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Regulation of Wnt/β-Catenin Pathway by cPLA$_2$α and PPARδ

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ABSTRACT

Cytosolic phospholipase A$_2$α (cPLA$_2$α) is a rate-limiting key enzyme that releases arachidonic acid (AA) from membrane phospholipid for the production of biologically active lipid mediators including prostaglandins, leukotrienes and platelet-activating factor. cPLA$_2$α is translocated to nuclear envelope in response to intracellular calcium increase and the enzyme is also present inside the cell nucleus; however, the biological function of cPLA$_2$α in the nucleus remains unknown. Here we show a novel role of cPLA$_2$α for activation of peroxisome proliferator-activated receptor-δ (PPARδ) and β-catenin in the nuclei. Overexpression of cPLA$_2$α in human cholangiocarcinoma cells induced the binding of PPARδ to β-catenin and increased their association with the TCF/LEF response element. These effects are inhibited by the cPLA$_2$α siRNA and inhibitors as well as by siRNA knockdown of PPARδ. Overexpression of PPARδ or treatment with the selective PPARδ ligand, GW501516, also increased β-catenin binding to TCF/LEF response element and increased its reporter activity. Addition of AA and GW501516 to nuclear extracts induced a comparable degree of β-catenin binding to TCF/LEF response element. Furthermore, cPLA$_2$α protein is present in the PPARδ and β-catenin binding complex. Thus the close proximity between cPLA$_2$α and PPARδ provides a unique advantage for their efficient functional coupling in the nucleus, where AA produced by cPLA$_2$α becomes immediately available for PPARδ binding and subsequent β-catenin activation. These results depict a novel interaction linking cPLA$_2$α, PPARδ and Wnt/β-catenin signaling pathways and provide insight for further understanding the roles of these key molecules in human cells and diseases. J. Cell. Biochem. 105: 534–545, 2008.

KEY WORDS: cPLA$_2$; PPAR-δ; ARACHIDONIC ACID; β-CATENIN; CHOLANGIOCARCINOMA

Mammalian cells contain a large number of phospholipases that hydrolyze phospholipids in a structurally specific manner for production of a variety of biologically active products. Phospholipase A$_2$s (PLA$_2$s, EC 3.1.1.4, phosphatide sn-2 acylhydrolases) are distinct families of enzymes that catalyze hydrolysis of the *sn*-2 ester bond of membrane glycerophospholipids, leading to the production of two classes of lipid mediators: fatty acid metabolites and lysophospholipid-related lipids [Capper and Marshall, 2001; Fitzpatrick and Soberman, 2001; Funk, 2001; Murakami and Kudo, 2002; Ghosh et al., 2006; Schaloske and Dennis, 2006]. Among the many types of mammalian PLA$_2$s, cytosolic PLA$_2$α (cPLA$_2$α) is the rate-limiting key enzyme for hormone, growth factor, and mitogen-induced eicosanoid synthesis, since the cPLA$_2$α selectively cleaves AA from substrate phospholipids and its enzyme activity is tightly controlled by several intracellular signaling events, including physiologically relevant concentrations of Ca^{++}, enzyme phosphorylation, S-nitrosylation, G-proteins and induction of gene expression [Balsinde et al., 1999; Capper and Marshall, 2001; Fitzpatrick and Soberman, 2001; Funk, 2001; Murakami and Kudo, 2002; Bonventre, 2004; Leslie, 2004; Ghosh et al., 2006; Kita et al., 2006; Schaloske and Dennis, 2006; Xu et al., 2008]. The free AA cleaved by cPLA$_2$α is subsequently converted to prostaglandins (PGs) and leukotrienes (LTs), whereas the lysophospholipid is converted to platelet-activating factor (PAF), lysophosphatidic acid (LPA), and sphingosine-1-phosphate (S1P) [Prescott et al., 2000; Fitzpatrick and Soberman, 2001; Funk, 2001; Tsuboi et al., 2002; Ghosh et al., 2006; Schaloske and Dennis, 2006]. These lipid products function as local hormones through binding to their cellular receptors in autocrine or paracrine fashions or serve as intracellular second messengers to mediate a myriad of physiological and pathophysiological functions such as inflammation, cell proliferation, and carcinogenesis. The essential role of

Abbreviations used: AACOCF$_3$, arachidonyltrifluoromethyl ketone; COX-2, cyclooxygenase-2; cPLA$_2$α, cytosolic phospholipase A$_2$α; GSK3β, glycogen synthase kinase 3β; LEF, lymphoid enhancer factor; PARP, poly(ADP)-ribose polymerase; PGE$_2$, prostaglandin E$_2$; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; siRNA, small interfering RNA; TCF, T cell factor.

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cPLA$_2\alpha$ in AA metabolism and in the pathogenesis of inflammatory diseases is highlighted by experiments utilizing cPLA$_2\alpha$ knock-out mice, in which the cells generated less AA-derived metabolites and PAF [Bonventre et al., 1997; Uozumi et al., 1997] and the animals showed significantly less inflammatory damage during disease processes [Bonventre et al., 1997; Uozumi et al., 1997; Fujishima et al., 1999; Nagase et al., 2000, 2002, 2003; Nakatani et al., 2000; Hegen et al., 2003; Tabuchi et al., 2003; Bonventre, 2004; Marusic et al., 2005].

An intriguing phenomenon in the regulation of cPLA$_2\alpha$ is its translocation from the cytosol to membranes to access substrate [Leslie, 1997; Ghosh et al., 2006]. The translocation is observed in the setting of increased intracellular calcium, which binds to the C2 domain of cPLA$_2\alpha$ and increases its affinity for membranes [Clark et al., 1991; Leslie, 1997; Ghosh et al., 2006]. In many cells exposed to calcium-mobilizing agonists, cPLA$_2\alpha$ has been shown to translocate from cytoplasm to nuclear envelope, endoplasmic reticulum (ER) and Golgi [Schievella et al., 1995; Peters-Golden et al., 1996; Leslie, 1997; Hirabayashi et al., 1999; Evans et al., 2001; Ghosh et al., 2006]. It is of note that the localization of cPLA$_2\alpha$ in cellular compartment is influenced by the status of cell confluence, that is, nonconfluent endothelial cells display homogeneous cPLA$_2\alpha$ staining throughout the cytoplasm and nucleus [Sierra-Honigmann et al., 1996; Grewal et al., 2002; Herbert et al., 2005], whereas at confluence cPLA$_2\alpha$ is redistributed to the juxtanuclear region [Herbert et al., 2005]. Additionally, cPLA$_2\alpha$ has also been shown to localize inside the nucleus [Sierra-Honigmann et al., 1996], although it remains debatable whether the cPLA$_2\alpha$ actually enters into the nucleus. The biological role of cPLA$_2\alpha$ nuclear association (or in the nuclei) remains to be further defined.

Recent studies from our laboratory show that the cPLA$_2\alpha$-controlled arachidonic acid metabolism in cell nucleus can activate the PPARδ, which belongs to one of three subtypes of the PPAR nuclear receptor family [Xu et al., 2006a,b]. PPARs belong to the superfamily of nuclear receptors that function as ligand-activated transcription factors, which regulate gene expression by binding with their heterodimeric partner retinoid X receptor to specific peroxisome proliferator response elements (PPREs) [Vamecq and Latruffe, 1999; Kliewer et al., 2001; Reddy and Hashimoto, 2001; Willson et al., 2001; Desvergne et al., 2004; Knouff and Auwerx, 2004; Michalik et al., 2004; Chinetti-Gbaguidi et al., 2005; Michalik et al., 2006]. The transcription activity of PPARs is controlled by specific ligands (including AA derivatives) and co-activator or co-repressor proteins [Forman et al., 1995; Kliewer et al., 1995; Devchand et al., 1996]. In addition to this canonical mechanism, PPARs can also function independently, in the absence of a hetero-partner [Tan et al., 2005]. There are also evidences that PPARs can regulate cell functions through PPRE-independent mechanisms such as interaction with other intracellular signaling molecules including AP-1, NF-κB, and STAT proteins [Chinetti et al., 1998; Ricote et al., 1998; Staels et al., 1998]. Therefore, PPARs may regulate diverse cellular functions through both PPRE-dependent and independent mechanisms in various cell types. We have shown that cPLA$_2\alpha$ overexpression or activation significantly increases PPARδ transcription activity and enhances the binding of PPARδ to its DNA response element in human liver cancer cells [Xu et al., 2006a,b]. In particular, our data reveal that AA directly binds to PPARδ in vitro and that addition of AA to isolated nuclear extracts or recombinant PPARδ protein enhances PPARδ DNA binding ability. These observations suggest that the effect of cPLA$_2\alpha$ on PPARδ activation may be mediated at least in part through increased AA in the nuclei. It is of note that the expression of PPARδ is regulated by β-catenin signal pathway [He et al., 1999]; however, it remains unknown whether the cPLA$_2\alpha$ and PPARδ signaling pathways interact with β-catenin at other levels.

β-catenin is a key mediator in Wnt regulation of multiple cellular functions in embryogenesis and tumorigenesis [Moon et al., 2004; Clevers, 2006; Gordon and Nusse, 2006; Hoppler and Kavanagh, 2007]. In adult tissues, β-catenin is a component of stable cell adherent complexes whereas its free form functions as a co-activator for a family of transcription factors termed T cell factor/lymphoid enhancer factor (TCF/LEF). Wnt proteins comprise a family of highly conserved secreted proteins that signal through the Frizzled receptors [Moon et al., 2004; Clevers, 2006; Gordon and Nusse, 2006; Hoppler and Kavanagh, 2007]. In the absence of a Wnt signal, β-catenin exists within a cytoplasmic complex (β-catenin destruction complex) along with glycogen synthase kinase 3β (GSK3β), adenomatous polyposis coli (APC), and axin, where it is phosphorylated and targeted for degradation by the proteasome. Activation of Wnt signaling perturbs this destruction complex, leading to cytoplasmic accumulation of β-catenin and allowing its translocation into the cell nucleus. In the nucleus, β-catenin associates with TCF/LEF that stimulate transcription of target genes important for proliferation, differentiation, and apoptosis [Moon et al., 2004; Clevers, 2006; Gordon and Nusse, 2006; Hoppler and Kavanagh, 2007].

Given that PPARδ and β-catenin are nuclear transcription factors or cofactors, we sought to further determine whether these two molecules might interact with each other in cell nucleus to modulate gene expression. In this study, we provide experimental evidence for a direct binding between PPARδ and β-catenin in human cholangiocarcinoma cells and show that this interaction is important for TCF/LEF transcription activity. Our data further reveal that the interaction between PPARδ and β-catenin and their transcription activity is regulated by cPLA$_2\alpha$.

MATERIALS AND METHODS

MATERIALS

Dulbecco's modified minimum essential medium (DMEM), minimum essential medium alpha (α-MEM), fetal bovine serum, glutamine, antibiotics, the Lipofectamine plus™ reagent and Lipofectamine™ 2000 reagent were purchased from Invitrogen (Carlsbad, CA). Arachidonic acid (AA), oleic Acid, prostaglandin E$_2$ (PGE$_2$), the cPLA$_2\alpha$ inhibitors arachidonyltrifluoromethyl ketone (AACOCF$_3$) and pyrolidine, the COX-2 inhibitor NS398, the COX inhibitor indomethacin, the p38 MAP kinase inhibitor SB203580 and the p42/44 MEK inhibitor PD98059 were purchased from Calbiochem (San Diego, CA). The PPARδ agonist GW501516 was purchased from Cayman Chemical (Ann Arbor, MI). The antibodies against human cPLA$_2\alpha$, c-myc, β-catenin, PPARδ and PARP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against

phospho-cPLA$_2\alpha$ (Ser^{505}) was purchased from Cell Signaling (Berverly, MA). The antibody against β-actin was purchased from Sigma (St. Louis, MO). Horseradish peroxidase-linked streptavidin and chemiluminescence detection reagents were purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). The TCF/LEF-Luc reporter vector was purchased from Panomics (Redwood City, CA). The siRNAs for cPLA$_2\alpha$, PPARδ or β-catenin were purchased from Dharmacon (Chicago, IL).

CELL CULTURE AND TRANSIENT TRANSFECTION

Two human cholangiocarcinoma cell lines were utilized in this study (CCLP1 and SG231). The cells were cultured according to our previously described methods [Wu et al., 2002; Han et al., 2004b]. The cells were cultured at 37°C in a humidified CO_2 incubator. For transient transfection assays, the cultured cells were transfected with the cPLA$_2\alpha$ expression plasmid (with MT-2 as control plasmid) or the PPARδ expression plasmid (with SG5 as control plasmid) using Lipofectamine plus™ reagent. The cells with optimal overexpression of either cPLA$_2\alpha$ or PPARδ were confirmed by immunoblotting and subsequently used for further experiments.

LUCIFERASE REPORTER ASSAY

The cultured cells were seeded at a concentration achieving 80% confluence in 12-well plates 18 h before transfection. The cells were transiently transfected with translucent TCF/LEF-Luc reporter vector using Lipofectamine plus™ reagent. After transfection, the cells were treated with specific reagent such as PPARδ agonist GW501516 in serum-free medium for 24 h. Then the cell lysates were obtained with 1× reporter lysis buffer (Promega). The luciferase activity was assayed in a Berthold AutoLumat LB 953 luminometer (Nashua, NH) by using the luciferase assay system from Promega. The relative luciferase activity was calculated after normalization of cellular proteins. All values are expressed as fold induction relative to basal activity.

PREPARATION OF CELLULAR PROTEIN

At the end of each indicated treatment, the cells were scraped off the plates and centrifuged, washed twice with cold phosphate-buffered saline (PBS) containing 0.5 mM PMSF and 10 μg/ml leupeptin and resuspended in fivefold volume of hypotonic buffer consisting of 50 mM HEPES pH 7.55, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail tablets (Roche Diagnostics GmbH). After sonication, the whole cell lysate was collected by centrifugation at the speed of 16,000*g* at 4°C for 10 min to remove cell debris and stored in aliquots at −20°C until use. The protein concentrations in the cell extracts were determined by the Bio-Rad protein assay (Bio-Rad, CA) using BSA as a standard. The cellular protein was verified by western blot with β-actin as a loading control.

PREPARATION OF NUCLEAR PROTEIN

The nuclear proteins from control or stimulated cells were extracted with the CelLytic™ NuCLEAR EXTRACTION KIT from Sigma (St. Louis, MI) according to the protocol provided by the manufacturer. Briefly, the cultured cells were washed and scraped into phosphate-buffered solution and centrifuged at 450*g* for 5 min; then washed twice by resuspending the cell pellets in PBS and centrifuged at 450*g* for 5 min. The final pelleted cells were suspended in 1× Lysis Buffer with 0.6% IGEPAL CA-630 (a nonionic, non-denaturing detergent) at approximately five times the packed cell volume and lysed by gentle pipetting. Nuclei were recovered by centrifugation at 8,000*g* for 20 min. The nuclear proteins were extracted by gentle resuspension of the nuclei at approximately two times the packed nuclear volume of Nuclear Extraction Buffer containing 0.6% IGEPAL CA-630 (a mild detergent to remove the outer membrane), followed by 30 min of platform rotation. The nuclear protein suspension was cleared by centrifugation at 16,000*g* for 5 min. The supernatants were collected and frozen at −80°C. All buffers contained DTT and protease inhibitor cocktail tablets (Roche Diagnostics GmbH). All the steps were carried out on ice or at 4°C. The protein concentrations in the nuclear extracts were measured by the Bio-Rad protein assay (Bio-Rad) using BSA as a standard. The nuclear fraction was verified by western blot with PARP as a loading control and no contamination of β-actin.

IMMUNOBLOTTING

Thirty micrograms of either cellular protein or nuclear protein was subjected to SDS–PAGE on 4–20% Tris-glycine gels (Invitrogen) for cPLA$_2\alpha$, c-myc, β-catenin., PPARδ, actin, and PARP. The separated proteins were electrophoretically transferred onto the nitrocellulose membranes (BioRad, CA). Nonspecific binding was blocked with PBS-T (0.5% Tween-20 in PBS) containing 5% non-fat milk for 1 h at room temperature. The membranes were then incubated overnight at 4°C with individual primary antibodies in PBS-T containing 1% non-fat milk at the dilutions specified by the manufacturers. Following three washes with PBS-T, the membranes were then incubated with the horseradish peroxidase-conjugated secondary antibodies at 1:10,000 dilution in PBS-T containing 1% non-fat milk for 1 h at room temperature. The membranes were then washed three times with PBS-T and the protein bands were visualized with the ECL Western blotting detection system.

RNA INTERFERENCE

cPLA$_2\alpha$ siRNA and PPARδ siRNA were purchased from Dharmacon. Cells with fifty percent confluence were transfected with either cPLA$_2\alpha$ siRNA or PPARδ siRNA or a 21-nucleotide irrelevant RNA duplex as a control using Lipofectamine™ 2000. Depletion of cPLA$_2\alpha$ or PPARδ was confirmed by immunoblotting.

BIOTINYLATED LIGONUCLEOTIDES PRECIPITATION ASSAYS

These experiments were performed as described previously with minor modification [Hata et al., 2000]. The sequences of biotinylated oligonucleotides corresponding to TCF/LEF binding site are forward: 5′-TGCTTCCCGAATTCCCGAATTCCCGAATTCCCGAATTCCCGAATTCCCGAACGT-3′; and reverse: 5′-ACGTTCGGGAAT TCGGGAATTCGGGAATTCGGGAATTCGGGAATTCGGGAAGCA-3′. The 5′-biotinylated oligonucleotides were synthesized by Sigma-Genosys (Woodland, TX). Nuclear extracts were prepared with the method as described above. Cell extracts were prepared by sonication in HKMG buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and 0.5% of NP-40) containing protease and phosphatase inhibitors. Each binding reaction in either nuclear extract or cell extract was carried out at 4°C for 16 h with 1 μg TCF/

LEF biotinylated double-strand oligonucleotides and 10 μg poly(dl-dC).poly(dl-dC). The DNA-bound proteins were precipitated using ImmunoPure streptavidin-agarose beads (Pierce, Rockford, IL) at 4°C for 1 h and subjected to detect β-catenin, PPARδ or cPLA$_2$α by Western blotting.

BINDING OF PPARδ TO β-CATENIN

The binding complexes of PPARδ and β-catenin in CCLP1 cells were determined by immunoprecipitation and western blot. CCLP1 cells with 80% confluence were transfected with the PPARδ expression plasmid or SG5 control plasmid for 24 h. The cell lysates were subsequently prepared for immunoprecipitation with antibody against either PPARδ or β-catenin. The immunoprecipitants were then subjected to SDS–PAGE and immunoblotted with either anti-β-catenin or anti-PPARδ antibodies.

BINDING OF cPLA$_2$α TO β-CATENIN THROUGH PPARδ

The protein complexes of cPLA$_2$α, PPARδ and β-catenin in CCLP1 cells were determined by immunoprecipitation and Western blot. The cells with 80% confluence were transfected with either cPLA$_2$α expression plasmid or MT-2 control plasmid for 24 h. The cell lysates were subsequently prepared for immunoprecipitation with antibodies against either cPLA$_2$α, PPARδ or β-catenin. The immunoprecipitants were then subjected to SDS–PAGE and immunoblotted with antibodies against either β-catenin, cPLA$_2$α, or PPARδ.

SITE-DIRECTED MUTAGENESIS

Human cPLA$_2$α expression vector cloned in pMT-2 was utilized for site-directed mutagenesis to replace the active site Ser-228 to alanine (SER 228 → ALA) as previously described [Sharp et al., 1994; Huang et al., 1996]. The oligonucleotides used for the mutagenesis is 5′-GCT GGT CTT **GCT** GGC TCC ACC-3′, which is synthesis by IDT (Coralville, IA). Site-directed mutagenesis was performed with QuickChange II Site-Directed Mutagenesis Kit from Stratagene (Lo Jolla, CA) and positive clones were identified by sequencing.

RESULTS

We first examined the effect of cPLA$_2$α on β-catenin-mediated transcription activity in a human cholangiocarcinoma cell line, CCLP1 [Xu et al., 2006b]. The cells were transfected with a cPLA$_2$α expression plasmid with cotransfection of a TCF/LEF luciferase reporter construct. As shown in Figure 1, overexpression of cPLA$_2$α significantly increased the TCF/LEF reporter activity ($P < 0.01$). This effect is dose-dependent, with approximately 6.5-fold increase of reporter activity in cells transfected with 0.5 μg cPLA$_2$α plasmid. Consistent with this, overexpression of cPLA$_2$α enhanced the binding of β-catenin to the TCF/LEF response element, as determined by the combined immunoprecipitation and immunoblotting assay (Fig. 2a). Since the protein levels of β-catenin and TCF/LEF were not altered by cPLA$_2$α overexpression, the effect is likely mediated through increased β-catenin binding affinity to TCF/LEF element. The involvement of cPLA$_2$α in β-catenin activation is further confirmed by the observations that inhibition

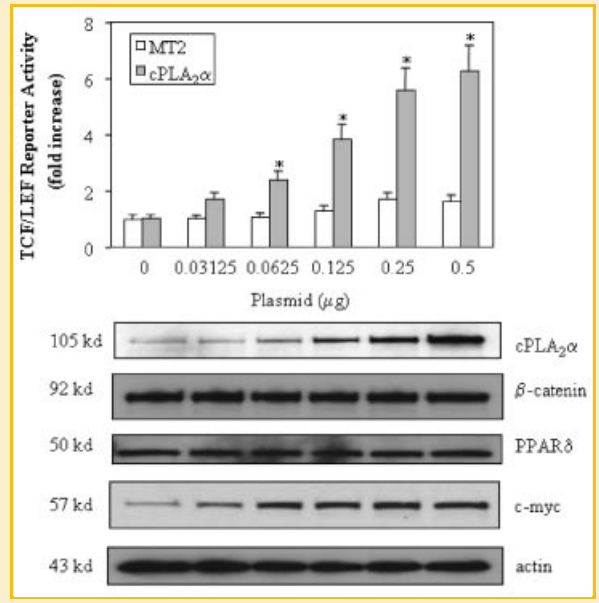

Fig. 1. Overexpression of cPLA$_2$α increases TCF/LEF reporter activity. The CCLP1 cells with 80% confluence were transiently transfected with different amounts of either cPLA$_2$α in MT-2 expression plasmid or MT-2 control plasmid with co-transfection of equal amount of pTCF/LEF-Luc reporter vector. After transfection, the cells were cultured in serum-free medium for 24 h; and then the cell lysates were obtained to determine the luciferase activity as described in the Materials and Methods Section. The data are presented as mean ± SD of three independent experiments. The cells with cPLA$_2$α overexpression confirmed by western blot showed significantly increased TCF/LEF luciferase reporter activity when compared with the cells transfected with the equal amount of control vector ($^*P < 0.01$) (upper panel). cPLA$_2$α overexpression had no effect on the protein levels of β-catenin or PPARδ; it increased the expression of c-myc, a β-catenin downstream gene (lower panel; whole cell lysates were used for Western blotting).

of cPLA$_2$α by specific siRNA or chemical inhibitors (pyrolidine and AACOCF3) significantly reduced the TCF/LEF reporter activity (Fig. 3A,B). Moreover, inactivation of cPLA$_2$α by site directed mutagenesis (Ser-228 → Alanine) abolished TCF/LEF transcription activity (Fig. 3C), suggesting that cPLA$_2$α activity is likely required for β-catenin activation. In contrast, the cyclooxygenase inhibitor, indomethacin, or the COX-2 inhibitor, NS-398, exhibited no significant effect on TCF/LEF reporter activity under the same experimental conditions (Fig. 3B). These results suggest that cPLA$_2$α initiated signaling cascade facilitates β-catenin association to TCF/LEF element and enhances transcription activity.

The cPLA$_2$α is a rate-limiting key enzyme for the release of arachidonic acid from membrane phospholipids and thus plays a central role in the production of bioactive eicosanoids (including prostaglandins and leukotrienes) as well as platelet-activating factor. In addition to this classical function, our recent studies reveal that the cPLA$_2$α-controlled arachidonic acid metabolism in cell nucleus can activate PPARδ and influence its transcription activity [Xu et al., 2006a,b]. We have shown that overexpression of cPLA$_2$α or activation of cPLA$_2$α by the calcium ionophore, A23187, significantly increases PPARδ transcription activity and enhances the binding of PPARδ to its DNA response element, which was

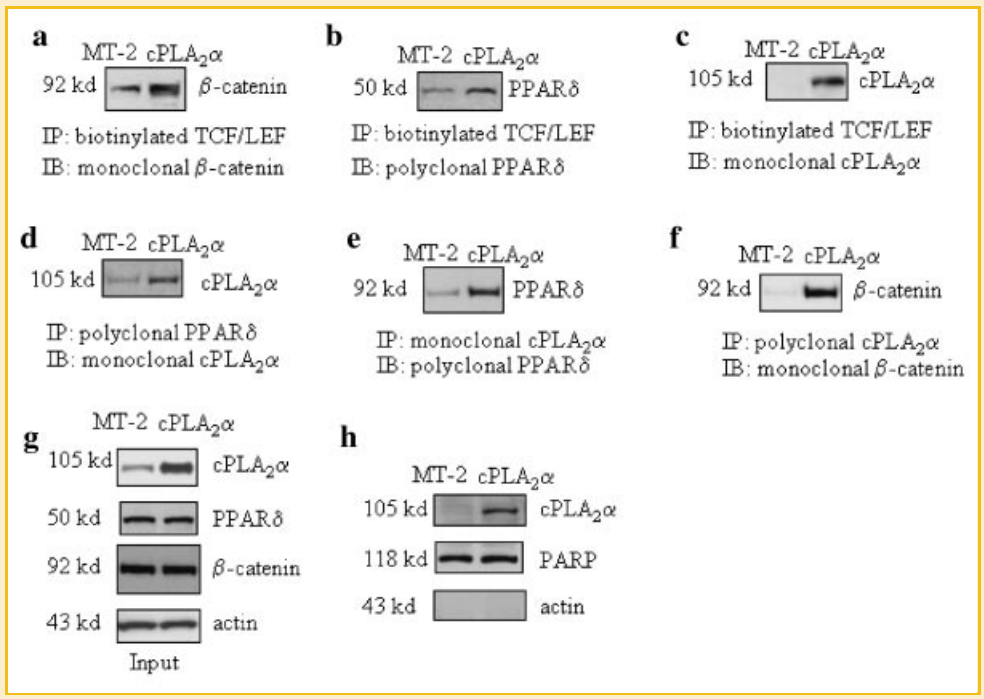

Fig. 2. Overexpression of cPLA$_2\alpha$ induces the association of PPARδ to β-catenin and their binding to TCF/LEF response element. CCLP1 cells were transfected with either 4 μg cPLA$_2\alpha$ in MT-2 expression plasmid or MT-2 control plasmid in the 100 mm dish for 24 h. The cell lysates were obtained for immunoprecipitation with biotinylated TCF/LEF oligonucleiotides followed by immunoblotting for β-catenin, PPARδ or cPLA$_2\alpha$ to determine their binding to TCF/LEF response element. The cell lysates were also utilized for immunoprecipitation with antibody against PPARδ or cPLA$_2\alpha$ followed by immunoblotting for cPLA$_2\alpha$, PPARδ or β-catenin. Panel a shows that overexpression of cPLA$_2\alpha$ increases β-catenin association with the TCF/LEF element. Panels b and c show that PPARδ and cPLA$_2\alpha$ are also present in the β-catenin–TCF/LEF binding complex. Panels d, e, and f show that overexpression of cPLA$_2\alpha$ increases its binding to PPARδ or β-catenin. Panel g is the input of the whole cellular proteins for the above experiments. Panel h shows increased cPLA$_2\alpha$ protein in the nuclear extract obtained from the cPLA$_2\alpha$ overexpressed cells (the purity of the isolated nuclear extract was confirmed by the presence of nuclear protein PARP and absence of the cytoplasmic protein β-actin). The results were confirmed in at least two independent experiments.

blocked by the specific cPLA$_2$ inhibitors, AACOCF$_3$ and pyrrolidine derivative [Xu et al., 2006a,b]. The effect of cPLA$_2\alpha$ on PPARδ activation is mediated at least in part through increased AA in the nuclei; this assertion is supported by the observations that AA directly bound to PPARδ in vitro and that addition of AA to isolated nuclear extracts or recombinant PPARδ protein enhanced the ability of PPARδ binding to its DNA response element, DRE (PPARδ response element) [Xu et al., 2006a,b].

Given the key role of cPLA$_2\alpha$-derived AA in PPARδ activation as indicated above, in the current study we sought to further determine whether cPLA$_2\alpha$-mediated PPARδ activation is implicated in β-catenin activity. As shown in Figure 2b, overexpression of cPLA$_2\alpha$ enhanced the association of PPARδ with the β-catenin-TCF/LEF complex, whereas it did not alter PPARδ protein level. Furthermore, siRNA suppression of PPARδ inhibited the cPLA$_2\alpha$-induced increase of TCF/LEF reporter activity (Fig. 4A). These results suggest a potential role for PPARδ in cPLA$_2\alpha$-mediated β-catenin activation.

We then carried out further experiments to examine the direct effect of PPARδ on β-catenin activation. Overexpression of PPARδ in CCLP1 cells significantly increased the TCF/LEF reporter activity; this effect is dose-dependent, with approximately 40-fold increase of reporter activity in cells transfected with 0.5 μg PPARδ expression plasmid (Fig. 5A). A similar effect was also observed in another human cholangiocarcinoma cell line (SG231). Overexpression of PPARδ did not alter the protein level of β-catenin; it increased the formation of β-catenin-PPARδ binding complex and enhanced their association with TCF/LEF response element (Fig. 5B). In addition, treatment with the selective PPARδ ligand, GW501516, also increased the TCF/LEF luciferase reporter activity (Fig. 5C). Moreover, RNAi knockdown of PPARδ reduced the TCF/LEF reporter activity (Fig. 6). The involvement of cPLA$_2\alpha$ and PPARδ in β-catenin activation is further supported by the observation that overexpression of cPLA$_2\alpha$ and PPARδ induced the expression of c-myc, a β-catenin downstream gene (Figs. 1 and 5A).

After the role of PPARδ in β-catenin activation is documented, we performed further experiments to determine whether the PPARδ ligand, GW501516, and AA might be able to alter the binding of β-catenin to TCF/LEF response element. As shown in Figure 7, both GW501516 and AA induced the binding of β-catenin to TCF/LEF response element when added to either intact cells or isolated nuclear extracts. The degree of β-catenin binding induced by AA is comparable to that by GW501516. In contrast, oleic acid, which has no effect on PPARδ activation, failed to alter β-catenin binding to TCF/LEF response element. These findings further support the role of PPARδ in β-catenin activation.

The importance of cPLA$_2\alpha$ in eicosanoid metabolism and intracellular signaling transduction is determined by several unique

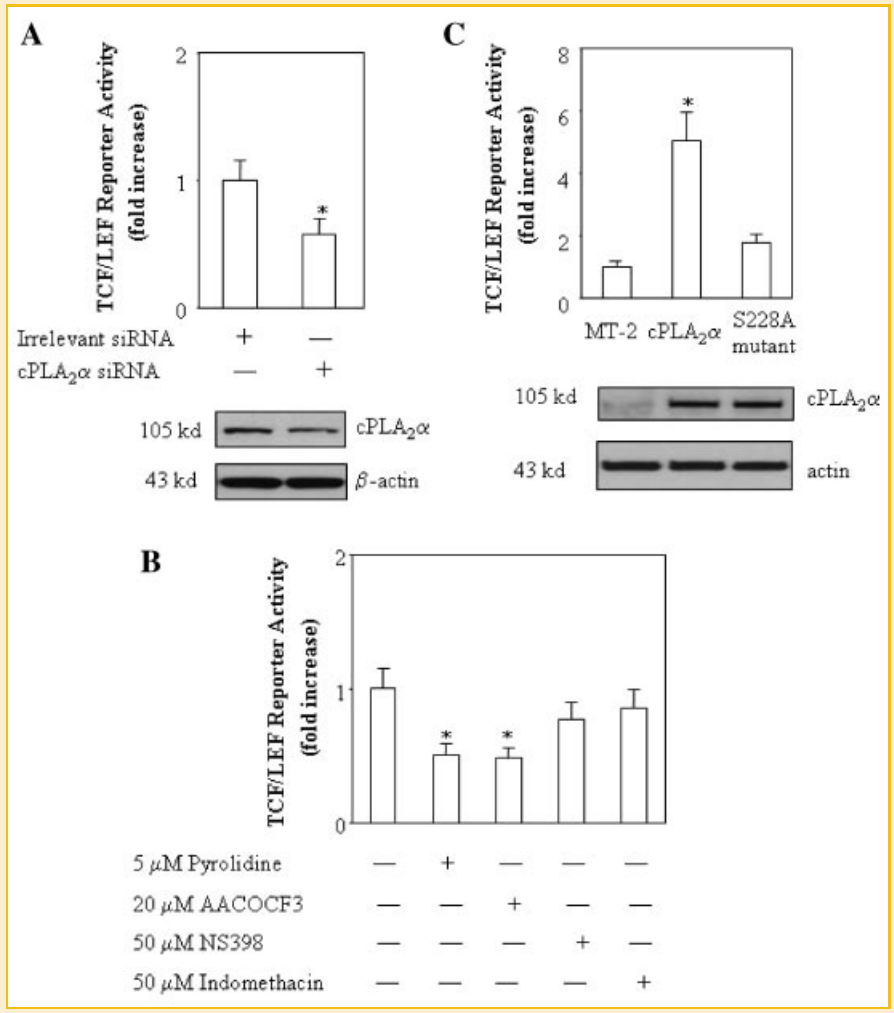

Fig. 3. Inhibition of $cPLA_2\alpha$ reduces TCF/LEF transcription activity. A: RNAi suppression of $cPLA_2\alpha$ decreases TCF/LEF reporter activity. CCLP1 cells cultured in 12-well plates were transiently transfected with either 20 μM $cPLA_2\alpha$ siRNA or the equal amount of irrelevant siRNA with co-transfection of 0.2 μg/well pTCF/LEF-Luc reporter vector. After transfection the cells were cultured in serum-free medium for 24 h and the cell lysates were obtained to determine the luciferase activity. The data are presented as mean ± SD of three independent experiments. RNAi suppression of $cPLA_2\alpha$ significantly decreases TCF/LEF luciferase reporter activity when compared with the cells transfected with irrelevant siRNA ($^*P < 0.05$) (upper panel). Western blot analysis for $cPLA_2\alpha$ was performed using total cellular proteins (lower panel, with β-actin as the loading control). B: The effect of $cPLA_2\alpha$ inhibitors (pyrolidine and $AACOCF_3$), COX-2 inhibitor (NS-398), and COX inhibitor (indomethacin) on TCF/LEF reporter activity. CCLP1 cells were transiently transfected with pTCF/LEF-Luc reporter vector (0.2 μg/well). After transfection the cells were cultured in serum-free medium for 24 h. The cells were then treated with vehicle as control, 5 μM pyrolidine, 20 μM $AACOCF_3$, 50 μM NS398, or 50 μM indomethacin in serum-free medium for 4 h. The cell lysates were subsequently obtained to determine the luciferase activity. The data are presented as mean ± SD of three independent experiments. The cells treated with either pyrolidine or $AACOCF_3$ showed significantly decreased TCF/LEF luciferase reporter activities when compared with the cells treated with vehicle ($^*P < 0.01$). Indomethacin or NS-398 treatment only slightly reduced the TCF/LEF reporter activity (the effect is not statistically significant). C: Overexpression of the S228A $cPLA_2\alpha$ mutant fails to increase TCF/LEF reporter activity. CCLP1 cells cultured in 12-well plates were transiently transfected with 0.5 μg/well $cPLA_2\alpha$ expression plasmid, S228A $cPLA_2\alpha$ mutant plasmid or MT-2 control plasmid with co-transfection of equal amount of pTCF/LEF-Luc reporter vector. After transfection the cells were cultured in serum-free medium for 24 h and the cell lysates were obtained to determine luciferase reporter activity as described in the Materials and Methods Section. The data are presented as mean ± SD of three independent experiments. Whereas overexpression of $cPLA_2\alpha$ significantly increased TCF/LEF reporter activity ($^*P < 0.01$ compared with control), overexpression of S228A $cPLA_2\alpha$ mutant had no significant effect (upper panel). Western blot analysis for $cPLA_2\alpha$ was performed using total cellular proteins (lower panel, with β-actin as the loading control).

characteristic of this enzyme, including its selective cleavage of arachidonic acid from membrane phospholipid and its nuclear localization. However, since $cPLA_2\alpha$ lacks classical nuclear localization signals, the mechanism for localization of $cPLA_2\alpha$ in the cell nucleus has not been fully elucidated. To further investigate the role of $cPLA_2\alpha$ in the nuclei, we sought to determine whether $cPLA_2\alpha$ itself might physically interact with PPARδ. Indeed, immunoprecipitation and immunoblotting analyses reveal the presence of $cPLA_2\alpha$ and PPARδ binding complex in CCLP1 cells (Fig. 2d,e). Whereas this binding complex is detected at a relatively low level in control cells, it becomes more abundant after transfection with the $cPLA_2\alpha$ expression vector. Furthermore,

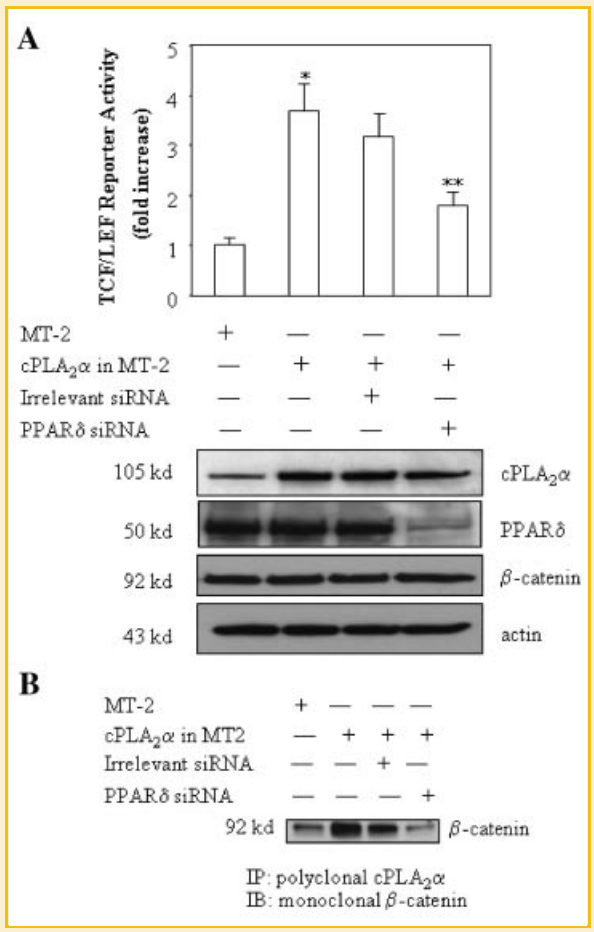

Fig. 4. RNAi suppression of PPARδ prevents cPLA$_2\alpha$ and β-catenin association and reduces TCF/LEF reporter activity. A: RNAi suppression of PPARδ inhibits cPLA$_2\alpha$-mediated increase of TCF/LEF reporter activity. CCLP1 cells cultured in 12-well plates were transiently transfected with 0.5 μg/well cPLA$_2\alpha$ expression plasmid together with 20 μM PPARδ siRNA or irrelevant siRNA with co-transfection of equal amount of pTCF/LEF-Luc reporter vector. After transfection, the cells were cultured in serum-free medium for 24 h and then the cell lysates were obtained to determine the luciferase activity as described in the Materials and Methods Section. The data are presented as mean ± SD of three independent experiments. RNAi suppression of PPARδ expression significantly blocks the increase of TCF/LEF reporter activity induced by cPLA$_2\alpha$ overexpression ($^{*}P < 0.01$ compared to cells transfected with MT-2 control vector; $^{**}P < 0.05$ compared to cells transfected with cPLA$_2\alpha$ expression vector) (upper panel). Western blot analysis was performed using total cellular proteins, with β-actin as the loading control (lower panel). B: RNAi suppression of PPARδ blocks cPLA$_2\alpha$ and β-catenin association. CCLP1 cells cultured in six-well plates were transiently transfected with 1 μg/well cPLA$_2\alpha$ expression plasmid together with either PPARδ siRNA or irrelevant siRNA for 24 h. The cell lysates were subsequently prepared for immunoprecipitation with the antibody against cPLA$_2\alpha$. The immunoprecipitants were then subjected to Western blotting using the antibody against β-catenin. The results were confirmed in at least two independent experiments.

cPLA$_2\alpha$ is also present in the PPARδ-β-catenin protein complex and associated with the TCF/LEF element (Fig. 2c,f); this association is likely mediated through PPARδ, since RNAi suppression of PPARδ prevented cPLA$_2\alpha$-β-catenin association (Fig. 4B). These data suggest that PPARδ is able to bind cPLA$_2\alpha$, which may facilitate the import of cPLA$_2\alpha$ into the nucleus. Phosphorylation of cPLA$_2\alpha$ is not required for its association with PPARδ, given that inhibition of the p42/44 and p38 MAP kinases (key enzymes for cPLA$_2\alpha$ phosphorylation) by PD98059 and SB203580 did not alter the formation of cPLA$_2\alpha$-PPARδ binding complex (Fig. 8A), despite that these inhibitors prevent cytokine/growth factor-induced cPLA$_2\alpha$ phosphorylation in these cells [Han et al., 2004a; Wu et al., 2002]. Similarly, cPLA$_2\alpha$ enzyme activity is also not required for cPLA$_2\alpha$ and PPARδ association, since their binding was not altered by site-directed mutation of cPLA$_2\alpha$ (S228A mutant) (Fig. 8B). Therefore, the association between cPLA$_2\alpha$ and PPARδ is likely mediated through their protein interactions. The close proximity between cPLA$_2\alpha$ and PPARδ provides a unique and efficient advantage for their functional coupling in the nucleus, where AA produced by cPLA$_2\alpha$ enzyme activity becomes immediately available for PPARδ binding and activation.

DISCUSSION

Activation of PPAR involves ligand-induced conformational change which alters the binding of PPAR with other nuclear proteins and the basal transcriptional machinery. Although AA metabolites represent the natural ligands for PPAR activation, the individual enzymes involved in the control of eicosanoid production for PPAR activation remain to be further defined. Recent studies from our laboratory have documented the role of cPLA$_2\alpha$ for the activation of PPARδ [Xu et al., 2006a,b]. In the current study, we provide further evidence for cPLA$_2\alpha$-mediated PPARδ activation in the regulation of β-catenin signaling pathway. Several notable observations are presented in the current paper.

First, our results reveal a novel role of cPLA$_2\alpha$ for β-catenin activation. This conclusion is based on the following observations: (1) overexpression of cPLA$_2\alpha$ increases TCF/LEF reporter activity; (2) overexpression of cPLA$_2\alpha$ enhanced the binding of β-catenin to the TCF/LEF response element; (3) inhibition of cPLA$_2\alpha$ by chemical inhibitors, siRNA and site-direct mutagenesis reduced the TCF/LEF transcription activity; and (4) AA induced the binding of β-catenin to TCF/LEF response element.

Second, our data indicate that PPARδ is implicated in cPLA$_2\alpha$-mediated β-catenin activity. This is based on the observations that (1) overexpression of cPLA$_2\alpha$ enhanced the binding of PPARδ and β-catenin to the TCF/LEF response element; (2) siRNA suppression of PPARδ inhibited the cPLA$_2\alpha$-induced or spontaneous TCF/LEF reporter activity; and (3) siRNA suppression of PPARδ prevented cPLA$_2\alpha$ association with β-catenin.

Third, our findings reveal a direct interaction between PPARδ and β-catenin. This conclusion is based on (1) overexpression of PPARδ enhanced the formation of β-catenin-PPARδ binding complex; (2) overexpression of PPARδ increased the association of β-catenin with TCF/LEF response element; (3) overexpression of PPARδ increased the TCF/LEF reporter activity; (4) activation of PPARδ by its ligand, GW501516, increased the TCF/LEF luciferase reporter activity; (5) RNAi knockdown of PPARδ decreased β-catenin binding to TCF/LEF response element and reduced TCF/LEF reporter activity.

Fourth, we show that $cPLA_2\alpha$ is present in the PPARδ and β-catenin protein complex. This association is likely mediated through PPARδ, since RNAi suppression of PPARδ prevents $cPLA_2\alpha$-β-catenin association. This finding provides a possible mechanistic explanation for the localization of $cPLA_2\alpha$ in the nuclei. Since $cPLA_2\alpha$ lacks a nuclear localization signal, it is likely that association with PPARδ may direct the import of $cPLA_2\alpha$ into the nucleus (this process does not appear to require $cPLA_2\alpha$ phosphorylation or enzyme activity). The close proximity between $cPLA_2\alpha$ and PPARδ provides a unique and efficient advantage for their functional coupling in the nucleus, where AA produced by $cPLA_2\alpha$ enzyme activity becomes immediately available for PPARδ binding and activation. One potential limitation of this part of the study is that the data were obtained from limited experimental approaches (immunoprecipitation, Western blotting and reporter activity assays). Further investigation is needed to verify this phenomenon by optical analysis (such as confocal immunofluorescence and electron microscopy), although it is beyond the scope of the current paper.

Recent studies have shown that PGE_2 treatment increased β-catenin accumulation in human colon cancer cells. Castellone et al. [2005] reported that PGE_2 activates its G protein-coupled receptor, EP_2, resulting in direct association of the G protein alphas subunit with the regulator of G protein signaling (RGS) domain of

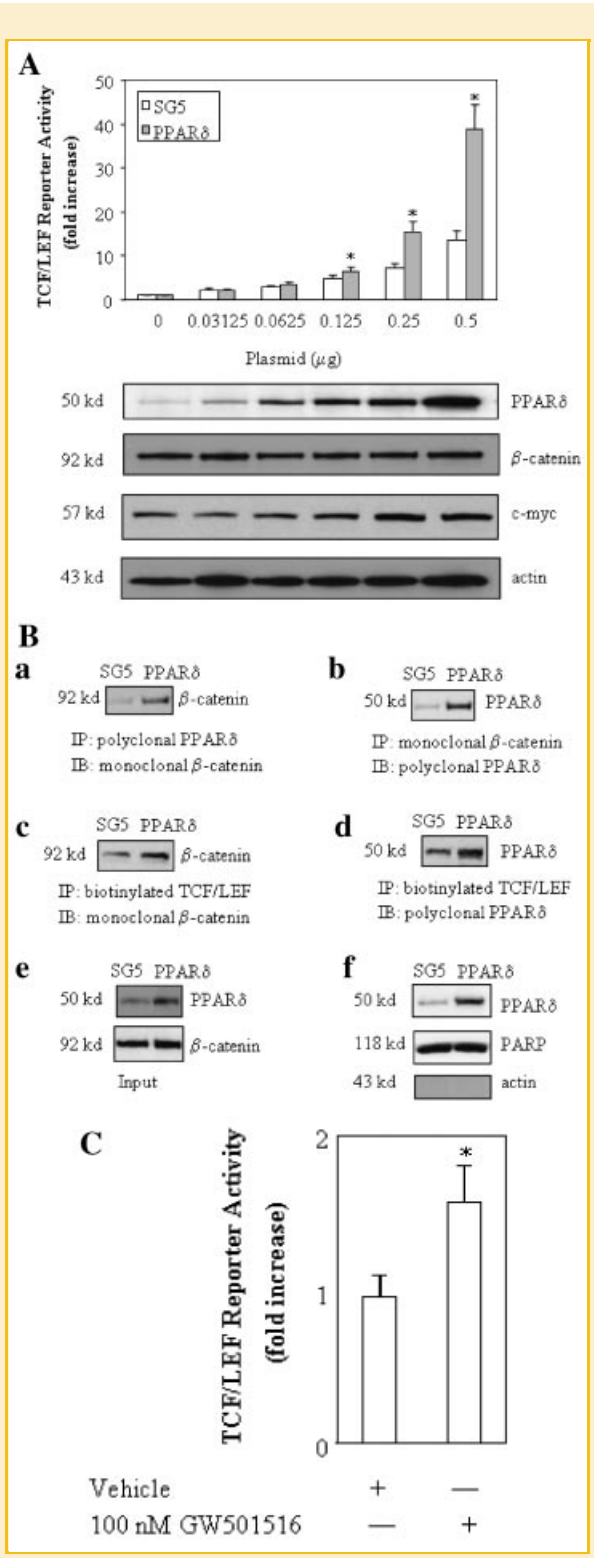

Fig. 5. The direct role of PPARδ in TCF/LEF transcription activity. A: Overexpression of PPARδ increases TCF/LEF reporter activity. CCLP1 cells were transiently transfected with different amounts of either PPARδ in SG5 expression plasmid or SG5 control plasmid with co-transfection of equal amount of pTCF/LEF-Luc reporter vector. After transfection, the cells were cultured in serum-free medium for 24 h and the cell lysates were obtained to determine the luciferase activity. The data are presented as mean ± SD of three independent experiments. The cells with PPARδ overexpression confirmed by Western blot showed significantly increased TCF/LEF luciferase reporter activity when compared with the cells transfected with equal amount of control vector ($^*P < 0.01$) (upper panel). PPARδ overexpression did not alter the protein level of β-catenin (lower panel). (Western blot analysis was performed using total cellular proteins, with β-actin as the loading control.) B: Overexpression of PPARδ induces its association with β-catenin and their binding to TCF/LEF response element. a,b: Overexpression of PPARδ increases the binding of β-catenin to PPARδ. CCLP1 cells were transfected with the PPARδ expression plasmid or SG5 control plasmid for 24 h. The cell lysates were subsequently prepared for immunoprecipitation with antibodies against either PPARδ or β-catenin; the immunoprecipitants were then immunoblotted with either anti-β-catenin (left panel) or anti-PPARδ (right panel) antibodies. c,d: Overexpression of PPARδ increases the binding of β-catenin or PPARδ to TCF/LEF response element. CCLP1 cells were transfected with the PPARδ expression plasmid or SG5 control plasmid for 24 h. The cell extracts were obtained and precipitated with biotinylated TCF/LEF oligonucleiotides followed by immunoblotting for β-catenin (left panel) or PPARδ (right panel). e: Western blots of the input of the whole cellular proteins for the above experiments. f: Western blot shows increased PPARδ protein in the nuclear extract obtained from the PPARδ overexpressed cells (the purity of the isolated nuclear extract was confirmed by the presence of nuclear protein PARP and absence of the cytoplasmic protein β-actin). The results were confirmed in at least two independent experiments. C: The PPARδ ligand, GW501516, increases TCF/LEF reporter activity. CCLP1 cells were transiently transfected with pTCF/LEF-Luc reporter vector. After transfection the cells were cultured in serum-free medium for 24 h. The cells were then treated with vehicle as control, or 100 nM GW501516 for 4 h and the cell lysates were obtained to determine the luciferase activity. The data are presented as mean ± SD of three independent experiments. The cells treated with GW501516 showed significantly increased TCF/LEF luciferase reporter activity when compared with the cells treated with vehicle ($^*P < 0.01$).

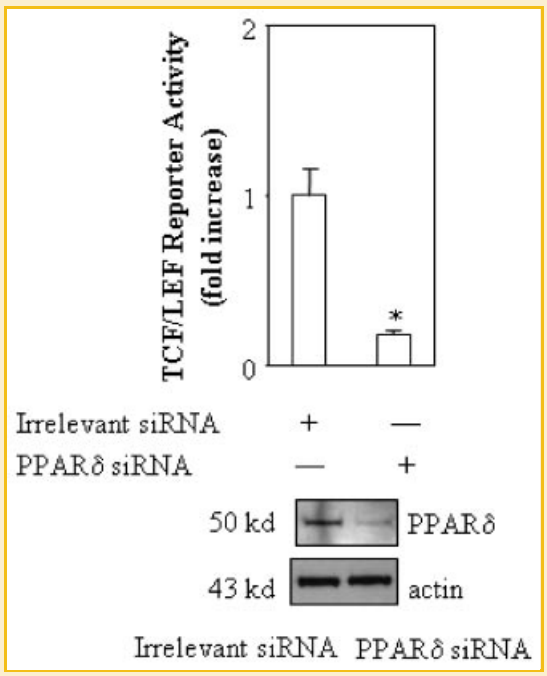

Fig. 6. RNAi suppression of PPARδ decreases TCF/LEF reporter activity. CCLP1 cells were transiently transfected with either PPARδ siRNA or irrelevant siRNA with co-transfection of pTCF/LEF-Luc reporter vector. After transfection, the cells were cultured in serum-free medium for 24 h and the cell lysates were obtained to determine the luciferase activity. The data are presented as mean ± SD of three independent experiments. RNAi suppression of PPARδ significantly decreases TCF/LEF reporter activity when compared with the cells transfected with irrelevant siRNA ($^*P < 0.05$) (upper panel). Western blot analysis for PPARδ was performed using total cellular proteins (lower panel, with β-actin as the loading control).

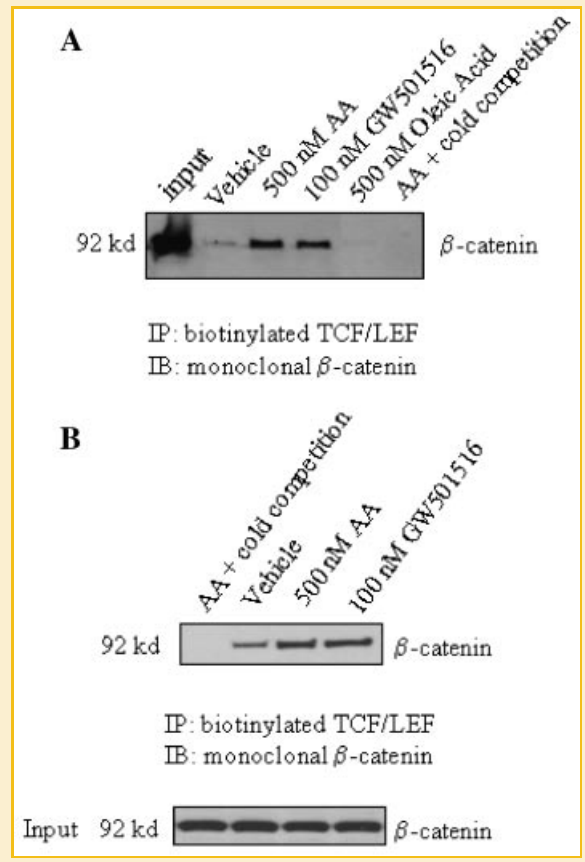

Fig. 7. AA and GW501516 increase the binding of β-catenin to TCF/LEF response element. A: AA and GW501516 increase the binding ability of β-catenin to TCF/LEF response element in a cell free system. Equal amounts of nuclear extracts from CCLP1 cells were incubated with vehicle as control, 500 nM AA, 100 nM GW501516 or 500 nM oleic acid for 30 min on ice and then precipitated with biotinylated TCF/LEF oligonucleiotides (with 20-fold cold unlabeled TCF/LEF oligonucleiotides as cold competition) followed by immunoblotting for β-catenin as described in the Materials and Methods Section. The results were confirmed in at least two independent experiments. B: AA and GW501516 increase the binding ability of β-catenin to TCF/LEF response element in intact cells. CCLP1 cells were serum-starved for 24 h and then treated with either 500 nM AA or 100 nM GW501516 for 4 h. The whole cell lysates were obtained and precipitated with biotinylated TCF/LEF oligonucleiotides (with 20-fold cold unlabeled TCF/LEF oligonucleiotides as cold competition) followed by immunoblotting for β-catenin (upper panel). The lower panel is the input. The results were confirmed in at least two independent experiments.

axin; this results in release of glycogen synthase kinase 3β from its complex with axin, thus leading to β-catenin accumulation. Shao et al. [2005] showed the involvement of cAMP/protein kinase A pathway in PGE_2-induced β-catenin accumulation in colon cancer cells. In the present study, we found that the COX inhibitor, indomethacin, and the COX-2 inhibitor, NS-398, failed to significantly alter TCF/LEF reporter activity, suggesting that COX enzyme may not be the principal mechanism for β-catenin activation in human cholangiocarcinoma cells. Instead, our data indicate that the $cPLA_2\alpha$-dervied AA represents a novel mechanism for activation of β-catenin and the effect is mediated via PPARδ and β-catenin binding and their association with the TCF/LEF response element.

Our findings in this study suggest that PPARδ is a key molecule that mediates β-catenin activation by $cPLA_2\alpha$. This is noteworthy in light of the observation that the transcription of PPARδ is directly activated by the Wnt/β-catenin signaling pathway [He et al., 1999]. We show that activation of PPARδ by $cPLA_2\alpha$ results in the formation of PPARδ-β-catenin complex, thus leading to β-catenin activation. The $cPLA_2\alpha$-induced PPARδ activation is mediated by arachidonic acid rather than PGE_2. The latter is supported by the observation that addition of AA, but not PGE_2, into nuclear extracts or recombinant PPARδ protein enhanced the ability of PPARδ binding to its DNA response element and that the COX-2 inhibitor, indomethacin, had no apparent influence on A23187-induced PPARδ DNA binding. It is of note that PGE_2 can activate PPARδ in cultured cells, but its effect is mediated through indirect mechanisms, including induction of $cPLA_2\alpha$ phosphorylation [Xu et al., 2006a,b] and activation of PI3/Akt pathway [Wang et al., 2004]. These observations further underscore the importance of PPARδ in $cPLA_2\alpha$-mediated β-catenin activation.

In summary, this study depicts a novel connection linking $cPLA_2\alpha$, PPARδ and Wnt/β-catenin signaling pathways in human cholangiocarcinoma cells. Given the documented involvement of

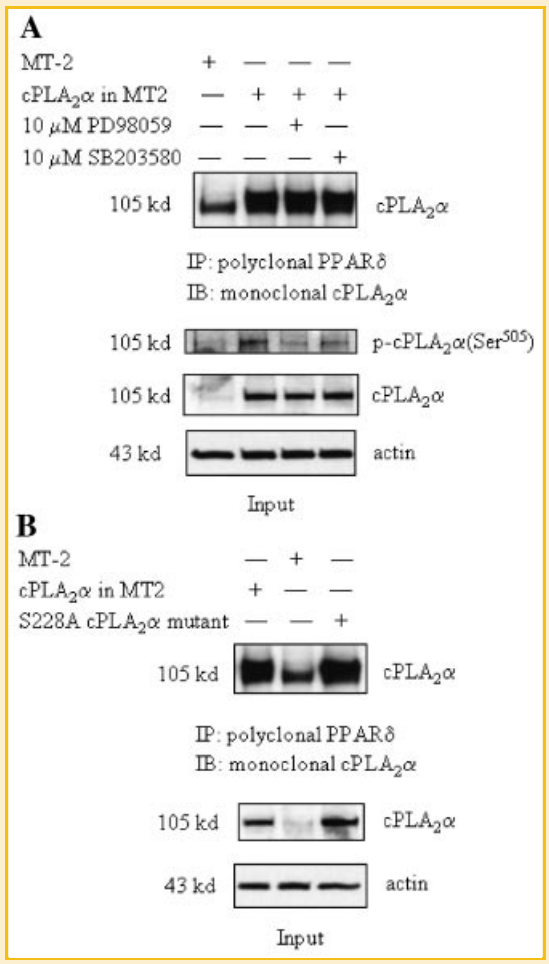

Fig. 8. The effect of $cPLA_2\alpha$ phosphorylation or mutagenesis on the association between $cPLA_2\alpha$ and PPARδ. A: The p38 MAP kinase inhibitor, SB203580, and the p42/44 MEK inhibitor, PD98059, do not affect $cPLA_2\alpha$-PPARδ association. CCLP1 cells were transfected with either $cPLA_2\alpha$ expression plasmid or MT-2 control vector for 24 h then treated with either 10 μM SB203580 or 10 μM PD98059 for 30 min as indicated. The cell lysates were precipitated with polyclonal PPARδ antibody followed by immunoblotting for $cPLA_2\alpha$ to determine their association. The lower panels are the input of the whole cellular proteins. The results were confirmed in at least two independent experiments. B: Inactivation of $cPLA_2\alpha$ by site-directed mutagenesis does not alter $cPLA_2\alpha$-PPARδ association. CCLP1 cells were transfected with the $cPLA_2\alpha$ expression plasmid, S228A $cPLA_2\alpha$ mutant, or MT-2 control vector for 24 h. The cell lysates were precipitated with polyclonal PPARδ antibody followed by immunoblotting for $cPLA_2\alpha$ to determine their association. The lower panels are the input of the whole cellular proteins. The results were confirmed in at least two independent experiments.

these molecules in bile duct inflammation and cancer, it is conceivable that activation of β-catenin by $cPLA_2\alpha$ and PPARδ may represent an important mechanism by which inflammatory process drives carcinogenesis. Furthermore, in light of the importance of $cPLA_2\alpha$ in various physiological and pathological processes, further studies are warranted to determine whether PPARδ and β-catenin is involved in the multifaceted actions of $cPLA_2\alpha$ and their potential implication in human diseases.

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