Interacting Proteins Dictate Function of the Minimal START Domain Phosphatidylcholine Transfer Protein/StarD2*  

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The Star (steroidogenic acute regulatory protein)-related transfer (START) domain superfamily is characterized by a distinct lipid-binding motif. START domains typically reside in multidomain proteins, suggesting their function as lipid sensors that trigger biological activities. Phosphatidylcholine transfer protein (PC-TP, also known as StarD2) is an example of a START domain minimal protein that consists only of the lipid-binding motif. PC-TP, which binds phosphatidylcholine exclusively, is expressed during embryonic development and in several tissues of the adult mouse, including liver. Although it catalyzes the intermembrane exchange of phosphatidylcholines in vitro, this activity does not appear to explain the various metabolic alterations observed in mice lacking PC-TP. Here we demonstrate that PC-TP function may be mediated via interacting proteins. Yeast two-hybrid screening using libraries prepared from mouse liver and embryo identified Them2 (thioesterase superfamily member 2) and the homeodomain transcription factor Pax3 (paired box gene 3), respectively, as PC-TP-interacting proteins. These were notable because the START domain superfamily contains multidomain proteins in which the START domain coexists with thioesterase domains in mammals and with homeodomain transcription factors in plants. Interactions were verified in pulldown assays, and colocalization with PC-TP was confirmed within tissues and intracellularly. The acyl-CoA thioesterase activity of purified recombinant Them2 was markedly enhanced by recombinant PC-TP. In tissue culture, PC-TP coactivated the transcriptional activity of Pax3. These findings suggest that PC-TP functions as a phosphatidylcholine-sensing molecule that engages in diverse regulatory activities that depend upon the cellular expression of distinct interacting proteins.

Phosphatidylcholine transfer protein (PC-TP)§ is a soluble lipid-binding protein with high specificity for phosphatidylcholines (1, 2). Whereas PC-TP promotes intermembrane exchange of phosphatidylcholines in vitro, its physiological function in vivo is not well understood (3). Studies in tissue culture and knock-out mice have demonstrated physiological roles for PC-TP in hepatobiliary lipid homeostasis, reverse cholesterol transport, and high density lipoprotein metabolism (3). However, these effects do not appear to be fully explained by the phosphatidylcholine transfer activity of PC-TP.

PC-TP (also known as StarD2) is a member of a steroidogenic acute regulatory protein-related transfer (START) domain protein superfamily (4). START domains are conserved motifs that bind hydrophobic ligands. Proteins that contain START domains participate in intracellular lipid transport, lipid metabolism, and cellular signaling (5–7). START domain proteins are expressed from bacteria to higher organisms but are most numerous in plants (8).

START domains largely reside within multidomain proteins, suggesting that binding of a hydrophobic ligand might regulate activity of another domain within the same protein. By contrast, PC-TP is an example of a minority of so-called START domain minimal proteins, the entire amino acid sequence of which comprises the START domain. Because of the apparent absence of other functional domains, we hypothesized that the biological activities of PC-TP might necessitate protein-protein interactions. PC-TP in mice is expressed in a variety of tissues of the adult animal (9, 10) and as early as embryonic stem cells (10). On this basis, we performed two yeast two-hybrid screens utilizing cDNA libraries prepared from adult mouse liver and from day 9.5–10.5 embryos. PC-TP-interacting proteins included Them2 (thioesterase superfamily member 2) from liver and the homeodomain transcription factor, Pax3 (paired box gene 3) from the embryo. These were noteworthy because the START superfamily contains multidomain proteins that consist of START plus thioesterase domains in mammals, as

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** The abbreviations used are: PC-TP, phosphatidylcholine transfer protein; CACH, cytosolic acetyl-CoA hydrolase; DHPE, lissamine rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethy lammonium salt; GST, glutathione S-transferase; GFP, green fluorescent protein; MITF, microphthalmia-associated transcription factor; NBD-PC, 2-[12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine; RFP, red fluorescent protein; START, steroidogenic acute regulatory protein (Star)-related transfer; HEK, human embryonic kidney; PBS, phosphate-buffered saline; HUVEC, human umbilical vein endothelial cells; DTNB, 5,5′-dithiobis(nitrobenzoic acid).

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well as START plus homeodomains in plants (5, 6, 8). Them2 and Pax3 were colocalized with PC-TP within tissues and cells. Functional analyses demonstrated that PC-TP increased the acyl-CoA thioesterase activity of Them2, as well as the transcriptional activity of Pax3. These findings suggest that interacting proteins play key roles in the biological functions of PC-TP.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screening to Identify PC-TP-interacting Proteins**—To identify liver-enriched PC-TP-interacting proteins, we utilized the Matchmaker GAL4 yeast two-hybrid system 3 (Clontech, Mountain View, CA). Briefly, the coding sequence of mouse PC-TP cDNA was cloned into pGBKKT7, which expresses GAL4BD to create a PC-TP:GAL4BD fusion protein. The PC-TP:GAL4BD construct was cotransformed into Saccharomyces cerevisiae AH109 cells with a mouse liver cDNA library cloned into the pACT2 GAL4AD reporter vector. Double transformants containing interacting proteins were identified according to growth on selective media. These were further screened under highest stringency conditions using three yeast reporter genes, one of which conferred survival on selective media, as well as two separate color-based selection genes. This approach offered the advantage of markedly reducing the likelihood of detecting false positive interactions. The main disadvantage was that low affinity protein-protein interactions were less likely to be detected.

In preliminary experiments, we verified robust PC-TP protein expression in mouse embryos at days 9.5 and 10.5 by immunoblot analysis, as well as representation of PC-TP by Southern blot analysis in a mouse embryonic day 9.5–10.5 cDNA library (11, 12), which was generously provided by Dr. Nicole Schreiber-Agus (Albert Einstein College of Medicine, Bronx, NY). The coding sequence of human PC-TP cDNA was cloned into pBTM116 containing the binding domain of the yeast transcription factor LexA (LexA<sub>AD</sub>). This was transformed into the S. cerevisiae L40 reporter strain together with mouse embryonic cDNA library cloned into pVP16 containing the LexA activation domain (LexA<sub>AD</sub>) (13). Double transformants containing interacting proteins were selected based on reporter gene activity that conferred survival on selective media, with care taken to exclude false positives (14). Purified cDNA:LexA<sub>AD</sub> plasmids were retransformed into L40 reporter strain expressing PC-TP:LexA<sub>AD</sub> to confirm positive interactions.

**Cell Culture and Transfection**—Human embryonic kidney (HEK) 293T cells were cultured in a controlled environment (37 °C, 5% CO₂) in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum. Transient transfection of plasmids was performed using Effectene transfection reagent (Qiagen) according to the manufacturer’s instructions.

**GST Pulldown Assay**—GST pulldown assays were carried out to confirm protein-protein interactions observed by yeast two-hybrid screening. Recombinant GST and GST-PC-TP fusion proteins were expressed using pGEX-KG as described previously (15) with minor modifications. Briefly, PC-TP coding sequences from human (hPC-TP) or mouse (mPC-TP) were cloned in frame to create fusion proteins with GST attached to the N termini. The pGEX-KG vector alone was used to express recombinant GST. Protein expression in Escherichia coli BL21(DE3) was induced by overnight culture at room temperature in LB supplemented with 1 mm isopropyl β-D-thiogalactopyranoside. For GST pulldown assays, bacteria (100 ml) were harvested by centrifugation and sonicated in 10 ml of lysis buffer consisting of 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. The supernatant containing recombinant proteins was collected following centrifugation at 14,000 × g at 4 °C for 15 min. Recombinant proteins were immobilized by the addition of 100 μl of glutathione-Sepharose 4B beads (Amersham Biosciences) for 2 h at 4 °C. The beads were collected by centrifugation at 1,500 × g for 1 min, and the beads were washed five times with PBS.

Both endogenously and heterologously expressed proteins in HEK 293T cells were pulled down. For heterologous protein expression, a cDNA encoding mThem2 (GenBank™ accession number NM 025790) was cloned into pEFF-N to create pEFF-mThem2, which encodes Them2 with an N-terminal FLAG tag driven by an EF1a promoter. The plasmid pC3NA-mThem2-HA (16) was a gift from Drs. Deborah Lang and Jonathan Epstein (University of Pennsylvania, Philadelphia, PA). Twenty-four hours after plasmid transfections, HEK 293T cells were lysed by the addition of 2 ml of lysis buffer/10-cm culture dish. Following 5 min of centrifugation at 14,000 × g to remove cellular debris, the lysates were incubated with GST or GST-PC-TP fusion proteins immobilized on glutathione-Sepharose 4B beads (100 μl) for 3 h at 4–37 °C. The beads were washed five times with lysis buffer, and then bound proteins were separated by SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride membranes, and subjected to immunoblotting. A monoclonal anti-FLAG mouse antibody was from Sigma, and a polyclonal anti-HA rabbit antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). A peptide designed to match the 14-amino acid sequence of the C terminus of human Them2 plus one cysteine residue added to the N terminus was synthesized and covalently attached to keyhole limpet hemocyanin. This protein-linked peptide was used as an antigen to produce rabbit anti-human Them2 polyclonal antiserum (Covance Research Products, Denver, PA). Detection of primary antibodies was with ECL (Amersham Biosciences) using goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Bio-Rad). Loading of GST or GST-PC-TP fusion proteins was assessed by Coo-massie Brilliant Blue staining.

**Quantitative Real Time PCR**—cDNA was synthesized from 1 μg of total RNA with GeneAmp Gold RNA PCR Core Kit (Applied Biosystems). Specific PCR primers were designed for hThem2 (forward, 5'-CAAGTCCTCGCTATGC-3'; reverse, 5'-ATTTTCTGTGCGCCTTGTT-3') using Primer 3 (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with nucleotide sequences obtained from GenBank™. Real time PCR was performed with Lightcycler 1.5 system using Lightcycler FastStart DNA Master plus SYBR Green 1 (Roche Applied Science). The expression data were normalized to the endogenous control, cyclophilin (17). The relative expression levels were calculated according to the formula 2⁻ΔC<sub>i</sub>, where ΔC<sub>i</sub> is
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the difference in threshold cycle (C\text{t}) values between Them2 and cyclophilin.

Tissue Distribution of Them2—To examine tissue-specific expression, a mouse multiple tissue Northern blot (Clontech) was hybridized with \(^{32}\)P-labeled full-length mThem2 and mPC-TP cDNAs. cDNAs were labeled with [\(\alpha\)-\(^{32}\)P]dCTP using a random primer DNA labeling kit (Invitrogen). Following autoradiography for each cDNA probe, the blot was stripped. A \(^{32}\)P-labeled β-actin cDNA was used as a loading control. The relative distribution of Them2 in cell types of the liver was assessed by quantitative real time PCR using cDNA prepared from HepG2 cells, LX1 stellate cells (gift from Dr. Scott Friedman, Mount Sinai School of Medicine, New York, NY), human peripheral blood monocytes (gift from Dr. Bruce Levy, Harvard Medical School), and human umbilical vein endothelial cells (HUVEC; gift from Dr. Jorge Plutzky, Harvard Medical School).

Distribution of PC-TP in Mouse Embryo—In situ hybridization was performed (18) on frozen sections of day 9.5 and 10.5 embryos using a digoxigenin-labeled antisense RNA probe that contained 373 bases of probe Pctp sequence. The controls were performed using an identical concentration of a sense probe.

Intracellular Localization of Proteins—Endogenously expressed Them2 and PC-TP were visualized in HEK 293T cells by immunofluorescence using confocal microscopy. The Them2 antibody was described above, and a rabbit anti-human PC-TP antibody was previously described (19). HEK 293T cells were rinsed three times with PBS and fixed for 20 min in 4% paraformaldehyde. The cells were then permeabilized and exposed to immune or preimmune antiserum at a 1:250 dilution. For visualization, the cells were incubated with a 1:500 dilution of Alexa Flour 495 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and mounted under coverslips in ProLong Gold mounting medium (Molecular Probes) containing 1:1,000 dilution of the nuclear stain TO-PRO-3 (Molecular Probes). The cells were imaged using a PerkinElmer Life Sciences confocal system that was driven by Intelligent Imaging Innovations software and included a Nikon TE2000 inverted microscope interfaced to a Yokogawa spinning disk confocal head paired with a Melles Griot Kr/Ar laser illumination source and a Hamamatsu Orca ERG cooled CCD camera for digitization. An Apple PowerMac G4 work station was used for image capture and processing with Slidebook software.

For visualization of fluorescent fusion proteins, the plasmids pAcGF-N1 (Clontech) and pflag195 (generous gift from Dr. Jagesh V. Shah, Harvard Medical School, Boston, MA), respectively, were used to prepare GFP-PC-TP (pAcGF-N1-mPC-TP) or monomeric red fluorescent protein (RFP)-Them2 (pflag195-mThem2) or RFP-Pax3 (pflag195-mPax3) by cloning the open reading frames of mPC-TP, mThem2, and mPax3, respectively. Them2 and Pax3 were cloned 3′ to the coding sequence of RFP to fuse the RFP protein to the N terminus of the proteins. A GFP-PC-TP fusion protein was created by cloning full-length mPC-TP 5′ to the coding sequence of GFP, so that the fluorescent protein was fused to the C terminus. HEK 293T cells were plated on glass coverslips at a density of 5 × 10\(^5\) cells/well in six-well plates. Twenty-four hours following transfection with either recombinant or empty plasmids, the cells were fixed. HEK 293T cells were rinsed three times with PBS, fixed for 20 min in 4% paraformaldehyde, and mounted under coverslips in VECTASHIELD mounting medium (Vector Laboratories, Inc., Burlingame, CA) containing TO-PRO-3. The cells were visualized under a Nikon E800 a fixed stage upright microscope controlled by Bio-Rad MRC 1024 confocal system. The images were acquired and processed using Lasersharp 2000.

Expression and Purification of Recombinant PC-TP and Them2—For bacterial expression of His tag recombinant proteins, the open reading frames of mouse Them2 and PC-TP were each cloned into pET19b (Novagen). Plasmids were transformed into E. coli BL21(DE3), and protein expression was induced by addition of 1 mM isopropyl β-d-thiogalactopyranoside to LB followed by 24 h of shaking (250 rpm) at room temperature. Bacteria were pelleted by centrifugation and lysed by sonication in PBS supplemented with EDTA-free protease inhibitor and centrifuged (100,000 \(g\) for 30 min at 4 °C). His tag proteins contained in the bacterial supernatants were adsorbed to a nickel affinity column composed of 1.0 ml of immobilized iminodiacetic acid resin (Pierce), which was packed into a C10/10 column (GE Healthcare, Piscataway, NJ) and chelated with 100 mM NiSO\(_4\). The proteins were eluted using a stepped imidazole gradient (6 mM \(\times\) 5 ml and 300 mM \(\times\) 5 ml). For further purification, fractions containing PC-TP or Them2 were pooled and applied to a XK16/100 column packed with Sepharose G-100SF (GE Healthcare) and equilibrated with buffer (20 mM NaH\(_2\)PO\(_4\), 0.5 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 0.2% Na\(_2\)EDTA). Following purification, His tag recombinant proteins yielded single bands as assessed by SDS-PAGE followed by Coomassie Brilliant Blue staining. Protein concentrations were determined according to their molar extinction coefficients at 280 nm, which were calculated based on amino acid sequences (www.expasy.org).

Influence of PC-TP on Enzymatic Activity of Them2—Acyl-CoA thioesterase activity of Them2 was measured as described (20) with modifications. Activity was determined using 5,5′-dithiobis(nitrobenzoic acid) (DTNB) to detect CoA release spectrophotometrically at 412 nm. The reaction buffer consisted of 50 mM KCl, 10 mM HEPEs, pH 7.4, 0.3 mM DTNB plus 100 μM myristoyl-CoA (Avanti Polar Lipids, Alabaster, AL) as substrate. Purified recombinant His tag mThem2 and mPC-TP were added at varying concentrations in a final volume of 200 μl. The reaction was monitored at 37 °C in a 96-well plate using a Molecular Devices Versamax plate reader (Molecular Devices, Sunnyvale, CA).

Influence of Them2 on Phosphatidylcholine Transfer Activity of PC-TP—Phosphatidylcholine transfer activity of PC-TP was measured by modification of a fluorescence assay (21). Donor phospholipids vesicles prepared by sonication of 0.5 μM phospholipids in 150 mM NaCl, 10 mM HEPEs, pH 7.4, contained egg phosphatidylcholine (Avanti Polar Lipids), 2-(12-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminocarbonyl]-sn-glycero-3-phosphocholine (NBD-PC) (Invitrogen), and Lissamine rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (rhodamine DHPE) (Invitrogen) (molar ratio egg phosphatidylcholine: NBD-PC:rhodamine DHPE of 94:1:5). Fluorescent phosphatidylincholines in these donor vesicles were quenched. Acceptor
vesicles (0.5 μM phospholipids in 150 mM NaCl, 10 mM HEPES, pH 7.4) were prepared by sonication of egg phosphatidylincholine and egg phosphatidic acid (Avanti Polar Lipids) in a ratio of 95:5. In 96-well plates, 30 μl of acceptor vesicles were added to 50 μl of buffer (0.9% NaCl, 10 mM HEPES, pH 7.4) in each well. Purified His tag mPC-TP and mThem2 were added to wells for varying final concentrations. Buffer was added to each well for a total volume of 135 μl. At time 0, 15 μl of donor vesicles were added to each well simultaneously using a multi-channel pipettor. The plates were immediately loaded into a Spectramax M5 fluorimeter (Molecular Devices). The excitation wavelength was 475 nm, and the emission wavelength was 535 nm. The plates were shaken for 2 s, and 10 readings of each well were averaged every 6 s for 10 min. Increases in fluorescence intensity reflected transfer of NBD-PC by PC-TP from quenched donor vesicles containing rhodamine DHPE to unquenched acceptor vesicles. Apparent rates of phosphatidylincholine transfer from donor and acceptor vesicles were determined by fitting fluorescence intensities (F(t)) to the function F(t) = Aexp(−kt) + B using Prism 4 (GraphPad Software, San Diego, CA), where k is the apparent first order rate constant, A is the amplitude, and B is a constant (22).

Influence of PC-TP on Transcriptional Activity of Pax3—The influence of PC-TP on transcriptional activity of Pax3 was examined using dual luciferase assay. The plasmids pCMV-mPax3 and pGL3-MITF, a promoter firefly luciferase reporter construct of MITF, a Pax3 transcriptional target gene, were provided by Dr. Jonathan Epstein (23, 24). The plasmid pcDNA3.1-hPC-TP was previously described (25). HEK 293T cells (2 × 10⁶) were seeded in 96-well plates. This was followed in 24 h by transfection with 73.5 ng of pGL3-MITF, 3.5 ng of pCMV-Pax3, 1.5 ng of pRL-TK (Renilla luciferase; Promega) as a control transfection for transfection efficiency, and variable amounts (0–35 ng) of pcDNA3.1-hPC-TP. Total amount of DNA was kept constant at 150 ng by the addition of empty pcDNA3.1 plasmid. After 72 h, firefly and Renilla luciferase activities were measured using a TRL17 Microplate Luminometer (Applied Biosystems, Bedford, MA) using a dual luciferase reporter assay system (Promega).

Statistical Analysis—The data are expressed as the means ± S.E. unless otherwise indicated. The statistical significances of the differences between the means of experimental groups was tested using Student’s t test. A difference was considered statistically significant for a two-tailed p < 0.05.

RESULTS

Identification of Them2 and Pax3 as PC-TP-interacting Proteins—To identify proteins binding to PC-TP, we screened cDNA libraries from mouse liver and from mouse day 9.5–10.5 embryos in yeast two-hybrid assays using PC-TP as the bait. The liver library screen utilized mPC-TP and yielded only 20 clones, which encoded 10 different PC-TP-interacting proteins. Two of the pACT2 GALAD clones encoded mThem2. The screen of the embryonic library was performed using hPC-TP and yielded 320 clones. A smaller subset of clones was sequenced, and this revealed three different nucleotide sequences, one of which encoded Pax3. Each of three different cDNAs was also used to prepare ³²P-labeled probes, which were separately hybridized to colony lifts of the original 320 clones. This revealed that the three different clones were represented in roughly equal proportions and accounted for 96% of all clones isolated in the screen. Them2 was not identified in the yeast two-hybrid screen of the embryonic library, and Pax3 was not identified in the screen of the liver library.

In Vitro Verification of PC-TP-Protein Interactions—We utilized GST pulldown assays to verify the interactions detected by the yeast two-hybrid screen. Fig. 1A demonstrates in transfected HEK 293T cells that FLAG tag Them2 interacted with a GST-mPC-TP fusion protein but not with GST alone, even when excess GST was utilized in the pulldown (Coomasie Brilliant Blue (CBB) staining were performed to assess the amounts of GST or GST-mPC-TP that were used in corresponding pulldown experiments (lower panel). B, GST-mPC-TP pulldown of endogenous Them2 in HEK 293T cells at 37°C, interaction between PC-TP and Pax3. GST pulldown assay was performed at 25°C using GST (negative control) or GST-hPC-TP bound to glutathione-Sepharose 4B beads when HA tag mPax3 was expressed in HEK 293T cells. Each panel represents one of three experiments that yielded the same result.
cipated with GST-hPC-TP conjugated to beads but not with an excess of GST-conjugated beads alone, which is demonstrated by the Coomassie Brilliant Blue staining panel. This was the case at both 4 and at 25 °C. For Them2 and Pax3, the same pulldown results were obtained whether we used GST-hPC-TP or GST-mPC-TP, indicating that PC-TP protein interactions were not species-dependent (data not shown). This is in keeping with the high degree of conservation of these three proteins, with percentages of identity/similarity for human and mouse PC-TP, Them2 and Pax3 of 81/86, 83/92, and 98/99%, respectively.

**FIGURE 2. Tissue and cellular distribution of mouse Them2.** A, Northern blot analysis of mouse poly(A)⁺ RNA (2 μg/lane) probed with 32P-labeled mouse Them2 (top panel) and 32P-labeled mouse PC-TP (middle panel). Loading of mRNA was assessed by hybridizing with 32P-labeled β-actin cDNA (bottom panel). The more intense, lower molecular weight actin band in the heart and muscle is attributed to high level expression of muscle actin in addition to cytoskeletal actin of these tissues. B, the cellular distribution of Them2 mRNA in liver was estimated by real time PCR using cell types representative of hepatocytes (HepG2) and nonparenchymal cells (LX1 stellate cells, monocytes, and HUVEC endothelial cells). The error bars represent S.E. and are too small to visualize for LX1 and HUVEC cells.

**FIGURE 3. Immunolocalization of Them2 and PC-TP in HEK293T cells.** HEK293T cells were fixed, immunostained for Them2 (A and B) and PC-TP (C and D) and visualized by confocal fluorescence microscopy. Locations of the nuclei are demonstrated using the nuclear stain TO-PRO-3 for cells immunostained for Them2 (B) and PC-TP (D). These images are representative of two independent experiments.

multiple tissue Northern blot revealed substantial expression of Them2 mRNA in heart, brain, liver, kidney, and skeletal muscle, in general agreement with recent observations by reverse transcription-PCR in human tissues (26). As confirmed here, PC-TP is expressed in mouse liver as well as other tissues (27). To explore whether enrichment of Them2 in liver was due principally to its expression in hepatocytes, we carried out quantitative real time PCR using pertinent available individual cell types (Fig. 2B). Them2 expression was abundant in HepG2 hepatoma cells, with much lower expression in LX1 stellate cells, human peripheral blood monocytes, and HUVEC endothelial cells.

The spatial distribution of Pax3 expression in the embryo is localized to the neural tube and somites (28). To determine whether PC-TP was coexpressed in these regions, we performed in situ hybridization, which revealed that PC-TP mRNA was diffusely and uniformly expressed within cells of day 9.5 and 10.5 embryos (not shown).

**Intracellular Distributions of PC-TP, Them2, and Pax3**—We next sought to determine whether the intracellular distributions of the proteins were suitable for interactions. Fig. 3 shows the intracellular distributions of endogenous Them2 and PC-TP. By immunofluorescence confocal microscopy, both Them2 (Fig. 3, A and B) and PC-TP (Fig. 3, C and D) were located both outside and within the nucleus. Not shown are the corresponding images using preimmune serum at the same titer, which revealed the absence of immunofluorescence staining for Them2 and only faint nonspecific staining for PC-TP. Because immunofluorescence of each endogenous protein utilized the same secondary anti-rabbit antibody, we utilized RFP and GFP fusion proteins to visually assess colocalization. Whereas RFP alone was equally distributed throughout the cytoplasm and nucleus (not shown), RFP-Them2 entered the nucleus to a lesser extent (Fig. 4, A–C). GFP-PC-TP was distributed throughout the cytoplasm and nucleus (Fig. 4D), so that in cotransfected cells, GFP-PC-TP and RFP-Them2 (Fig. 4E) appeared to colocalize in the cytoplasm (Fig. 4F). By contrast,
RFP-Pax3 was restricted to the nucleus (Fig. 5, A–C). In cells cotransfected with GFP-PC-TP and RFP-Pax3 (Fig. 5, D–F), the yellow color in the merged panels was consistent with colocalization in the nucleus. Because endogenous Pax3 is not expressed in HEK 293T cells, its nuclear localization could not be independently confirmed by immunofluorescence. However, in separate experiments, Pax3-HA transfected into HEK 293T cells was shown by immunofluorescence to localize within the nucleus (not shown).

**Stimulation of Them2 Activity by PC-TP**—We next sought to determine whether interactions between PC-TP and Them2 might influence the acyl-CoA thioesterase activity of Them2. Fig. 6A demonstrates that purified recombinant His tag Them2 exhibits thioesterase activity when myristoyl-CoA is utilized as the substrate. By contrast, there was no activity of His tag PC-TP in this assay. However, when increasing concentrations of PC-TP were added to Them2 in the assay, there was a progressive increase in activity of Them2. Fig. 6B shows the time-dependent differences in thioesterase activity attributable
to the addition of PC-TP, which were calculated by subtracting the $A_{412}$ values obtained for Them2 alone from values for PC-TP plus Them2. These curves were then integrated to quantify the PC-TP-induced increases in activity as AUC. As shown in Fig. 6C, AUC values increased sharply as functions of PC-TP:Them2 molar ratio and then leveled off in the range of 1:2, as denoted by the arrow.

Influence of Them2 on Phosphatidylcholine Transfer Activity of PC-TP—Fig. 7 demonstrates the influence of Them2 on the phosphatidylcholine transfer activity of PC-TP. In the absence of Them2, PC-TP facilitates the rapid transfer of NBD-PC from donor to acceptor vesicles. Whereas Them2 alone did not promote NBD-PC transfer (not shown), its addition to PC-TP resulted in modest increases in activity over a range of Them2:PC-TP molar ratios up to 8 (Fig. 7A). Analysis of these time-dependent increases in activity indicates that the maximum fluorescence was increased, but there were no changes in the rates of transfer (0.00398 ± 0.00005 s$^{-1}$). As shown in Fig. 7B, the increase in maximum fluorescence attributable to added Them2 leveled off at a Them2:PC-TP molar ratio of ~2:1.

**PC-TP Coactivates Pax3 Transcriptional Activity**—By binding to its response element on the promoter of MITF, a transcription factor that is essential for melanocyte development and differentiation, Pax3 transactivates MITF expression (16). Because PC-TP does not contain a DNA-binding domain, we postulated that PC-TP binding to Pax3 might regulate the transcriptional activity of Pax3. Consistent with the weak transcriptional activity of Pax3 alone for the MITF promoter (16), the plasmid DNA concentration utilized in this experiment did not yield significant Pax3-mediated activation of MITF when compared with the PC-TP control (Fig. 8). By contrast, cotransfection of Pax3 with increasing amounts of pcDNA3.1-hPC-TP increased activity up to a 1:1 ratio of PC-TP to Pax3 but was not further increased by a 10-fold excess of PC-TP.

**DISCUSSION**

As illustrated in Fig. 9, this study demonstrates that PC-TP interacts with Them2 and Pax3. These two proteins comprise domains similar to those observed in multidomain START proteins. In mammals, Them1 (also known as brown fat-inducible thioesterase, thioesterase adipose-associated, and StarD14) and cytosolic acetyl-CoA hydrolase (CACH; also known as StarD15) each contain a START domain plus...
In plants, START domains are predominantly associated with homeodomain transcription factors (8).

Them2 is a newly described 140-amino acid hot dog fold thioesterase (26, 29), with a tissue distribution (Fig. 2A and Ref. 26) that overlaps with PC-TP (9, 27). Recently, a GFP-hThem2 construct was localized to microtubules in a U2OS osteosarcoma cell line (26). In our studies, both endogenous and RFP-labeled Them2 displayed a more diffuse pattern in HEK 293T cells. Although a proteomic analysis of mitochondria has suggested that Them2 is mitochondrial-associated (30), we did not observe colocalization of RFP-mThem2 with a mitochondria-specific fluorescent dye (Mitotracker, Invitrogen) (data not shown).

The interaction of PC-TP with Them2 is intriguing in part because of the characteristics of Them1 and CACH, which are illustrated in Fig. 9. Each of these comprise two ~140-amino acid thioesterase domains plus a C-terminal START domain. Them1 is highly induced in brown adipose tissue by cold exposure and apparently functions as an acyl-CoA thioesterase with substrate specificity for medium (C12-CoA) to long chain (C16-CoA) fatty acyl-CoAs (31). CACH is an acetyl-CoA hydrolase that is enriched in liver (32).

When confirming PC-TP-Them2 interactions by GST-pull-down assays, we observed that GST-PC-TP did not precipitate Them2 at 4 °C. However, an interaction was observed at 25 °C and became increasingly robust as the temperature was raised to 37 °C. In support of a critical role for temperature, CACH is most active as a multimer at 37 °C, becomes less active as a dimer at 25 °C and loses activity completely at 4 °C because of dissociation of dimers to inactive monomers (33–35).

These observations suggested that PC-TP might modulate acyl-CoA thioesterase activity of Them2 in liver. Indeed, we observed that the addition of PC-TP to Them2 increased the acyl-CoA thioesterase activity of Them2. It is noteworthy that the molar ratio of 0.5 (i.e. PC-TP:Them2 1:2) falls within the region of the more gradual increase (arrow in Fig. 6C). This ratio corresponds to the 1 START and 2 thioesterase domains that are contained within both StarD14 and StarD15 (Fig. 9).

Although a mechanism by which PC-TP increases Them2 activity is not yet known, the recently reported crystal structures of human (29) and mouse (www.pdb.org/pdb/cgi/explore.cgi?dbid=2CY9) Them2 homologs provide some suggestions. Them2 multimerizes so that the active site of the enzyme is apparently formed by the interface of asymmetric dimers. Moreover, the gradual increase of Them2 activity above a PC-TP:Them2 molar ratio of 1:2 suggests the possibility that higher order assemblies further increase enzymatic activity, such as is observed for CACH as a function of increasing temperature. Interestingly, Them2 also increased the maximum amount of fluorescent phospholipid transferred by PC-TP from donor to acceptor vesicles. This maximum value is determined principally by the proportion of fluorescent phosphatidylcholine molecules that reside in the outer monolayer of donor vesicles. These are the molecules that can be accessed by PC-TP in solution and transferred to acceptor vesicles (1). A higher value for maximum fluorescence suggests increased access of PC-TP to fluorescent molecules, which presumably reside in the inner monolayer of donor vesicles. These data suggest that interactions between PC-TP and Them2 create a protein complex that may facilitate transbilayer phosphatidylcholine movement, with optimal activity at a molar ratio of 2 Them2 molecules to 1 PC-TP (arrow in Fig. 7B).

Although a recent survey of potential substrates indicated that recombinant hThem2 preferentially hydrolyzes CoA thioesters that are functionalized with polar aromatic substituents (29), this may not be representative of function in vivo. Importantly, these experiments were carried out at 25 °C and in the absence of PC-TP. Based on our current data and its possible association with mitochondria (30), Them2 appears more likely to function in fatty acid metabolism as an acyl-CoA thioesterase.

A potential limitation of these studies is the use of purified recombinant proteins to examine the functional consequences of PC-TP-Them2 interactions. We chose this approach because other proteins with the same in vitro activities are broadly present in tissues. Acyl-CoA thioesterase activity is ubiquitously expressed, shows localization in most cellular compartments, and is exhibited by a family of proteins (36). In addition to PC-TP, phosphatidylcholine transfer activity is mediated by sterol carrier protein 2, StarD10, and phosphatidylinositol transfer protein (37, 38). The identification of specific cellular consequences related to the activities of PC-TP and/or Them2 should enable the use of knockdown approaches to dissect their interactions in vivo.

An interaction between PC-TP and the transcription factor Pax3 is of interest because START domain proteins in plants commonly contain homeodomains, suggesting that they function as lipid-responsive transcription factors (8). Pax3 plays a key role in neural and cardiac development (39). Observations...
here and by de Brouwer et al. (40) have suggested that fluorescent PC-TP fusion proteins are present within the nucleus, which is supported by immunolocalization of the endogenous protein in HEK 293T cells. Considering that PC-TP has no apparent DNA-binding domain, we tested the possibility that it might coactivate or corepress Pax3 transcriptional activity. Indeed, a modest but reproducible increase in Pax3-mediated transcription of MITF was consistent with coactivation. An important limitation of this experiment is that it did not include other coactivators that up-regulate activity of Pax3, including Sox10 (16). Unlike mutations in Pax3 (39), it did not include other coactivators that up-regulate activity of Pax3-mediated transcription of MITF was consistent with

cional activity. Indeed, a modest but reproducible increase in PC-TP has no apparent DNA-binding domain, we tested the

nucleus, which is supported by immunolocalization of the

PC-TP/StarD2-interacting Proteins

interacting proteins.

domain minimal proteins provides the flexibility for a single position. These findings suggest that the evolution of START domain proteins in plants may function as other domains of a multidomain protein. Considering that plants synthesize a variety of sterol molecules, it has been speculated that START domain proteins in plants may function as transcription factors that respond to the cellular sterol levels. PC-TP binds phosphatidylcholines exclusively. However, the protein does exhibit differential binding of phosphatidylcholines depending upon acyl chain composition (1). If the acyl chain composition of a bound phosphatidylcholine were to modulate the interaction between PC-TP and its binding partner, this would provide a flexible mechanism for sensing and responding to changes in membrane phosphatidylcholine composition. These findings suggest that the evolution of START domain minimal proteins provides the flexibility for a single lipid-binding domain to mediate more than one biological activity, depending upon the cellular expression of specific interacting proteins.

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