-terminus in the cytoplasm places tri-lysine residues (K373/K374/K375) in position to serve as recognition markers for packaging into COPI vesicles for return to the ER [19]. To see if these residues function in this way, we first attempted to express a truncation mutant that was missing most of the cytoplasmic C-terminus; however, this construct produced an unstable protein that was rapidly degraded (data not shown). Alternatively, the lysine residues were all changed to alanines in the

context of GFP tagged full length AGPAT3/LPAAT3. When expressed in HeLa cells, changing lysines 373–375 to alanines did not change the intracellular distribution of AGPAT3/LPAAT3, which was found in a diffuse ER pattern and also in the juxtanuclear Golgi complex where it co-localized with GPP-130 (Fig. 3D–F).

3.5 Conclusions

These data are consistent with the following topology model (Fig. 4A). The amino terminus of human AGPAT3/LPAAT3 contains a cytoplasmic domain from residues 1–125, which contains motif I (residues 95–101). This cytoplasmic domain is interrupted by a strongly predicted transmembrane domain, which extends approximately from residues 126–143. A relatively long luminal loop follows from residues 143–317 and contains motifs II-IV. Interestingly, motif II (residues 141–146) may be partially buried in the membrane as part of the first transmembrane domain. This single luminal loop ends at another strongly predicted transmembrane domain somewhere between residues 308–339 (different programs predict different start and stop residues). Starting around residues 331–339 and extending to residue 376 is a cytoplasmic C-terminal tail.

Mapping motifs I and II of AGPAT3/LPAAT3 to opposite sides of the membrane is consistent with recent data for human AGPAT1/LPAAT1 topology and activity [11], i.e., motifs I and II are separated by a transmembrane domain and disruption of either one of the two motifs completely abolishes catalytic activity. Yamashita et al., have hypothesize that AGPAT1, and by extension here AGPAT3/LPAAT3, may require both motifs for either catalysis or substrate binding. Motif I most likely plays a direct role in the catalytic reaction. Site directed mutagenesis has shown that both His104 and Asp109 of human AGPAT1 are critical for enzymatic activity [11], and other studies have shown that equivalent motif I residues in other lysophospholipid acyltransferases, including AGPAT3/LPAAT3, are required for catalysis [1,4,7]. Interestingly, motif II partially overlaps with the first transmembrane domain and therefore could bind substrates in the membrane and channel them to motif I. Indeed, other studies have suggested that motif II of human AGPAT1 binds to lysophosphatidic acid [11]. Finally, we hypothesize that AGPAT3/LPAAT3 may also have activities on both sides of the membrane, such as transferring acyl chains from one leaflet to another.

The disposition of the N-terminus of AGPAT3/LPAAT3 is less clear. In some experiments we detected a cytoplasmic signal with the Myc4 tag, whereas in others the signal was weak (data not shown). One possibility that we have not ruled out is that the N-terminus is cleaved, but not efficiently, leading to a variable signal. Alternatively, the variable signal may reflect a situation in which one or both of the hydrophobic stretches (roughly from residues 11–31 or 15–39 and 49–73) (Fig. 2A) may be inserted into the cytoplasmic leaflet as hairpin loops but as not transmembrane domains (Fig. 4B). This topology would explain why Myc41 is variably seen with cytoplasmic probes. Like AGPAT3/LPAAT3, human AGPAT1 has two similar hydrophobic stretches at the N-terminus, and one model suggests that both are transmembrane domains separated by a very short luminal loop (equivalent to where the AGPAT3/LPAAT3 Myc41 tag would be found). However, this result is not consistent with our results because Myc41 is most reliably found on the cytoplasmic side of the membrane. Although the predicted locations of the N-terminal hydrophobic amino acid sequences of human AGPAT1 and AGPAT3/LPAAT3 are similarly placed, the sequence similarity between these proteins is only 31% (14% identical; 17% strongly similar) for residues encompassing this region. Therefore, it is possible that human AGPAT3/LPAAT3 and AGPAT1 have a different N-terminal topology. In any case, the structural organization of the two transmembrane domains and hydrophobic loops is unknown, but likely assumes a complex 3-dimensional channel or pocket that allows entrance of substrates (lysophosphatidic acid and acyl-CoA) and exit of product (PA).

Our previous studies have revealed a novel role for LPATs, and specifically AGPAT3/LPAAT3, in regulating the structure and function of the Golgi complex [7,22,23]. AGPAT3/LPAAT3 appears to negatively regulate the formation of Golgi membrane tubules that likely serve as trafficking intermediates for plasma membrane delivery and for maintenance of an intact Golgi ribbon. Generation of PA by AGPAT3/LPAAT3 could be used to negatively regulate tubule formation by ensuring the proper fission of membrane vesicles from these tubules—a process that may be facilitated by the intrinsic negative curvature of PA that could be localized at the neck of the budding vesicle [24]. In addition, PA and its metabolite diacylglycerol could be used to recruit effector proteins such as CtBP-BARS50, BAR domain containing proteins, and protein kinase D to the cytoplasmic side of the Golgi complex, thereby facilitating the fission process [5,25,26,27]. Therefore, our data placing motif I on the cytoplasmic surface is consistent with PA being generated on the appropriate side to influence membrane curvature and effector protein recruitment.

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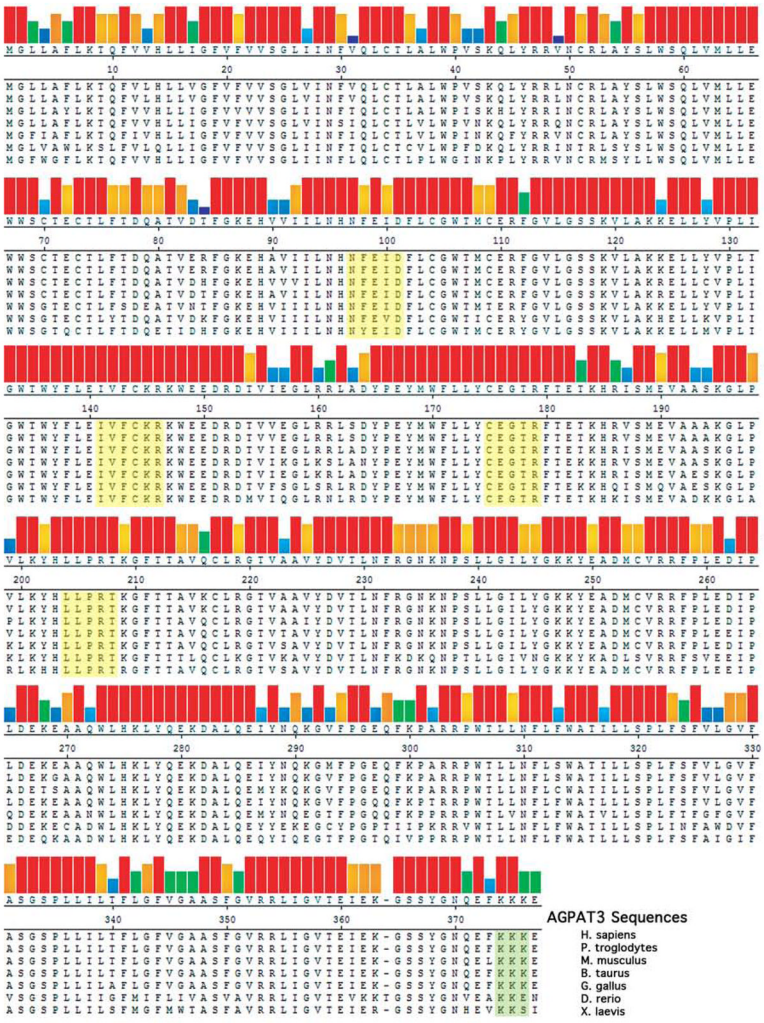

Figure 1.

AGPAT3/LPAAT3 sequence alignment. The primary amino acid sequences for multiple eukaryotes were aligned using Clustaw V. AGPAT3/LPAAT3 is highly conserved between species. Black arrows indicate potential transmembrane regions. Horizontal blue boxes indicate two conserved consensus motifs found in all AGPAT3/LPAAT3 sequences, all LPAAT family members, *E. coli* PlsC, and *S. cerevisiae* SLC4 orthologs.

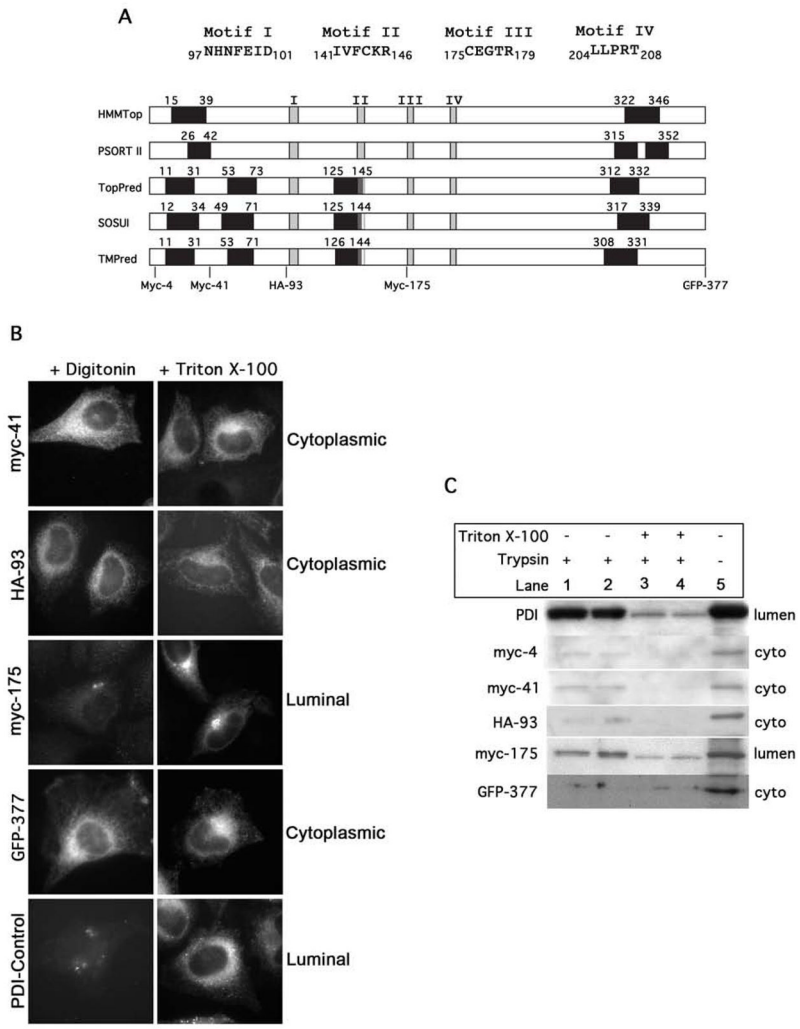

Figure 2.

(A) AGPAT3/LPAAT3 domains. Like other LPAATs, AGPAT3/LPAAT3 is predicted to contain transmembrane domains (dark bars) and an acyltransferase or PLsC domain. Topology prediction programs are shown on the left and numbers indicate residues predicted to be transmembrane domains. Within the PLsC domain, four conserved motifs (I–IV) are shown that contain acidic and basic amino acid residues possibly involved in catalyis and/or substrate recognition. The locations of inserted epitope tags or C-terminally appended GFP are shown on the TMPred model. (B) The use of immunofluorescence to determine the membrane topology of AGPAT3/LPAAT3. Antigenic amino acid tags were inserted into the AGPAT3/LPAAT3 amino acid sequences at specific locations indicated by the tag and number. These were individually transfected into HeLa cells and the cells were permeabilized with either digitonin or Triton X-100 and treated with corresponding antibodies. Cells that showed fluorescence with digitonin processing are inferred to contain that amino acid tag in the cytoplasm. If cells are not fluorescent with digitonin, the tag is inferred to reside in the lumen. (C) Protease protection assay to determine AGPAT3/LPAAT3 topology. Organelles from cells transfected with AGPAT3/LPAAT3 genes encoding antigenic tags were isolated and subjected to trypsin proteolysis. Antigenic tags were detected by western blot. In samples with Triton X-100 all tags are expected to be digested, in samples with digitonin only cytosolic tags are expected to be digested, and in

samples with no trypsin tags are expected to be preserved. Lanes 1 and 2 are duplicates, and lanes 3 and 4 are also duplicates.

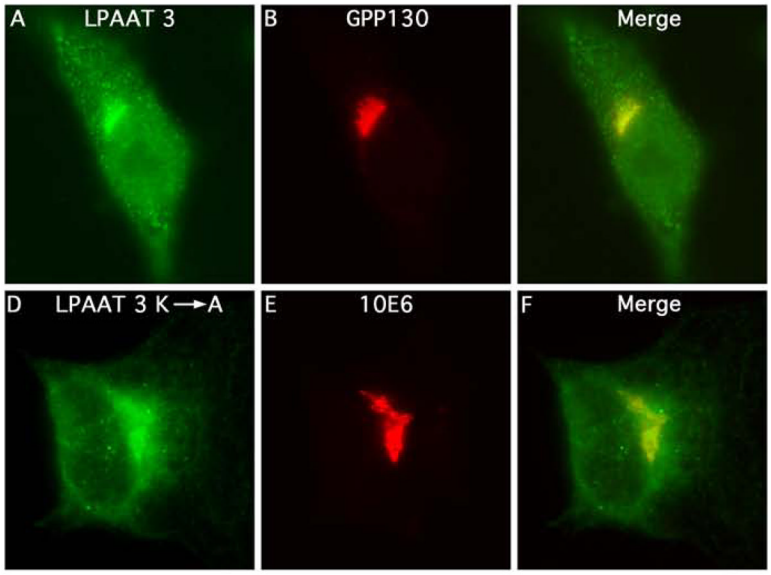

Figure 3.

Mutation of C-terminal tri-lysine motif (K373/K374/K375) to alanines does not effect the intracellular distribution of human AGPAT3/LPAAT3. (A–C) Immunofluorescence localization AGPAT3/LPAAT3 and the Golgi marker GPP130. (D–F) Localization of expressed GFP-tagged AGPAT3/LPAAT3 (KKK to AAA mutant) in HeLa cells labeled by immunofluorescence with cis-Golgi marker 10E6.

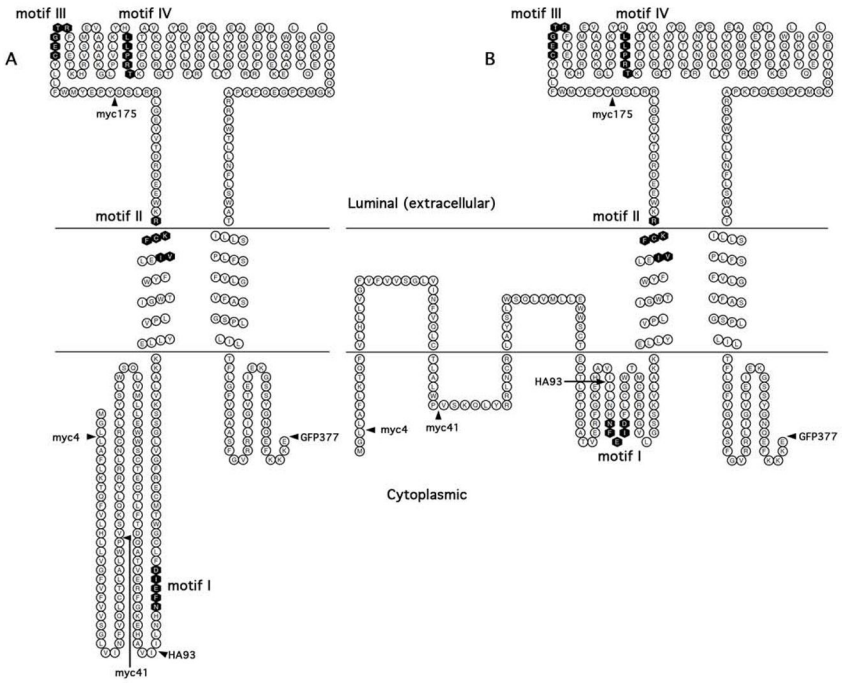

Figure 4.

Models for AGPAT3/LPAAT3 topology. (A) Based on redundant methods for determining membrane topology, it has been demonstrated that AGPAT3/LPAAT3 likely contains two transmembrane domains. The first transmembrane domain bisects the acyltransferase domains and places each of the conserved catalytic motifs I and II on opposing sides of the membrane. (B) An alternative model places two N-terminal hydrophobic stretches as membrane insertion loops, which does not change the orientation of the two transmembrane domains. Shown in the diagram are the locations of epitope and GFP tags used to determine the topological orientation of domains.