Nucleolin regulates c-Jun/Sp1-dependent transcriptional activation of cPLA2α in phorbol ester-treated non-small cell lung cancer A549 cells

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ABSTRACT

The expression of cPLA2 is critical for transformed growth of non-small cell lung cancer (NSCLC). It is known that phorbol 12-myristate 13-acetate (PMA)-activated signal transduction pathway is thought to be involved in the oncogene action in NSCLC and enzymatic activation of cPLA2. However, the transcriptional regulation of cPLA2α in PMA-activated NSCLC is not clear. In this study, we found that PMA induced the mRNA level and protein expression of cPLA2α. In addition, two Sp1-binding sites of cPLA2α promoter were required for response to PMA and c-Jun overexpression. Small interfering RNA (siRNA) of c-Jun and nucleolin inhibited PMA induced the promoter activity and protein expression of cPLA2α. Furthermore, PMA stimulated the formation of c-Jun/Sp1 and c-Jun/nucleolin complexes as well as the binding of these transcription factor complexes to the cPLA2α promoter. Although Sp1-binding sites were required for the bindings of Sp1 and nucleolin to the promoter, the binding of nucleolin or Sp1 to the promoter was independent of each other. Our results revealed that c-Jun/nucleolin and c-Jun/Sp1 complexes play an important role in PMA-regulated cPLA2α gene expression. It is likely that nucleolin binding at place of Sp1 on gene promoter could also mediate the regulation of c-Jun/Sp1-activated genes.

INTRODUCTION

The production of lipid mediators, the eicosanoids (i.e. prostaglandins and leukotrienes) are derived from metabolism of arachidonic acid, which has been implicated in the regulation of cell growth, inflammation, thrombosis and tumor progression (1–3). There is much evidence indicating that eicosanoids, particularly prostaglandins, are involved in the etiologies of cancer (4). Increased levels of eicosanoids occur in a number of different types of human cancer, including colon, pancreatic, breast and lung. In the case of lung cancer, increased prostaglandin biosynthesis has been found to occur mainly in non-small cell lung cancer (NSCLC), which comprises 80% of lung cancers rather than small cell lung cancer (SCLC) (5,6). In addition, the increase of cPLA2 is correlated with the eicosanoid synthesis that participates in NSCLC transformation (7).

Cytosolic phospholipase A2 (cPLA2) is the major intracellular form of PLA2, which preferentially hydrolyzes membrane phospholipids at the sn-2 position to release arachidonic acid (8). cPLA2 activity is regulated by intracellular Ca2+ and phosphorylation. Increase in Ca2+ results in translocation of cPLA2 to the nuclear envelope and activation (9,10). But it has been shown that phorbol 12-myristate 13-acetate (PMA) provides cPLA2-activating signals without inducing Ca2+ influx (11,12) and by a Rac-p38 kinase-dependent pathway (13). The maximal activation of cPLA2 requires sustained phosphorylation of Ser505, Ser727 and Ser515 by mitogen-activated protein kinases (MAPKs), MAPK-activated protein kinases (MAP2Ks), MAPK-activated protein kinases and by calcium/calmodulin-dependent protein kinase II, respectively (14–16). In addition to acute regulation, expression of cPLA2 through changes in gene transcription is mediated by a number of agents including cytokines, thrombin and growth factors (17–21). The promoter for cPLA2 has been isolated from both human (18) and rat (22). A number of putative binding sites for possible regulatory elements have been identified within the promoter, including AP-1 sites, nuclear factor κB sites.

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and glucocorticoid regulatory elements. Truncation of a 2.4 kb region of the promoter fragment down to the last 58 bp of the 5′-untranslated region has been identified in three regulatory regions and indicates that the transcription factor Sp1 can bind to two of these regions (23,24). In the regulation of cPLA2 promoter, LKLF (lung Krüppel-like factor) as a transcriptional activator also binds to the cPLA2 promoter and may interact with the Sp1 family (25).

Although Sp1 is required for the transcriptional activation of cPLA2 gene, only a limited number of studies have addressed the mechanisms by which cPLA2 gene expression is controlled. In addition to enzymatic activation, in this study, we also clarified that PMA could activate the transcription via the Sp1-binding sites of cPLA2 promoter in NSCLC A549 cells. We demonstrated that transcription factors c-Jun and Sp1 form a complex to regulate PMA-induced gene expression of cPLA2 as the manner as the c-Jun/Sp1-regulated genes including 12(E)-lipoxygenase, keratin 16, p21(WAF1/CIP1) and glucocorticoid regulatory elements. Truncation of a untranslated region has been identified in three regulatory regions and indicates that the transcription factor Sp1 can bind to two of these regions (23,24).

In the regulation of cPLA2 promoter, LKLF (lung Krüppel-like factor) as a transcriptional activator also binds to the cPLA2 promoter and may interact with the Sp1 family (25).

Northern blot analysis
Total RNA was isolated using the TRIzol RNA extraction kit (Invitrogen, Grand Island, NY), and 20 μg of RNA were used for electrophoresis and transferred to a nylon membrane as previously described (32). The cDNA probes used were prepared from RT-PCR. Probes were labeled with [γ-32P]dCTP by using a RediprimeTMII random prime labeling system (Amersham, Bucks, UK). The nylon membranes were washed three times at room temperature in 2× standard saline/ phosphate/EDTA buffer (300 mM NaCl, 20 mM NaH2PO4 and 2 mM EDTA) containing 0.1% SDS. Each wash was carried out for 15 min. Autoradiography was then performed.

Plasmid construction
A 619 bp of human cPLA2 promoter region was PCR-amplified from human genomic DNA and subcloned into luciferase plasmid pXP1 as the pPLA599 plasmid. Respective additions of KpnI to forward primer 5′-GA AATTCCAACTTGAATTCAATTTCTTCCCT-3′ and of HindIII to reverse primer 5′-GATCTTTCCTCAGC-3′ facilitated subcloning. 5′-Deletions of various lengths, pPLA393, pPLA241, pPLA140, pPLA53, pPLA35 and pPLA27 were generated using reverse primer 5′-GATCTTTCCTAGTCCGGA-3′ and the forward primers 5′-AATTTTGCCCTTITATATGATGCA-3′, 5′-ACAGAAAATCCGACACAGACTC-3′, 5′-CATTTA CATTCAATATTAGC-3′, 5′-GGAGACACACGCCA CATTTTAG-3′, 5′-TAGCCCTCCTCTACTCAGG-3′ and 5′-CTACTCAGAATAAGACT-3′, respectively. The mutants at Sp1 site (pLAm) were constructed by the site-directed mutagenesis method. Synthetic primers were shown in the following: pLAm1 mutant primer 5′- GG AGACCAGTTCCACATTATTAG-3′, and PLaM2 mutant primer 5′- TAGTCTTCCCTCTACTCAGG-3′. Mutated positions in the sequence of the primers were underlined. All DNA fragments were directly subcloned into pXP1 using KpnI and HindIII. The vector sequence was confirmed by DNA sequencing. To generate the luciferase plasmid UTR-1-446, a 447 bp of human cPLA2 mRNA 3′UTR was PCR-amplified from cDNA and inserted between luciferase gene and SV40 late poly(A) signal coding regions of luciferase plasmid pGL3. Respective additions of XbaI to forward primer 5′-TTCATG TACTGAAAATGGCAAGC-3′ and of FseI to reverse primer 5′-CATGTGATATGATATGAC-3′ facilitated subcloning. The nucleotide sequences of constructs were confirmed by automatic DNA sequencing.

Transfection of cells with plasmids
Cells were transfected with plasmids by lipofection using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instruction with a slight modification. Cells were replated 24 h before transfection at an optimal cell density in 2 ml of fresh culture medium in a 3.5 cm plastic dish. For use in transfection, 0.5 μl of Lipofectamine 2000 was incubated with 0.5 μg of pPLA,
DNA affinity precipitation assay (DAPA)

This assay was performed according to the method reported previously (35) with a slight modification. The binding assay was performed by mixing 200 μg of nuclear extract proteins, 2 μg of biotinylated specific wild-type and mutated Sp1 oligonucleotides of cPLA2 gene, 12(S)-lipoxygenase and gastrin promoters 5'-CA AGA GAC CAG CCC ACA TTT TAG CCC CTC CTA CTC AGG-3', 5'-CA GGA GAC CAG CTT ACA TTT TAT CCC TCT CTA CTC AGG-3', 5'-TAA AAC TTG CGA GGA GGG CGG GGC CGC AG-3' and 5'-CAG GTG AGG GGC GGG GTG GGG GGA CA-3', respectively, and Sp1 and NFκB consensus oligonucleotides 5'-ATT CGA TCG GGG CGG GGC CAG C-3' and 5'-AGT CTA GGT AGG GGC GGG GTG GGG GGA CA-3', respectively, and 20 μl of streptavidin-agarose beads (4%) with 50% slurry. Mutated positions in the sequence were underlined. The mixture was incubated at room temperature for 1 h with rotating. Beads were pelleted and washed three times with PBS. The binding proteins were eluted by loading buffer and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by western blot analysis probed with specific antibodies.

Chromatin immunoprecipitation (ChiP) assay

Chromatin immunoprecipitation assay was done according to previous report (36) with minor modification. Briefly, A549 cells were treated with 1% formaldehyde for 15 min. The cross-linked chromatin was sonicated to 400–500 bp fragments. Lysates were precleared with protein A beads and incubated overnight at 4°C with antibodies specific to nucleolin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), c-Jun (Santa Cruz, CA, USA) and Sp1 (Santa Cruz, CA, USA). Immune complex was precipitated with protein A beads preabsorbed with sonicated salmon sperm DNA and BSA. After reversal of cross-linking, levels of precipitated cPLA2 promoter DNA were determined by PCR using oligonucleotides spanning the Sp1-binding sites (sense, 5'-ACA GAA ATC CGC AAC AGC ACT C-3'; antisense, 5'-GAT CCT TTT TCA GCT CCG GA-3'). The PCR products were separated by 1% agarose-gel electrophoresis and visualized with ethidium bromide staining.

Western blotting

The cytoplasmic fractions and nuclear extracts of cells were prepared for western blot analysis according to the method described (37). An analytical 10% SDS–PAGE was performed, and 30 μg of protein of each sample were analyzed, unless stated otherwise. For immunoblotting, proteins in the SDS gels were transferred to a polyvinylidene difluoride membrane by an electroblot apparatus. Antibodies against human Sp1 (Santa Cruz, CA, USA), c-Jun (Santa Cruz, CA, USA), nucleolin (Santa Cruz, CA, USA) and β-actin (Santa Cruz, CA, USA) were used as the primary antibodies. Mouse or rabbit IgG antibodies coupled to horseshadish peroxidase were used as secondary antibodies. An enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) was used for detection.

Coimmunoprecipitation

Two hundred micrograms protein of nuclear extracts and lysate were incubated under gentle shaking at 4°C overnight with a mixture of anti-c-Jun or anti-Sp1 antibodies and protein A agarose in 300 μl of immunoprecipitation buffer [20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2 and 25% glycerol (v/v), 0.5 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 2 μg/ml pepstatin A and 2 μg/ml leupeptin. Beads were pelleted at 7500g for 2 min and washed three times with RIPA buffer [50 mM Tris–HCl, pH 7.5, 1% IGEPAL CA-630 (v/v), 150 mM NaCl, and 0.5% sodium deoxycholate]. Protein was removed from the beads by boiling in sample buffer (120 mM Tris–HCl, pH 6.8, 10% glycerol, 3% SDS, 20 mM DTT, and 0.4% bromophenol blue) for 5 min and subjected to 10% SDS–PAGE. Western blot analysis was carried out as described above.

RESULTS

Effect of PMA on the transcription of cPLA2 gene

Expression of cPLA2 was detected by northern blot and immunoblot analysis in PMA-treated A549 cells. PMA induced the expression of cPLA2 mRNA (Figure 1A) and protein (Figure 1B) in a time-dependent manner. To confirm that PMA enhanced the expression of cPLA2 through the transcriptional activation of the gene, the vector containing human cPLA2 gene promoter was used in the reporter assay. PMA induced the promoter activity of cPLA2 in a time-dependent manner (Figure 2A). The maximum effect of PMA on the promoter activity was observed in cells treated with PMA for 18 h. To identify the promoter elements responsible for PMA induction, DNA constructs with 5'-deletions of cPLA2 promoter were transiently transfected into cells. The effect of PMA on the luciferase reporter activity was then studied and summarized in Figure 2B. The luciferase activities of vectors pPLA599, pPLA393, pPLA241, pPLA140 and pPLA53 were stimulated by PMA treatment. The stimulation was about 5-fold, obtained by comparing the luciferase activity in PMA-treated cells with that in control cells. An apparent decrease in the stimulatory response of PMA was observed in vectors bearing a
promoter with a deletion from −53 (pPLA 53) to −27 bp (pPLA 27), indicating that a promoter region ranging from −53 to −27 bp was required for the PMA-induced expression of cPLA2β. To further confirm whether two Sp1-binding sites (−45 to −42 bp and −33 to −29 bp) located in the region were important for PMA response, DNA constructs with mutations on Sp1-binding sites were transfected into cells. Compared to pPLA599, a significant decrease in PMA-stimulated response was observed in pPLA m1 and pPLA m2, and a complete elimination of PMA response was detected in pPLA m1/2. In addition, overexpression of Sp1 also induced the promoter activity of cPLA2β (Figure 2C). These results indicated that two Sp1 sites were essential for PMA-induced transcriptional activation of cPLA2β gene and Sp1 was involved in the regulation of gene expression.

Effect of c-Jun/Sp1 on the PMA-induced transcriptional activation of cPLA2β gene

Since overexpressing Ha-Ras leads to induction of c-Jun protein and drives rat cPLA2β promoter activity (23), we then studied the possibility that whether c-Jun also cooperated with Sp1 in the regulation of PMA-induced expression of human cPLA2β gene. To confirm the role of c-Jun in PMA-regulated gene expression, c-Jun siRNA oligonucleotides were transfected into cells. As shown in Figure 3A, c-Jun siRNA inhibited PMA-induced promoter activity and protein expression of cPLA2β. Furthermore, overexpression of c-Jun also induced the promoter activity of cPLA2β gene (Figure 3B). Consistent with the results of Figure 2B, the luciferase activity of vectors pPLA 599, pPLA 393, pPLA 241, pPLA 140 and pPLA 53 but not pPLA 35 and pPLA 27 were stimulated by pRSVjun (Figure 3B). A significant decrease in pRSVjun response was observed in pPLA m1 and pPLA m2, whereas the response was completely abolished in pPLA m1/2. These results indicated that the two Sp1 sites play an important role in c-Jun-induced transcription of cPLA2β gene as well as the PMA response (Figure 2B). Since there is no AP1 site within the promoter region ranging from −53 to −27 bp, we therefore speculated that the effect of c-Jun on gene expression was mediated by cooperation with Sp1.

We then studied the functional interaction between c-Jun and Sp1 in the regulation of PMA-induced gene expression of cPLA2β by immunoprecipitation, DAPA and ChIP assay. Expression of Sp1 and c-Jun in nuclear extracts prepared from cells treated with PMA for 1–6 h was studied by using immunoblot analysis. No difference of Sp1 expression between control and PMA-treated cells was observed (Figure 4A). PMA induced the expression of c-Jun (Figure 4A), and enhanced the interaction between c-Jun and Sp1 (Figure 4B). To directly examine whether the c-Jun/Sp1 complex bound to cPLA2β promoter in PMA-treated cells, the probes containing two Sp1-binding sites of cPLA2β promoter were used in DAPA. The binding of c-Jun and phospho-c-Jun but not Sp1 to the promoter under the PMA stimulation was increased (Figure 4D and E). In addition, phospho-c-Jun binding to the promoter was significantly increased by over-expressed Sp1 in cells (Figure 2C). In ChIP assay, the binding of c-Jun but not Sp1 to the promoter in cells under the PMA treatment was also enhanced (Figure 4F). These results suggested that PMA induced the formation of c-Jun/Sp1 complex, resulting in facilitating the access of activated c-Jun to the promoter and enhanced the transcriptional activation.

Nucleolin mediates the PMA-induced transcriptional activation of cPLA2β gene

To gain insight into the PMA-regulated transcriptional mechanism of cPLA2β gene, factors bound to Sp1 sites of cPLA2β promoter were analyzed by DAPA. The bound proteins were subsequently analyzed by 2D SDS–PAGE. Proteins on the gel were identified by MALDI-TOF. One of the proteins, nucleolin bound to the probe was then clearly identified (data not shown). As shown in Figure 4D analyzed by DAPA, the binding of nucleolin to the promoter was not changed in PMA-treated cells as well as Sp1 binding. In addition, the constant interaction between nucleolin and Sp1 was observed in control or PMA-treated cells (Figure 4C). However, the interaction of nucleolin with c-Jun was increased in PMA-treated condition (Figure 4C). We further confirmed the binding of nucleolin to cPLA2β promoter by ChIP assay (Figure 4F), which was consistent with the results shown in Figure 4D. Since the formation and binding of c-Jun/nucleolin complex to cPLA2β promoter was increased in PMA-treated cells, it is likely that nucleolin is involved in the regulation of PMA-induced cPLA2β gene expression. We then studied the role of nucleolin in the regulation of PMA-induced gene expression. As shown in Figure 5A, PMA-induced promoter activity and protein expression of cPLA2β were inhibited in nucleolin knockdown cells. Nucleolin siRNA oligonucleotides also significantly inhibited the PMA-induced increase of cPLA2β mRNA (Figure 5B). These results revealed that c-Jun/nucleolin complex played an important role in PMA-induced...
cPLA₂gene expression. Tay et al. (38) have shown that epidermal growth factor, platelet-derived growth factor, serum and PMA exert their effects on cPLA₂expression through post-translational mechanisms involving stabilization of the mRNA. In order to confirm that whether nucleolin-regulated PMA-induced cPLA₂gene expression through transcriptional activation pathway, pGL3 vector containing SV40 promoter and luciferase cDNA without cPLA₂promoter was used. The sequence of SV40 promoter was analyzed by the computer.

Figure 2. Analysis of PMA-responsive element in the promoter region of cPLA₂gene in A549 cells. (A) Cells were transfected with 0.5 μg of luciferase plasmid bearing cPLA₂gene promoter by lipofection, incubated for 24 h and then treated with 5 nM PMA for a different time period as indicated. The luciferase activities and protein concentrations were determined and normalized. The results shown represent the means ± SEM of three determinations. Blank column: non-treatment; black column: PMA-treated cells. (B) Truncated promoter fragment was ligated into a luciferase plasmid as described in the Materials and Methods section. Numbers indicate the distance in base pairs from the translation start site. Cells were transfected with 0.5 μg of luciferase plasmid bearing cPLA₂gene promoter by lipofection, incubated for 24 h and then treated with 5 nM PMA for 18 h. The luciferase activities and protein concentrations were determined and normalized. The expression ratio of PMA treated to control cells is indicated. The results shown represent the means ± SEM of 3 to 8 independent experiments in triplicate wells for each construct. (C) Cells were transfected with 0.5 μg of luciferase plasmid bearing cPLA₂gene promoter by lipofection, incubated for 6 h and then infected with adenovirus carrying GFP-Sp1 (Ad-GFP-Sp1) at 50 MOI. Cells infected with Ad-GFP were used as control. The luciferase activities and protein concentrations were determined. The results shown represent the means ± SEM of three determinations. Cell lysates were prepared and DNA affinity precipitation assay was performed as described under Materials and Methods section. Binding of Sp1 and phospho-c-Jun (Ser73) to cPLA2x Sp1 probes was analyzed by western blot.
The luciferase activities and protein concentrations were determined and normalized. The results shown represent the means ± SEM of three determinations. Expressions of c-Jun, cPLA_2 and β-actin proteins were analyzed by western blot analysis using anti-c-Jun, cPLA_2 and β-actin antibodies, respectively. SC: scramble oligonucleotides. Statistical significance (* * * P < 0.001) between PMA with c-Jun siRNA and PMA alone was analyzed by Student’s t-test. (B) Cells were transfected with 0.5 μg of pRSVjun expression vector and 0.5 μg of luciferase plasmid bearing cPLA_2 gene promoter by lipofection, incubated further for 36 h and the luciferase activities and protein concentrations were determined. The expression ratio of pRSVjun treated to control cells is indicated. The results shown represent the means ± SEM of 3 to 7 independent experiments in triplicate wells for each construct.

Nucleolin and Sp1 are individually essential for the recruitment of c-Jun to GC-rich promoter

Several lines of evidence reveal that the motif (U/G)CCCG(A/G) is important for nucleolin RNA binding (39). However, it is interesting to note that cPLA_2 promoter, containing two potential Sp1-binding sequences, AGCCCA and AGCCCGCT might also be considered as the nucleolin recognition element. We then clarified whether the binding of nucleolin to cPLA_2 promoter was Sp1-dependent. In Sp1 knockdown cells (Figure 7A), the amount of c-Jun on DNA was reduced to 50% (Figure 7B). However, Sp1 siRNA had no significant effect on the binding of nucleolin to DNA. We further studied whether nucleolin was required for Sp1–DNA interaction. Although the interaction of Sp1 with DNA was not changed in nucleolin siRNA-treated cells, the binding of c-Jun to DNA was almost abolished (Figure 7B). To clarify that whether nucleolin bound to DNA through Sp1-binding sites, we studied the binding of nucleolin to wild-type Sp1 probes, compared to mutated probes. As shown in Figure 7C, mutated Sp1 probes abolished both bindings of Sp1 and nucleolin to DNA, resulting in complete inhibition of the interaction between c-Jun and DNA. These results indicated that the bindings of nucleolin and Sp1 to DNA were independent of each other. Furthermore, in addition to Sp1, nucleolin might also be an anchor protein to recruit c-Jun to Sp1 sites and activated cPLA_2 gene expression in PMA-treated cells. In order to further dissect that whether the binding of nucleolin to Sp1 site was mediated by recognizing the canonical or non-canonical Sp1 sequence, the promoters...
with consensus Sp1 sequence of 12(S)-lipoygenase (33) and gastrin (40) or consensus Sp1 oligonucleotides were used in DAPA. Although the binding of Sp1 to Sp1-binding site was observed as expectations, the interaction between nucleolin and DNA was only observed in cPLA2α promoter, 12(S)-lipoygenase promoter and consensus Sp1 oligonucleotide. However, no interaction between nucleolin and DNA was observed in gastrin promoter or consensus NFκB site (Figure 7D). The binding of phospho-c-Jun to DNA was increased in all oligonucleotide probes in PMA-treated cells (Figure 7D). These results revealed that not all the Sp1 or Sp1-like binding sites were in favor of nucleolin binding, indicating that the binding of nucleolin to Sp1 site was not determined by canonical or non-canonical Sp1-binding sequence. To further confirm that nucleolin was essential for c-Jun/Sp1-regulated gene expression resulted from the interaction of nucleolin and Sp1, the luciferase assay was performed using anti-nucleolin, cPLA2 and GAPDH antibodies, respec-

Figure 4. PMA induces the complex formation and the binding of c-Jun, nucleolin and Sp1 to cPLA2α gene promoter in A549 cells. (A) Cells were starved for 18 h in serum-free culture medium and then treated with 5 nM PMA for a different time period as indicated. The Sp1, nucleolin (NCL) and c-Jun proteins were detected by anti-Sp1, anti-nucleolin and anti-c-Jun antibodies, respectively. (B and C) Nuclear extracts were immunoprecipitated (IP) with antibodies (Ab) against Sp1 and c-Jun. The proteins were subjected to SDS–PAGE and analyzed by western blotting with antibodies against c-Jun, NCL and Sp1. IgG: negative control of antibodies. (D and E) Cells were starved for 18 h in serum-free culture medium and then treated with 5 nM PMA for a different time period as indicated. Nuclear extracts were prepared and DNA affinity precipitation assay was performed as described under Materials and Methods section. Binding of Sp1, NCL, c-Jun and phospho-c-Jun (Ser73) to Sp1 probes were analyzed by western blot. The streptavidin-agarose beads were used to serve as a nonspecific binding control. (F) Cross-linked chromatin derived from PMA-treated cells was immunoprecipitated with c-Jun, NCL and Sp1 antibodies and analyzed by PCR with specific primers for the region from −239 to +19 bp of cPLA2α promoter. Input: nonimmunoprecipitated cross-linked chromatin.

Figure 5. Effect of nucleolin on PMA-induced expression of cPLA2α in A549 cells. (A) Cells were transfected with nucleolin siRNA oligonucleotide (siNCL) by lipofection, incubated for 6 h and then transfected with 0.5 μg of pPLA 599 again. Cells were incubated for 24 h and then treated with 5 nM PMA for 18 h. The luciferase activities and protein concentrations were determined and normalized. The results shown represent the means ± SEM of three determinations. Expressions of nucleolin, cPLA2α and β-actin proteins were analyzed by western blot analysis using anti-nucleolin, cPLA2α and β-actin antibodies, respectively. SC: scramble oligonucleotides. Statistical significance (**P < 0.01 and ***P < 0.001) between PMA with nucleolin siRNA and PMA alone was analyzed by Student’s t-test. (B) Cells were transfected with nucleolin siRNA oligonucleotide by lipofection for 36 h and then treated with 5 nM PMA for 18 h. Total RNA was extracted for RT-PCR with nucleolin, cPLA2α and GAPDH primers. The relative density of PCR products was quantified as indicated. SC: scramble oligonucleotides.
recruitment of c-Jun to Sp1 sites-dependent transcriptional activation, we studied the effect of nucleolin on the induction of 12(S)-lipoxygenase which is Sp1-dependent, and cyclooxygenase-2 which is Sp1-independent in cells under EGF treatment (33,34). Under these experimental conditions, c-Jun is essential for the EGF-induced expression of 12(S)-lipoxygenase and cyclooxygenase-2. Consistent with that observed in the regulation of cPLA2\alpha gene, nucleolin siRNA abolished the EGF-induced transcriptional activation of 12(S)-lipoxygenase gene (Figure 8A). Contrary to c-Jun/sp1-regulated genes, nucleolin was not involved in EGF-induced transcriptional activation of cyclooxygenase-2 gene (Figure 8B).

**DISCUSSION**

The expression of cPLA2 can be regulated in NSCLC (7) and is critical for transformed growth of NSCLC. When these cells are treated with a specific inhibitor of the enzyme, or blocking downstream production of prostaglandins with cyclooxygenase inhibitors, resulting in inhibition of anchorage-independent growth of these cells (7). These results suggest that induction of cPLA2 is critical for tumorogenesis. Consistent with this finding, lung tumorigenesis is inhibited in mice that are deficient in cPLA2 (41). In this study, we first found that PMA induced gene expression of cPLA2 in NSCLC. It has been known that PMA-activated PKC signal transduction pathway is thought to be involved in the oncogene
action in NSCLC and enzymatic activation of cPLA2. Activation of PKC with PMA impairs progression of lung adenocarcinoma cells from early G1 phase into S phase (42). The broad-range PKC inhibitor staurosporine analog, PKC 412, induces apoptosis in SCLC cells and sensitizes NSCLC cells to apoptosis induced by DNA-damaging agents (43). Taking these results together, we concluded that PMA-induced expression of cPLA2 might be related to either a cause or a consequence of PMA-regulated tumorigenesis of NSCLC.

In the regulation of gene expression, previous studies have reported that c-Jun/Sp1 complex is critical for various different gene expressions, e.g. 12(S)-lipoxigenase, keratin 16, p21$^{WAF1/CIP1}$ and neuronal nicotinic acetylcholine receptor β4 (26-29). Although overexpression of c-Jun and Sp1 promotes a synergistic increase in the rat cPLA2 promoter activity, no evidence shows that the formation of c-Jun/Sp1 complex and the complex binding to the rat cPLA2 promoter are observed (23). However, we first clarified that PMA induced c-Jun/Sp1 interaction and the complex bound to Sp1-binding sites of human cPLA2 promoter, resulting in the induction of transcriptional activity. In addition, we found that nucleolin was also a coactivator interacting with c-Jun to regulate PMA-induced transcriptional activation of cPLA2 gene. Nucleolin is a ubiquitous, nonhistone nucleolar phosphoprotein of exponentially growing eukaryotic cells, which is directly involved in the regulation of ribosome biogenesis, the processing of ribosomal RNA, mRNA stability, transcriptional regulation and cell proliferation, and it is also a downstream target of several signal transduction pathways (30). Matching our results, the functional role of nucleolin involved in the transcriptional activation of gene expression has been found. Nucleolin binds acetylated interferon regulatory factor-2 (IRF-2) to enhance H4 promoter activity (44) and plays a key activator of HPV18 oncoprotein transcription in cervical cancer (45). Nucleolin also binds transcription factors Myb and tumor suppressor Rb to regulate Myb transcriptional activity and tumor development (46,47). These findings suggest that nucleolin may act as a transcriptional regulator via interacting with activators. Indeed, in the experiments of immunoprecipitation and DAPA, we found that nucleolin bound and bridged c-Jun to Sp1-binding sites of cPLA2 promoter (Figures 4C and 7B). These results suggested the possibility that nucleolin participated in the regulation of c-Jun/Sp1-regulated genes, e.g. 12(S)-lipoxigenase (Figure 7D) and p21$^{WAF1/CIP1}$ to regulate cell growth (48). From our study, we can conclude that for PMA-induced cPLA2 gene expression, and nucleolin acts as a coactivator via cooperation of c-Jun transcriptional factor. However, the nucleolin/c-Jun complex had no effect on AP1-regulated promoter activation (Figures 6C and 8B). The different binding affinity between nucleolin/c-Jun to Sp1-binding site and to AP1-binding site might be caused by the stereo-recognition of complex to the Sp1 binding but not AP1-binding sequences. Consistent with the role of nucleolin in the regulation of mRNA stability, we found that nucleolin also contributed to the stabilization of cPLA2 mRNA. Since without identical nucleolin-binding site UCCCGA but with HuR-binding element AUUUA is found within the cPLA2 3′UTR sequence, the effect of nucleolin on the stabilization of cPLA2 mRNA might result from the cooperation of nucleolin with RNA-binding proteins as well as the basal expression of GADD45α mRNA is regulated by association of nucleolin and HuR (49). Indeed, we found that RNA-binding protein HuR bound to AUUUA of cPLA2 3′UTR sequence and enhanced the mRNA stability (our unpublished data). Although PMA had no effect on the stabilization of cPLA2 mRNA, our data showed that nucleolin was also involved in the regulation of PMA-induced transcriptional activation of cPLA2 gene. These results revealed that nucleolin plays dual functions of transcriptional and post-transcriptional activity in the regulation of cPLA2 gene.

It is known that the motif (U/G)CCC(A/G) is responsible for the binding of nucleolin to RNA (39). However, we found that the binding of nucleolin to the GC-rich Sp1 site within cPLA2 promoter was in an Sp1-independent manner. Thus, nucleolin might gain access to DNA through two pathways. First, since Sp1 siRNA had no effect on the binding of nucleolin to Sp1 element, indicating that nucleolin might directly bind to DNA and recruit c-Jun transcriptional factor to Sp1 sites and then activate gene expression. Nucleolin is a matrix attachment region (MAR)-binding protein to provide a link between DNA and nuclear matrix scaffolding (50). In addition, nucleolin was also found to bind to the NFκB DNA-binding motif and the KLF2 promoter (51,52). Thus, nucleolin may interact with DNA and other transcriptional factors, such as c-Jun, to regulate transcription. It is very interesting to note that whether the binding of nucleolin to GC-rich Sp1 site has the sequence specificity. Contrary to the binding of nucleolin to cPLA2 promoter, we found that SV40 promoter containing Sp1-binding sites was not regulated by nucleolin. Furthermore, we also found that the interaction between nucleolin and Sp1-binding site was not occurred in all Sp1-regulated

Figure 8. Nucleolin is essential for c-Jun/Sp1-regulated gene expression. (A and B) A431 cells were transfected with nucleolin siRNA oligonucleotides by lipofection, incubated for 6h and then transfected with 0.5μg of pXLO-7-1 (A) and pXC918 (B) vectors bearing the 12(S)-lipoxigenase and cyclooxygenase-2 promoters, respectively. Cells were incubated for 24 h and pXLO-7-1- and pXC918-transfected cells were treated with EGF (50 ng/ml) for 18 and 6h, respectively. The luciferase activities and protein concentrations were determined and normalized. The results shown represent the means± SEM of three determinations. SC: scramble oligonucleotides. Statistical significance (**P < 0.001) between EGF with nucleolin siRNA and EGF alone was analyzed by Student’s t-test.
promoters such as gastrin. It is possible that the nucleotide sequence surrounding the AGCCC and UCCCGA can modulate the interaction of nucleolin with DNA and RNA (39), respectively. Second, access of nucleolin to modulate the interaction of nucleolin with DNA and sequence surrounding the AGCCC and UCCCGA can promote such as gastrin. It is possible that the nucleotide sequence surrounding the AGCCC and UCCCGA can modulate the interaction of nucleolin with DNA and RNA (39), respectively. Second, access of nucleolin to DNA might have occurred concomitant with Sp1-like proteins binding. In addition, in spite of nucleolin and Sp1 bound to DNA was dependent on Sp1-binding sites, the binding of Sp1 to DNA was in a nucleolin-independent manner. These results suggested that Sp1/c-Jun and nucleolin/c-Jun complexes could together or independently bind to Sp1-binding sites and regulate PMA-induced transcriptional activation of cPLA2α gene.

In conclusion, we have identified the functional interaction between c-Jun, nucleolin and Sp1 to mediate cPLA2α expression in PMA signal-activated human tumor cells. Our results demonstrate that the activation of the PMA signaling pathway leads to the binding of c-Jun/ nucleolin and c-Jun/Sp1 complexes to the cPLA2α promoter, resulting in the transcriptional activation of cPLA2α gene. Based on the expression of cPLA2α is critical for transformed growth of NSCLC and tumorigenesis (7,41), our study suggested that nucleolin, in addition to contribute to the process of tumor development by linking to major tumor suppressors, Rb and p53 (47,53), could associate with transcription factor c-Jun and up-regulate cPLA2α expression to increase lipid mediators, resulting in the tumorigenesis. Our data also suggested that nucleolin binds to GC-rich elements. It is likely that nucleolin binding at place of Sp1-binding sequences could also mediate the expression of c-Jun/Sp1-activated genes.

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