Enzymological analysis of the tumor suppressor A-C1 reveals a novel group of phospholipid-metabolizing enzymes

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Abstract A-C1 protein is the product of a tumor suppressor gene negatively regulating the oncogene Ras and belongs to the HRASLS (HRAS-like suppressor) subfamily. We recently found that four members of this subfamily expressed in human tissues function as phospholipid-metabolizing enzymes. Here we examined a possible enzyme activity of A-C1. The homogenates of COS-7 cells overexpressing recombinant A-C1s from human, mouse, and rat showed a phospholipase A₁/₂ (PLA₁/₂) activity toward phosphatidylcholine (PC). This finding was confirmed with the purified A-C1. The activity was Ca²⁺ independent, and dithiothreitol and Nonidet P-40 were indispensable for full activity. Phosphatidylyethanolamine (PE) was also a substrate and the resultant lysophospholipids were also reacylated by various acyltransferases (5). Deacylation of phospholipids also leads to the generation of many classes of lipid mediators, including eicosanoids and lysophospholipids, which show their diverse cellular actions principally through G protein-coupled receptors (6, 7).

Lecithin retinol acyltransferase (LRAT) is an enzyme responsible for the metabolic cycle of retinol (8, 9). This enzyme transfers an acyl chain at the sn-1 position of phosphatidylcholine (PC) to all-trans-retinol, resulting in the formation of retinyl ester, and constitutes the LRAT family, together with various proteins such as Caenorhabditis elegans Egl-29 and several mammalian tumor suppressors (10). In human, five tumor suppressor proteins are included in this family, and they form the HRAS-like suppressor (HRASLS) subfamily or the H-rev107 subfamily (11). The members are H-rev107 (also known as HRASLS3, HRSL3, or H-REV107-1) (12, 13), TIG3 (also known as RARRES3, RIG1 or HRASLS4) (14, 15), A-C1 (also known as HRASLS) (16), HRASLS2 (11) and Ca²⁺-independent N-acyltransferase (iNAT, also known as HRASLS5 or HRLP5) (17). Recently we found that H-rev107 (18, 19), TIG3 (19), HRASLS2 (19) and iNAT (17, 20) have PLA₁/₂ and/or acyltransferase activities.

The metabolism of phospholipids is a dynamic event regulated by numerous enzymes. This includes remodeling of the acyl chains of glycerophospholipids by deacylation and reacylation (1–3). The deacylation is catalyzed by a series of phospholipase A₁s (PLA₁s) and PLA₁s (1, 2, 4), whereas the resultant lysophospholipids are reacylated by various acyltransferases (5).

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EXPERIMENTAL PROCEDURES

Materials

[1,14C]palmitic acid, 1,2-[1,14C]dipalmitoyl-PC, 1-palmitoyl-2-[1,14C]arachidonoyl-PE, and 1-[1,14C]palmitoyl-lyso PC were purchased from PerkinElmer Life Science. 1-Palmitoyl-2-[1,14C]palmitoyl-PC, horseradish peroxidase-linked anti-mouse IgG, Hybond P, and an ECL Plus kit were from GE Healthcare. 1,2-Dipalmitoyl-PC, 1,2-dioleoyl-PE, 1-palmitoyl-lyso PC, anti-FLAG monoclonal antibody M2, anti-FLAG M2 affinity gel, FLAG peptide, snake venom PLA2 and Rhizopus arrhizus lipase were from Sigma. Dulbecco’s modified Eagle’s medium, Lipofectamine 2000, fetal calf serum, pEF6/myc-His vector, TRIzol, and Moloney murine leukemia virus reverse transcriptase were from Invitrogen Life Technologies. 1-Palmitoyl-2-arachidonoyl-PE was from Avanti Polar Lipids (Alabaster, AL). Human Testis Marathon-Ready™ cDNA and human MTC™ Panels I and II were from Clontech. Nonidet P-40 was from Nacalai Tesque, Inc. (Kyoto, Japan). Rhizopus delemar lipase was from Seikagaku Corp. (Tokyo, Japan). Random hexamer and Ex Taq DNA polymerase were from TaKaRa Bio, Inc. (Ohtsu, Japan). KOD-Plus DNA polymerase was from TOYOBO (Osaka, Japan). Protein assay dye reagent concentrate was from Bio-Rad, and precoated Silica Gel 60 F254 aluminum sheets (20 × 20 cm, 0.2 mm thick) for TLC were from Merck (Darmstadt, Germany). N-[14C]palmitoyl-PE was prepared from [14C]palmitic acid and 1,2-dioleoyl-PE according to the method of Schmid et al. (27). 2-Palmitoyl-lyso PC was prepared from 1,2-dipalmitoyl-PC using R. delemar lipase as described previously (28). 2-[14C]palmitoyl-lyso PC was prepared from 1-palmitoyl-2-[14C]palmitoyl-PC using R. arrhizus lipase. 1-[14C]palmitoyl-2-palmitoyl-PC was prepared from 2-palmitoyl-lyso PC and [14C]palmitic acid.

Construction of expression vectors

The cDNAs encoding C-terminally FLAG-tagged A-C1s of human, mouse, and rat were amplified by PCR with Human Testis...
Enzyme assay

For the PLAc2 assay, the enzyme was incubated with 200 μM 1,2-[14C]dipalmitoyl-PC (45,000 cpm) in 100 μl of 50 mM Tris-HCl (pH 8.0), 2 mM DTT, and 0.1% Nonidet P-40 at 37°C for 30 min. The reaction was terminated by the addition of 200 μl of a mixture of chloroform-methanol (2:1; v/v) containing 5 mM 3(2)-ethyl-4-hydroxyanisole. After centrifugation, 100 μl of the lower fraction was spotted onto a silica gel thin-layer plate (10 cm height) and developed at 4°C for 25 min either in chloroform-methanol-28% ammonium hydroxide (80:20:2; v/v) for the PE N-acylation assay or in chloroform-methanol-H2O (65:25:4; v/v) for PLAc2 and lys POC-acylation assays. The distribution of radioactivity on the plate was quantified using a BAS1500 bioimaging analyzer (FUJIX Ltd., Tokyo).

Western blotting

Samples (20 μg protein) were separated by SDS-PAGE on 14% gel and electrotransferred to a hydrophobic polyvinylidene difluoride membrane (Hybond P). The membrane was blocked with PBS containing 5% dried milk and 0.1% Tween 20 (buffer B) and then incubated with anti-FLAG antibody (1:2,000 dilution) in buffer B at room temperature for 1 h, followed by incubation with HRP-labeled secondary antibody (1:4,000 dilution) in buffer B for 1 h. FLAG-tagged proteins were visualized using an ECL Plus kit and analyzed using an LAS-1000plus lumino-imaging analyzer (FUJIX Ltd.).

PCR

To examine tissue distribution of human A-C1 mRNA, human MTC™ Panels I and II were used as templates for PCR amplification with Ex Taq DNA polymerase. For the analyses of rat and mouse A-C1 mRNAs, total RNAs were isolated from various organs of Wistar/ST rats and C57BL/6 mice (Japan SLC, Inc.) using TRIzol. CDNs were prepared from 5 μg of total RNA using Moloney murine leukemia virus reverse transcriptase and random hexamer, and subjected to PCR amplification by Ex Taq DNA polymerase. The primers used for human A-C1 were the forward primer 5′-CCCCAGGAATGAGAAGACACCAACAGC-3′ and the reverse primer 5′-CTCCTCCCGTCATGTGTGCTC-3′ containing an in-frame FLAG sequence and the BamHI site and the reverse primer 5′-CGCAATTTTCATCTCATGCTGTCATCTCGTATAATTGA-3′ (mouse A-C1), and 5′-CGCGCGGCCGCTACTTATCGTCGTCATCCTTGTAATCAGATCTTTTGGTCTTTTGGAGAAC-3′ (human A-C1), 5′-CGCGGCGGCCGCTACTTATCGTCGTCATCCTTGTAATCA-TATTTTGGTCTTTTGGG-3′ (mouse A-C1), and 5′-CGC-GCGCCGGCCGCTACTTATCGTCGTCATCCTTGTAATCATTTTGGTCTTTTGGG-3′ (rat A-C1). The cDNA encoding C-terminally FLAG-tagged recombinant proteins was subcloned into the corresponding restriction enzyme sites of pEF6/myc-His vector. All constructs were sequenced in both directions using an ABI 3130 Genetic Analyzer (Applied Biosystems Life Technologies; Carlsbad, CA).
RESULTS

Functional expression of A-C1 proteins

We previously cloned cDNA of A-C1 (tentatively termed RLP-2) from rat testis (GenBank™ accession number AB510983) (17). In the present study, we also cloned cDNAs of the counterparts from human testis and mouse brain (AB510981 and AB510982) based on the reported nucleotide sequences. Their sequences we determined were completely identical to those reported previously. The primary structures of A-C1 proteins were composed of 168 (human), 167 (mouse), and 167 (rat) amino acid residues, respectively (Fig. 2A). The alignment revealed their high homology to each other (85, 83, and 96% identity at amino acid level between human and mouse, between human and rat, and between mouse and rat, respectively). The putative catalytic dyad was completely conserved as histidine-30 and cysteine-119. We previously failed in the functional expression of rat A-C1 with pcDNA3.1(+) as an eukaryotic expression vector (17). We therefore constructed the pEF6/myc-His expression vector harboring the A-C1 cDNA of either human, mouse, or rat with a FLAG tag at the C terminus, and transiently expressed recombinant proteins in COS-7 cells. Based on the amino acid sequences, the molecular masses of the tagged proteins of A-C1s were calculated to be 19,745 (human), 19,778 (mouse), and 19,516 (rat) Da, respectively. When analyzed by Western blotting using anti-FLAG antibody, each cell homogenate exhibited an immunopositive band around 19–20 kDa (Fig. 2B). Although the band of human A-C1 consistently migrated a little faster than expected, the reason remained unclear.

We next assayed the homogenates for PLA1/2 activity. When the samples were incubated with 1,2-[14C]dipalmitoyl-PC for the PLA1/2 assay, followed by separation of the products by TLC, the radioactive bands corresponding to palmitic acid and lyso PC were detected (Fig. 2C). The activities of the homogenates containing human, mouse, and rat A-C1 were 1.65, 0.47, and 0.75 nmol/min/mg of protein, respectively, whereas the endogenous activity of mock transfectant was 0.16 nmol/min/mg of protein (Fig. 2D). These results suggested that A-C1 possesses PLA1/2 activity. We examined a possible secretion of recombinant human A-C1 into the culture medium by measuring PLA1/2 activity. Consistent with the lack of the signal sequence for the secretory pathway in its primary structure, the activity was not detected in the culture medium of COS-7 cells expressing human A-C1 (data not shown). Although A-C1 was found as a tumor suppressor gene (16), its transient expression in COS-7 cells did not show an obvious effect on cell proliferation and viability (data not shown).

Characterization of the purified human A-C1

To further analyze the enzymatic properties of A-C1, we prepared cytosolic fractions from the COS-7 cell homogenate by ultracentrifugation and purified the C-terminally FLAG-tagged human A-C1 protein from the cytosol by anti-FLAG antibody-conjugated column chromatography. As analyzed by SDS-PAGE, a nearly homogenous protein band was seen around 19 kDa (Fig. 3A). The specific PLA1/2 activity of the purified protein was 182 nmol/min/mg of protein, which was 110-fold higher than that of the A-C1-expressing cell homogenate. The optimal pH was around 8 (Fig. 3B). The PLA1/2 activity increased up to 246 nmol/min/mg of protein, depending on the concentrations of the substrate PC, with an apparent K_m at 80 μM (Fig. 3C). We also examined the effects of several factors on the PLA1/2 activity. The addition of 1 mM and 5 mM Ca^{2+} reduced the activity by 9.9% and 21.7%, respectively. On the other hand, 1 mM EDTA increased the activity by 14.3% (Fig. 3D). In the absence of the sulfhydryl reducing reagent DTT, the activity was hardly detected (Fig. 3E). In agreement with this stimulatory effect of DTT, 5 mM iodoacetate, an irreversible sulfhydryl blocker, acted as an inhibitor. The standard reaction mix also contained 0.1% Nonidet P-40 (a nonionic detergent). Removal of the detergent decreased the activity by 94.5%. These effects of
A-C1 showed a remarkable preference of sn-1 position over sn-2 position. A similar result was obtained using 1-[14C]palmitoyl-2-palmitoyl-PC as a substrate (data not shown). 1-Palmitoyl-2-[14C]arachidonoyl-PE was also an active substrate, and PLA1 activity was again higher than PLA2 activity (Table 1). On the other hand, lysophospholipase activities for 1-[14C]palmitoyl-lyso PC or 2-[14C]palmitoyl-lyso PC were not detected.

We next examined transacylation activities of the purified A-C1. When 1-[14C]dipalmitoyl-PC and nonradioactive dioleoyl-PE were used as a donor substrate and an acceptor substrate, respectively, a radioactive band corresponding to N-palmitoyl-PE was detected on the TLC plate (Fig. 4A). In the absence of nonradioactive PE, this band was not detected (not shown). These results showed that A-C1 possesses an N-acyltransferase activity for PE. Moreover, when the protein was allowed to react with nonradioactive dipalmitoyl-PC and radioactive lyso PC (either 1-[14C]palmitoyl-lyso PC or 2-[14C]palmitoyl-lyso PC) as a donor substrate and an acceptor substrate, respectively, 14C-labeled PC was formed from both of the lyso PCs (Fig. 4B). 2-[14C]palmitoyl-lyso PC was a much more active substrate than 1-[14C]palmitoyl-lyso PC, as shown in Fig. 4C. With the aid of our previous results (19, 20), we compared catalytic properties of human A-C1, iNAT, H-rev107, HRASLS2, and TIG3, all of which belong to the HRASLS subfamily (Fig. 4C). All the purified recombinant proteins showed PLA1/2 activity, the highest being with H-rev107 and the lowest with iNAT. As for the PE N-acyltransferase activity, iNAT, HRASLS2, and A-C1 showed similar levels of activities, whereas H-rev107 and TIG3 were much less active. These five proteins also showed O-acyltransferase activities toward both 1-[14C]palmitoyl-lyso PC and 2-[14C]palmitoyl-lyso PC. The latter lyso PC was consistently a more-active acceptor substrate than the former lyso PC. In particular, A-C1 and HRASLS2 showed high O-acyltransferase activities.

Metabolic labeling of A-C1-expressing cells

We metabolically labeled the COS-7 cells transiently expressing human A-C1 with [14C]palmitic acid. When the extracted lipids were analyzed by TLC, we detected a clear radioactive band corresponding to N-palmitoyl-PE (Fig. 5A, C). This band was not observed in the control COS-7 cells. These results suggested that A-C1 actually functions as N-acyltransferase in the living cells. On the other hand, we did not see an obvious change in the levels of radioactive bands corresponding to free palmitic acid and lyso PC (expected products of PLA1/2) (Fig. 5A–C). The levels of bands corresponding to PC and PE (potential substrates of Ca2+, DTT, iodoacetate, and Nonidet P-40 were similar to the catalytic properties of H-rev107, TIG3, and HRASLS2, which we reported previously (19).

Because [14C]dipalmitoyl-PC that to this point we used as a substrate was radiolabeled on both sn-1 and sn-2 palmitoyl chains, we referred to the hydrolysis activity as PLA1/2 activity. To distinguish PLA1 activity from PLA2 activity, we next used [14C]PC radiolabeled only on the sn-2 palmitoyl chain (1-palmitoyl-2-[14C]palmitoyl-PC). As shown in Table 1, A-C1 showed a remarkable preference of sn-1 position over sn-2 position. A similar result was obtained using 1-[14C]palmitoyl-2-palmitoyl-PC as a substrate (data not shown). 1-Palmitoyl-2-[14C]arachidonoyl-PE was also an active substrate, and PLA2 activity was again higher than PLA1 activity (Table 1).

### Table 1. PLA1 and PLA2 activities of human A-C1

| PLA1 activity (nmol/min/mg) | PLA2 activity (nmol/min/mg) | PLA2/PLA1 ratio |
|-----------------------------|-----------------------------|-----------------|
| 1-Palmitoyl-2-[14C]palmitoyl-PC | 142.2 ± 5.9 | 16.0 ± 9.0 | 8.9 |
| 1-Palmitoyl-2-[14C]arachidonoyl-PE | 91.1 ± 6.3 | 13.5 ± 0.6 | 6.7 |

The purified recombinant human A-C1 (0.13 μg protein) was allowed to react with the indicated glycerophospholipids at 200 μM. Mean values ± SD are shown (n = 3).
examined by semi-quantitative real-time PCR. The highest A-C1/GAPDH ratio was found in testis (53.5), followed by skeletal muscle (2.4), brain (1.1), and heart (0.7). Other human tissues showed lower levels. The dominant expression in these four tissues was also observed with mouse and rat. Low levels of its expression were detected in lung, stomach, kidney, and colon of mouse, and thymus, lung, and small intestine of rat. Such a relatively high expression in the limited tissues was similar to the dominant expression of iNAT in testis (17, 20, 30), but was different from ubiquitous expressions of H-rev107 and TIG3 (19).

Lack of PLA₁/₂ activity in FAM84B

FAM84A and FAM84B were found as human genes up-regulated in some tumors (31, 32). The deduced amino acid sequences of these two genes are homologous to those of the HRASLS subfamily members as shown in a phylogenetic tree (Fig. 7A) and by comparison with the sequence of human A-C1 (Fig. 7B). The two sequences exhibit 43.5% identity to each other (Fig. 7B). However, serine is substituted for the cysteine residue forming the catalytic dyad in these proteins. We cloned cDNA of FAM84B from human testis and constructed the expression

Fig. 4. PE Nacylation and lyso PC O-acylation activities of human A-C1 and comparison of catalytic activities among the HRASLS subfamily members. A, B: The purified human A-C1 (0.13 μg) was assayed for PE Nacylation activity with 200 μM 1,2-[¹⁴C]dipalmityl-PC and 100 μM nonradioactive PE (lane 2) or for PC O-acylation activity with 200 μM nonradioactive PC and 100 μM of either 1-[¹⁴C]palmityl-lyso PC (lane 4) or 2-[¹⁴C]palmityl-lyso PC (lane 6). The substrates were also incubated with buffer alone as controls (lanes 1, 3, and 5 for lanes 2, 4, and 6, respectively). The mobile phases used for TLC were chloroform-methanol-28% ammonium hydroxide (80:20:2; v/v) (A) and chloroform-methanol-H₂O (65:25:4; v/v) (B), respectively. The positions of authentic compounds on the TLC plate are indicated by arrows. C: The purified human A-C1 (black), iNAT (dark gray), H-rev107 (light gray), HRASLS2 (white), and TIG3 (stripe) were allowed to react with 200 μM 1,2-[¹⁴C]dipalmityl-PC alone for PLA₁/₂ activity (panel a), with 200 μM 1,2-[¹⁴C]dipalmityl-PC and 100 μM nonradioactive PE for PC Nacylation activity (panel b), with 200 μM nonradioactive PC and 100 μM 1-[¹⁴C]palmityl-lyso PC for lyso PC O-acylation activity (panel c), or with 200 μM nonradioactive PC and 100 μM 2-[¹⁴C]palmityl-lyso PC for lyso PC O-acylation activity (panel d). Mean values ± SD are shown (n = 3). The symbols * and ‡ indicate that the data are cited from our previous articles (20) and (19), respectively. NPPE, N-palmitoyl-PE; C₁₆:₀, palmitic acid.

PLA₁/₂ were also unaltered. These findings may be explained by a low PLA₁/₂ activity of A-C1 in the living cells. Another possibility is that the produced [¹⁴C]palmityl acid and [¹⁴C]lyso PC are quickly incorporated into phospholipids.

Tissue distribution of A-C1

To examine tissue distribution of A-C1 in human, mouse, and rat, reverse transcriptase-PCR was employed (Fig. 6). In human, the levels of A-C1 mRNA were by far the highest in testis and skeletal muscle, followed by brain and heart. The expression levels of human A-C1 were also}

Fig. 5. Metabolic labeling of A-C1-expressing cells with [¹⁴C] palmitic acid. The COS-7 cells transiently expressing human A-C1 (lane 2) and control COS-7 cells (lane 1) were grown to 80% confluence and were metabolically labeled with [¹⁴C]palmitic acid (1.6 μCi) for 18 h. Total lipids were then extracted and separated by TLC using chloroform-methanol-28% ammonium hydroxide (80:20:2; v/v) (A) or chloroform-methanol-H₂O (65:25:4; v/v) (B) as mobile phase. The positions of authentic compounds on the TLC plate are indicated. NPPE, N-palmitoyl-PE; C₁₆:₀, palmitic acid. Relative radioactivities of the indicated compounds are shown (mean values ± SD, n = 3) (C). The radioactive band corresponding to authentic NPPE might include not only N-[¹⁴C]palmityl-PE but also N-acyl-[¹⁴C]palmityl-PE.

examined by semi-quantitative real-time PCR. The highest A-C1/GAPDH ratio was found in testis (53.5), followed by skeletal muscle (2.4), brain (1.1), and heart (0.7). Other human tissues showed lower levels. The dominant expression in these four tissues was also observed with mouse and rat. Low levels of its expression were detected in lung, stomach, kidney, and colon of mouse, and thymus, lung, and small intestine of rat. Such a relatively high expression in the limited tissues was similar to the dominant expression of iNAT in testis (17, 20, 30), but was different from ubiquitous expressions of H-rev107 and TIG3 (19).

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The dominant PLA₁ activity over PLA₂ activity, as well as the preference of 2-acyl-lyso PC in the lyso PC O-acylation, suggested the involvement of A-C1 in the remodeling at the sn-1 position of glycerophospholipids in a CoA-independent manner. As for tissue distribution, mRNA of AC-1 was highly expressed in testes and skeletal muscles of human, rat, and mouse. In addition, its moderate expression was seen in heart and brain. Our results were in agreement with previous reports that A-C1 was predominantly expressed in skeletal muscle, heart, brain, and bone marrow of mouse (16), and skeletal muscle, testis, heart, and brain of human (24). Such a tissue distribution of A-C1 distinguishable from those of other members of the HRASLS subfamily suggests a unique physiological role of this protein.

The present study and our previous studies (17–20) revealed that all proteins of the HRASLS subfamily possess phospholipid-metabolizing activities. Similarity among their enzymatic properties is in good agreement with high homology among their primary structures (Fig. 1). The histidine and cysteine residues corresponding to the catalytic dyad of LRAT are completely conserved throughout the five proteins. Because subtle structural differences of vector. Although transient expression of the FLAG-tagged recombinant protein in COS-7 cells was confirmed by Western blotting using anti-FLAG antibody, the cell homogenates did not show a significant PLA₁/₂ activity. The same procedure was applied to cDNA cloning and expression of human FAM84A. However, we failed in its expression for unknown reasons.
the members should explain different availabilities of acyl acceptor substrates, further investigation will be required to elucidate the structure-function relationship. Contribution of the catalytic activities to their tumor-suppressive activities currently remains unclear. The tumor-suppressing activity was mostly implicated in Ras-transformed cells (11, 13, 16, 33), and the mutants of TIG3 addressed to the conserved asparagine and cysteine residues failed to induce the apoptosis of HTA cervical cancer cells, which was caused by the wild-type (34). Because this cysteine residue functions as the catalytic center, it is possible that the phospholipid-metabolizing activity of the tumor suppressors regulates the function of Ras by altering the membrane structures of microdomains where Ras is specifically localized (35).

All the HRASLS subfamily members contain the sequence NCEHFV (amino acids 118–123 in the case of A-C1) (Fig. 1). Human FAM84A and FAM84B show homology to LRAT and the HRASLS subfamily members (Fig. 6A). Although both of the proteins contain a sequence similar to the sequence NCEHFV, serine is substituted for the cysteine residue. The lack of PLA1/2 activity in FAM84B may be related to this substitution. As shown in the phylogenetic tree (Fig. 6A), the distinct evolution of LRAT, FAM84B, and the HRASLS subfamily members appears to explain the difference in their catalytic properties. However, we cannot rule out a possibility that FAM84B has another enzyme activity.

To date, various names have been used for each member of the HRASLS subfamily (Table 2). According to the nomenclature proposed by the HUGO Gene Nomenclature Committee, HRASLS1-5s are assigned to genes for A-C1, HRASLS2, H-rev107, TIG3, and iNAT, respectively. Considering that all these proteins possess PLA1/2 and acyltransferase activities, here we propose to term the products of HRASLS1-5 genes as phospholipase A/acyltransferase (PLA/AT)-1 to -5, respectively (Table 2).

In conclusion, we characterized for the first time the tumor suppressor protein A-C1 as a phospholipid-metabolizing enzyme. Considering that five human members of the HRASLS subfamily, including A-C1, share similar catalytic properties, these proteins appear to form a novel class of enzymes showing PLA1/2 and acyltransferase activities.

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