Activation of Protein Kinase C-δ (PKC-δ) by All-trans-retinoic Acid

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Running Title: RA activates protein kinase C-δ
Abstract

All-trans-retinoic acid is a potent inhibitor of leukemic cell proliferation and induces differentiation of acute promyelocytic leukemia cells in vitro and in vivo. In order for RA to induce its biological effects in target cells, binding to specific retinoic acid nuclear receptors is required. The resulting complexes bind to RA responsive elements in the promoters of RA-inducible genes, to initiate gene transcription and to generate of protein-products that mediate the biological effects of RA. In the present report, we provide evidence that a member of the protein kinase C family of proteins, PKC-δ, is activated during RA-treatment of the NB-4 and HL-60 acute myeloid leukemia cell lines, as well as the MCF-7 breast cancer cell line. Such RA-dependent phosphorylation is also observed in primary acute promyelocytic leukemia cells and results in activation of the kinase domain of PKC-δ. In studies aimed to understand the functional relevance of PKC-δ in the induction of RA-responses, we found that pharmacological inhibition of PKC-δ, but not other PKC-isoforms, diminishes RA-dependent gene transcription via RARE elements. On the other hand, overexpression of a constitutively active form of the kinase, strongly enhances RA-dependent gene transcription via RARE-elements. Gel shift assays and chromatin immunoprecipitation (ChIP) studies demonstrate that PKC-δ associates with RARα and is present in a RA-inducible protein-complex that binds to RARE elements. Pharmacological inhibition of PKC-δ-activity abrogates the induction of cell-differentiation and growth inhibition of NB-4 blasts, demonstrating that its function is required for such effects. Altogether, our data provide strong evidence that PKC-δ is activated in an all-trans-retinoic acid-dependent manner and plays a critical role in the generation of the biological effects of all-trans-retinoic acid in malignant cells.
**Introduction**

All-trans-retinoic acid (RA)\(^1\) is a potent inducer of cellular differentiation and growth arrest of malignant cells *in vitro* and *in vivo* (1-6). This agent has potent effects against acute promyelocytic leukemia blasts, and its introduction in the clinical management of the disease has dramatically changed the outcome of this historically fatal subtype of acute leukemia (5). All-trans-retinoic acid and other retinoids have been shown to inhibit cell growth or to promote programmed cell death of neoplastic cells of diverse origin (7-14). The molecular mechanisms that regulate the induction of the biological effects of retinoids, include a series of signaling events, which are initiated by the binding of retinoids to specific receptors in the nucleus of target cells. Two families of retinoid receptors have been so far identified; RAR (types \(\alpha, \beta, \gamma\)) which are activated by both all-trans retinoic acid and 9-cis retinoic acid, and RXRs (types \(\alpha, \beta, \gamma\)) which are activated only by 9-cis retinoic acid (15-17). RA binds to the RAR nuclear receptors and induces the formation of RAR/RXR heterodimers, which associate with specific DNA binding sequences present in the promoters of RA-responsive genes, called retinoic acid response elements (RARE). Such binding of RA-nuclear receptor complexes to RARE promoter elements results in initiation of transcription of genes whose protein products mediate RA-biological responses (15-17).

In addition to the induction of formation of RAR/RXR complexes, RA induces a variety of other cellular effects that appear to play a role in the generation of its effects on target cells. Such mechanisms via which retinoids induce their biological effects on malignant cells include inhibition of activation of the AP-1 protein via a CBP-regulated mechanism (18, 19), modulation of histone acetylation (20), and up-regulation of TGF-\(\beta\)2 and insulin-like growth factor binding protein-3 expression (21).
Retinoids also regulate the activation of members of different groups of Map kinases. It has been previously shown that they inhibit activation of the c-Jun N-terminal kinase (22), and such inhibition appears to be required for the induction of retinoid-responses (22). On the other hand, all-trans-retinoic acid induces activation of the extracellular signal-regulated kinase (Erk2) mitogen-activated protein kinase (23), as well as activation of the p38 Map kinase (24). The activation of Erk2 mediates positive regulatory effects in the induction of retinoid-responses, and its function appears to be essential for RA-dependent differentiation of HL-60 cells (23). On the other hand, activation of the p38 Map kinase exhibits negative regulatory effects on the induction of differentiation of NB-4 cells by RA (24), and pharmacological inhibitors of this kinase promote the antileukemic effects of RA in vitro (24).

The PKC-family of proteins is a multi-gene family of at least 12 serine/threonine kinases that participate in signal transduction events and are classified into three groups based on the differences in their structure and regulatory domains, as well as differences in their activation requirements (25). The protein-members of this family of kinases exhibit serine-kinase activities and upon their activation regulate phosphorylation/activation of other serine kinases, resulting in signals that ultimately mediate multiple biological responses. The tissue distribution of PKC isoforms varies considerably, with PKC-α, -δ, -ζ being widely-expressed, whereas most of the other isoforms are selectively expressed in different types of cells and tissues (25). PKC-δ belongs to the group of novel PKC isoforms, which are Ca^{2+}-independent and are activated by phorbol-esters, diacylglycerol and phosphatidylserine (26). Previous studies have shown that this kinase plays important roles in the induction of anti-proliferative and pro-apoptotic responses in response to DNA-damaging agents and ionizing radiation (27, 28). Consistent with
this, it has been demonstrated that overexpression of its catalytically active fragment is capable of inducing apoptosis of target cells (29).

In the present study we provide evidence that PKC-δ is activated during treatment of acute promyelocytic leukemia and breast cancer cell lines with all-trans-retinoic acid. Our data demonstrate that this PKC-isoform forms complexes with RARα and binds to RARE elements. Such a function of PKC-δ plays a critical role in RA-dependent transcriptional regulation, as evidenced by the fact that inhibition of PKC-δ kinase activity blocks all-trans-retinoic acid-dependent gene transcription via RARE elements. Consistent with this, pharmacological inhibition of PKC-δ diminishes induction of cell-differentiation of APL blasts by RA and blocks RA-dependent suppression of cell-growth, underscoring the critical role that this PKC-isoform plays in the induction of RA-responses.
Experimental Procedures

**Cells and Reagents** The all-trans-retinoic acid-sensitive human acute promyelocytic leukemia NB-4 cell line and the acute myeloid leukemia HL-60 cell line were grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. MCF-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Polyclonal antibodies against PKC-δ and Stat1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against the phosphorylated/activated form of PKC-δ at threonine 505 was obtained from New England Biolabs (Beverly, MA). An antibody against the phosphorylated form of Stat1 on serine 727 (anti-Ser-727Stat1) was obtained from Upstate Biotechnology, Inc. The PKC-δ inhibitor rottlerin and the PKC-α inhibitor G06976 were obtained from Calbiochem Inc.

**Cell Lysis, Immunoprecipitations and Immunoblotting** Cells were treated with all-trans-retinoic acid (final concentration 1 µM) for the indicated times, and lysed in phosphorylation lysis buffer as previously described (30-32). Immunoprecipitations and immunoblotting using an ECL (enhanced chemiluminescence) method were performed as previously described (30-32).

**PKC-δ Kinase Assays** Immune complex kinase assays to detect PKC-δ activation were performed as previously described (33, 34). Briefly, cells were treated for the indicated times with retinoic acid and were then lysed in phosphorylation lysis buffer. Cell lysates were immunoprecipitated with an anti-PKC-δ antibody, and immunoprecipitates were washed three times with phosphorylation lysis buffer and two times with kinase buffer (25 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 20 µg of phosphatidylinerine, 20 µM
ATP) and were resuspended in 30 μl of kinase buffer containing 5 μg of histone H1 as an exogenous substrate, to which 20-30 μCi of [γ-32P]ATP was added. The reaction was incubated for 15-30 min at room temperature and was terminated by the addition of SDS-sample buffer. Proteins were analyzed by SDS-PAGE, and the phosphorylated form of histone H1 was detected by autoradiography.

**Mobility Shift Assays** Gel shift and supershift assays were performed as previously described (35-37). Briefly, nuclear extracts from untreated or RA-treated cells were incubated with or without double-stranded oligo-deoxynucleotide corresponding to a DR5-RARE sequence (AGGGTAGGGTTCACCGAAAGTTCACTC), in the presence or absence of cold-oligonucleotide. Supershifts using antibodies against PKC-δ or RARα were performed as previously described (35-37).

**Luciferase Reporter Assays** MCF-7 cells were transfected with a β-galactosidase expression vector and an RARE-luciferase plasmid (38) using the superfect transfection reagent as per the manufacturer's recommended procedure (Qiagen). Forty eight hours after transfection, triplicate cultures were either left untreated or treated with RA for 16 h in the presence or absence of pharmacological inhibitors of the different PKC-isoforms. The cells were pre-incubated with Go 6976 (2.5 nM), LY 3791196 (50 nM), rottlerin (5 μM), and PKC-ζ pseudosubstrate (50 μM), specific inhibitors for PKC-α (39), PKC βI/βII (40), PKC-δ (33, 34) and PKC-ζ (41), respectively, prior to the addition of all-trans-retinoic acid to the cultures. The cells were then washed twice with cold phosphate-buffered saline, and after cell lysis, luciferase activities were measured using the protocol of the manufacturer (Promega). The measured luciferase activities were
normalized for β-galactosidase activity for each sample. In other experiments, MCF-7 cells were transfected with an 8X-GAS luciferase construct, and forty-eight hours after transfection, triplicate cultures were either left untreated or treated with IFN-α (5000 U/ml), RA, rottlerin, or combinations of these agents. In the experiments in which the effects of overexpression of a wild type or constitutively active PKC-δ were determined on RARE-dependent gene transcription were evaluated, the cells were transfected with the pCDNA3-PKC-wt construct (42) or the pCDNA3-PKC-CAT construct, which encodes a truncated protein in which the catalytic domain (CAT) of PKC is preserved and the regulatory N-terminal domain (REG) is deleted in thereby generating a constitutively active catalytic domain, provided by Dr. J-W. Soh (Columbia University College of Physicians and Surgeons, New York, NY). (42).

**Cell Proliferation Assays** Cell proliferation assays using an MTT assay system were performed as in previous studies (43).

**Chromatin Immunoprecipitation (ChIP) Assays** Chromatin immunoprecipitation (ChIP) assays were performed essentially as previously described (44, 45). ChIP DNA was used as a template for polymerase chain reaction (PCR) using DR5-RARE forward (FP) (5′-CAC TGC AGA AAC AGC CAG -3') and reverse (RP) 5′-CAT GGG CAG GCT GAT AAG -3' primers.

**Flow Cytometric Analysis** Flow cytometric studies were performed as in our previous studies (24). Briefly, NB-4 cells were treated with DMSO or RA in the presence or absence of rottlerin 1 μM for 5 days, and cell differentiation was determined by staining with the anti-CD11b
monoclonal antibody. The anti-CD11b monoclonal antibody and a matched isotype control were purchased from Coulter Immunotech.
Results

We first determined whether treatment of cells with all-trans-retinoic acid induces activation of PKC-δ in the NB-4 acute promyelocytic leukemia cell line, which expresses the t(15;17) translocation. NB-4 cells were incubated in the presence or absence of RA for different times, and were subsequently lysed in phosphorylation lysis buffer. After cell lysis, total lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of PKC-δ on threonine 505. As shown in Fig. 1, RA-treatment of NB-4 cells induced strong phosphorylation of PKC-δ, which was time-dependent, with the intensity of the signal being strong at 12 hours of RA-treatment and gradually declining to baseline levels at 48 to 72 hours (Fig. 1A). Stripping and re-probing the same blot demonstrated that equal amounts of PKC-δ protein were detectable prior to and after RA- treatment, indicating that RA-treatment does not affect the levels of PKC-δ protein expression (Fig. 1B). Similarly, phosphorylation of PKC-δ protein was inducible by in vitro treatment of primary leukemia cells, isolated from the peripheral blood of a patient with APL with the t (15;17) translocation (Fig. 1C). To directly determine whether the phosphorylation of PKC-δ results in induction of its kinase activity, NB-4 cells were treated with RA, cell lysates were immunoprecipitated with an anti-PKC-δ antibody, and in vitro kinase assays were carried out on the immunoprecipitates, using histone H1 as an exogenous substrate. PKC-δ, immunoprecipitated from lysates of cells treated with RA, induced strong phosphorylation of H1 in the in vitro kinase assay (Fig. 1D), indicating that the catalytic activity of PKC-δ is induced in a RA-dependent manner during its phosphorylation on threonine 505.
In subsequent studies, we sought to determine whether phosphorylation/activation of PKC-δ occurs in other all-trans-retinoic acid sensitive-cell lines. We performed experiments using the HL-60 acute myeloid leukemia and the MCF-7 breast carcinoma cell lines, both of which are sensitive to the growth inhibitory effects of all-trans-retinoic acid (23, 46-48). Treatment of HL-60 (Fig. 2A and B) or MCF-7 (Fig. 2C and D) cells with RA resulted in strong phosphorylation/activation of PKC-δ, indicating that the RA-inducible activation of this serine kinase is not restricted to APL cells expressing the t(15;17) translocation, but also occurs in other RA-sensitive neoplastic cells.

It is well established that retinoids induce their biological effects by regulating gene-transcription for proteins that mediate cell differentiation, cell cycle arrest and/or apoptosis of target neoplastic cells (15-17). Such RA-dependent gene transcription is regulated by binding of retinoid:retinoid receptor complexes to RARE elements, present in the promoters of sensitive-genes (15-17). As our data demonstrated that PKC-δ is activated during treatment of cells with RA, we sought to determine whether it plays a role in RA-dependent transcriptional regulation. We first examined whether H7, a non-specific pan-PKC inhibitor, inhibits RA-dependent gene transcription. We performed experiments in which MCF-7 cells were transfected with a plasmid containing an RARE-luciferase construct and treated with RA, in the presence or absence of H7. As shown in Fig. 3A, H7 significantly abrogated RA-RARE-mediated luciferase activity, suggesting that protein kinase C-activity is required for RA-dependent gene transcription. We subsequently determined whether rottlerin, a specific inhibitor of PKC-δ (25, 33, 34, 49-51), exhibits negative regulatory effects on RA-inducible transcriptional activation. MCF-7 cells were transfected with the RARE-luciferase construct and were treated with RA, in the presence or absence of rottlerin, or inhibitors that exhibit specificity towards other PKC-
isoforms, but do not inhibit PKC-δ. The RA-dependent increase in RARE-dependent gene transcription was blocked when cells were pre-treated with rottlerin (Fig. 3B). On the other hand, the G06976 inhibitor which selectively inhibits PKC-α (39) and the LY379196 inhibitor which selectively inhibits PKC-β (40) had no effects on RARE-dependent luciferase activity (Fig. 3B). Similarly, a PKC-ζ pseudosubstrate (41), had no significant effects on transcriptional regulation via RARE elements (Fig. 3B), further establishing the specificity of the process.

Previous studies have established that Stat1 is up-regulated in a RA-dependent manner, and such up-regulation of Stat1 appears to be responsible for the induction of the synergistic effects that RA and interferons exhibit in malignant cells (52-55). As pharmacological inhibition of PKC-δ blocked RARE-dependent gene transcription, we sought to determine whether such inhibition also blocks up-regulation of Stat1-protein expression by RA. NB-4 cells were incubated with RA for 24 or 48 hours, the cells were lysed, and total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the serine phosphorylated form of Stat1 on ser727 (Fig. 4A) or against Stat1 (Fig. 4B). Consistent with previous reports (50-53), significantly higher levels of Stat1 were detectable in RA-treated samples (Fig. 4B). Also, there was an increase in the level of Stat1 phosphorylated on ser727 (24) (Fig. 4A), likely reflecting the increase in the levels of Stat1 protein induced by RA. Treatment of cells with rottlerin decreased the RA-dependent serine phosphorylated form of Stat1 (Fig. 4A), as well as the levels of total Stat1-protein (Fig. 4B). Thus, based on these findings, it is likely that PKC-δ is required for the induction of RA-dependent expression of Stat1, suggesting that it plays a role in the induction of the synergistic effects of RA and interferons.

To definitively establish the role of PKC-δ in RARE-dependent gene transcription, we determined whether overexpression of wild-type PKC-δ or a constitutively-active form of PKC-
δ, enhances RA-dependent transcriptional regulation. MCF-7 cells were transfected with constructs for either wild-type PKC-δ (pCDNA3-PKC-δ-Wt) (Fig. 5A) or a constitutively active form of PKC-δ (pCDNA3-PKC-δ-CAT) (Fig. 5B), and the DR-5-RARE luciferase plasmid. The cells were subsequently incubated in the presence or absence of RA and luciferase assays were performed. Overexpression of wild-type PKC-δ resulted in substantial enhancement of RA-dependent gene transcription (Fig. 5A). Such an enhancement was abrogated when cells were treated with rottlerin, demonstrating the specificity of the process (Fig. 5A). On the other hand, overexpression of the constitutively-active form of PKC-δ increased luciferase activity at baseline (prior to RA-treatment) (Fig. 5B), and resulted in further enhancement of RA-dependent RARE-mediated gene transcription (Fig. 5B).

As our data indicated a role for PKC-δ in RA-mediated gene transcription and modulation of Stat1 protein-expression, we sought to determine whether its function is essential for the induction of the synergistic effects of RA and IFNα. We have previously shown that PKC-δ is activated by the Type I IFN receptor and that such activation is required for Type I IFN-dependent gene transcription via ISRE or GAS elements (33). As RA upregulates Stat1 expression in a PKC-δ-dependent manner, we examined whether pre-treatment of cells with RA enhances IFNα-inducible gene transcription via GAS elements, and if so, whether PKC-δ-activity is required for such effects. MCF-7 cells were transiently transfected with an 8X-GAS luciferase construct, and were subsequently treated with IFNα or the combination of IFNα and RA. As expected, treatment of cells with IFNα resulted in induction of GAS-driven luciferase activity (Fig. 6). Combined treatment of the cells with RA and IFNα resulted in substantially higher levels of luciferase activity, while concomitant treatment of cells with rottlerin abrogated
the IFNα+RA synergistic effects (Fig. 6), strongly suggesting that PKC-δ activity is required for the generation of such responses.

To further understand the mechanisms by which PKC-δ regulates RA-dependent gene transcription, we examined whether during RA-stimulation PKC-δ associates with and forms complexes with other proteins that bind to RARE elements. We performed gel-shift assays using a double-stranded RARE/DR-5 oligonucleotide. As expected, treatment of NB-4 cells with all-trans-retinoic acid resulted in the induction of several complexes that bound RARE elements (Fig. 7A). Such complexes were competed by cold-oligonucleotide (Fig. 7A), demonstrating the specificity of the binding. Some of the bands detected in the gel-shift assay were supershifted by an anti-PKC-δ antibody, but not control non-immune rabbit immunoglobulin (RIgG), indicating that the PKC-δ protein participates in the formation of RARE-binding regulatory complexes (Fig. 7A). As expected, the RA-dependent DNA-binding complexes were also supershifted by an anti-RARα receptor antibody (Fig. 7B). Consistent with these findings, in studies using nuclear extracts from RA-treated NB-4 cells, we found that the PML-RARα fusion protein was co-immunoprecipitated by the anti-PKC-δ antibody, in a RA-dependent manner (Fig. 8A and B). Most importantly, when chromatin immunoprecipitation (ChIP) assays were performed, we found that PKC-δ was present in a complex that binds to RARE elements in a RA-dependent manner in NB-4 cells (Fig. 9). These findings provide very strong evidence that PKC-δ associates with retinoic acid receptors (RARs), and likely modulates RA-dependent gene transcription via direct interaction with the RA:RARα complex.

In further studies, we sought to examine the biological relevance of RA-dependent activation of PKC-δ in cells of acute promyelocytic leukemia origin. We determined the effects
of inhibition of the PKC-δ pathway on the induction of RA-dependent cell differentiation of NB-4 cells, using an approach that we employed in previous studies (24). Cells were treated with RA in the presence or absence of rottlerin, and the induction of differentiation was determined by staining the cells with an anti-CD11b antibody, the expression of which is a marker for RA-induced myeloid differentiation to the granulocytic stage (24). As expected, RA treatment induced up-regulation of CD11b expression. Concomitant treatment with rottlerin partially reversed the RA-dependent CD11b expression (Fig. 10), indicating that PKC-δ activity is essential for the induction of differentiation of NB-4 blasts to granulocytes.

In parallel studies, we examined whether pharmacological inhibition of PKC-δ reverses the induction of the suppressive effects of RA on cell-proliferation. NB-4 cells were incubated with RA, in the presence or absence of rottlerin or pharmacological PKC-inhibitors that selectively block activation of other isoforms. Consistent with previous reports (24), all-trans-retinoic acid inhibited the growth of NB-4 cells in a dose-dependent manner. Such an inhibition was reversed by concomitant treatment of cells with rottlerin (Fig. 10). On the other hand, G06976 and LY37916 had no significant effects, indicating that PKC-α and PKC-β do not play a role in the generation of the growth inhibitory effects of RA in NB-4 cells (Fig. 11).
Discussion

The protein kinase C (PKC) family of serine-threonine kinases includes several members that play important roles in signaling for various cytokine-receptors in mammalian cells (25, 51, 55-58). The classification of distinct members of the protein kinase C family in different isoform-groups, relies on the requirements that the different isoforms exhibit for activation of their kinase-domains. One group includes PKC-isoforms that require increases in intracellular calcium for their activation. The members of this group, which are also activated by the traditional PKC-activators, the phorbol-esters, are defined as the conventional PKC (cPKC) isoforms. The three known conventional PKC-isoforms are PKC-α, PKC-β, and PKC-γ (25, 51, 55-58). The second group of PKC-isozymes is the group of novel PKCs (nPKCs), which do not require Ca²⁺ for their activation, but are activated by phorbol esters (25, 51, 55-58). PKC-δ, PKC-ε, PKC-θ, PKC-η, and PKC-μ are included in this group. Finally, a third group of atypical PKCs (aPKC) exists, whose members are Ca²⁺-independent and are insensitive to phorbol-esters. PKC-ζ, and PKC-λ are the two known atypical PKC-isoforms (25, 51, 55-58).

The different isoforms of the PKC family participate in signaling cascades for various cytokine- and growth factor-receptors. Extensive studies have shown that these kinases play critical roles in the regulation of several important cellular responses, such as differentiation, cell growth, and apoptosis (25, 51, 55-58). It is of interest that different PKC-isoforms mediate different responses and, in some cases, appear to exhibit opposing effects on cell proliferation and apoptosis. For instance, PKC-ε exhibits oncogenic properties and promotes cell proliferation (59), while PKC-δ mediates antiproliferative and pro-apoptotic signals (28, 59-65). Similarly, PKC-α and PKC-δ exhibit antagonistic effects on the transformation of cells by the
epidermal growth factor receptor, with PKC-\(\alpha\) promoting EGF-transforming activity, and PKC-\(\delta\) inhibiting such a transformation and functioning as a tumor-suppressor gene (64).

Our finding, that PKC-\(\delta\) participates in the generation of all-\textit{trans}-retinoic acid responses and regulates induction of cell-differentiation and antiproliferative effects, is consistent with the previously described capacity of this PKC-isoform to mediate growth-suppressive signals. It is of particular interest that this kinase is also activated by interferons and regulates IFN-dependent gene transcription via modulation of serine phosphorylation of Stat1 (33). Interferons are growth inhibitory cytokines that exhibit synergistic effects with retinoids in the generation of cell-differentiation and growth suppression (66-70). It is noteworthy that RA not only augments the transcription of interferon responsive genes, but also causes increased synthesis and secretion of IFN\(\alpha\) itself (71), raising the possibility of an autocrine-loop mediating Stat1-activation. Our data indicate that, in addition to its involvement in the induction of RA-dependent responses, PKC-\(\delta\) is required for the generation of the synergistic effects of IFN\(\alpha\) and RA on gene transcription. Such regulatory effects on transcription via GAS elements are likely mediated by the RA-inducible, PKC-\(\delta\)-dependent, up-regulation of Stat1 protein-expression. Such effects, beyond mediating IFN\(\alpha\) and RA synergy, may be important for retinoic acid-sensitivity, as a recent study demonstrated that in certain cases retinoic acid resistance is associated with lack of IFN\(\alpha\)–synthesis and Stat1 induction (69).

Our data also establish that PKC-\(\delta\) is present in RA:RAR nuclear complexes that bind to RARE elements. This is demonstrated by gel shift and supershift assays, co-immunoprecipitation experiments, as well as chromatin immunoprecipitation (ChIP) assays. Previous studies had implicated a PKC-isoform in retinoic acid-dependent gene transcription, as evidenced by the fact that depletion of cellular PKC by prolonged treatment with TPA leads to
loss of ligand-dependent transcription (72). Such an effect could be directly linked to loss of DNA-binding activity of complexes containing RARα, but the identity of the PKC-isoform involved was unknown at the time (72). Other studies have demonstrated that PKC-α– or PKC-γ–dependent phosphorylation of RARα on serine 157 correlates with decreased ability of hRARα to heterodimerize with hRXRα, resulting in decreased transcriptional activity (73). As other studies have established that different PKC-isoforms have opposing effects in the induction of certain responses, it is possible that PKC-δ acts as a positive modulator of RARE-dependent gene transcription and opposes the effects of PKC-α and/or PKC-γ. A similar phenomenon appears to occur in the regulation of the RXR receptors in T-lymphocytes, in which case PKCδ synergizes with calcineurin to induce RXR-dependent activation, while such activation is antagonized by the PKC-α isoform (74). Independent of the precise mechanisms involved, our findings provide strong evidence for a novel function of PKCδ in the induction of all-trans-retinoic acid-responses. Future studies should examine whether induction of PKC-δ activity also occurs in response to other retinoids, and whether other PKC-isoforms antagonize the effects of PKC-δ on RA-dependent transcriptional regulation.

At this time the precise upstream regulatory events that ultimately result in PKC-δ-activation are not known. The phosphorylation/activation of PKC-δ by RA may reflect engagement of an inside-out signaling loop, following the formation of RA:RAR complexes, or could be regulated by other early biochemical cellular events induced by RA. There is accumulating evidence that serine-threonine kinases regulate activation of RARs via modulation of their phosphorylation status, and recently, the PI 3’-kinase pathway was shown to exhibit effects on the phosphorylation, degradation, and transcriptional activity of retinoic acid receptor γ2 (RAR γ2) (75). Interestingly, retinoic-acid-dependent neuronal tissue differentiation (76), as
well as induction of expression and activation of the tissue transglutaminase, is PI 3'-kinase-dependent (77). Studies in other systems have also shown that PKC-δ is activated downstream of the PI 3'-kinase via the kinase PDK1 (78, 79). It is therefore possible that the RA-dependent pathway, that ultimately facilitates RARE-dependent transcription, involves sequential activation of a PI 3'-kinase→PDK1→PKC-δ cascade, but this hypothesis remains to be determined in future studies.

**Footnotes**

1The abbreviations used are: RA, all-trans-retinoic acid; PKC, protein kinase C; IFN, interferon; Stat, signal transducer and activator of transcription; GAS, IFNγ activated site; ChIP, chromatin immunoprecipitation; RARE, retinoic acid responsive element; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
References


**Figure Legends**

**Figure 1:** All-trans-retinoic acid (RA) induces phosphorylation and activation of PKC-δ in acute promyelocytic leukemia cells.  

**A.** NB-4 cells were treated with RA for the indicated times. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of PKC-δ on threonine 505.  

**B.** The blot shown in A was stripped and reprobed with an antibody against PKC-δ.  

**C.** Isolated peripheral blood mononuclear cells, from a patient with acute promyelocytic leukemia, were incubated in the presence or absence of RA for the indicated times. The cells were lysed and equal amount of total cell lysates (100 μg/lane) were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of PKC-δ on threonine 505.  

**D.** NB-4 cells were incubated for 24 hours in the presence or absence of 1μM of RA, as indicated. Cell lysates were immunoprecipitated with an anti-PKC-δ antibody and immunoprecipitates were subjected to an *in vitro* kinase assay, using histone H1 as an exogenous substrate. Phosphorylated proteins were detected by autoradiography.

**Figure 2:** All-trans-retinoic acid-dependent phosphorylation of PKC-δ in HL-60 and MCF-7 cells.  

**A.** HL-60 cells were incubated with RA for forty-eight hours, as indicated. Cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of PKC-δ.  

**B.** The blot was subsequently stripped and re-probed with an anti-PKC-δ antibody to control for loading.  

**C.** MCF-7 cells were incubated with RA for the indicated times in hours. Cell lysates were analyzed by SDS-PAGE, and total lysates were immunoblotted with
an antibody against the phosphorylated form of PKC-δ. **D.** The blot shown in **C** was stripped and reprobed with an anti-PKC-δ antibody to control for loading.

**Figure 3:** PKC-δ is required for RA-dependent gene transcription via RARE elements. **A.** MCF-7 cells were transfected with an RARE-luciferase construct. Forty-eight hours after transfection, the cells were pre-incubated for 60 min in the presence or absence of the pan-PKC kinase inhibitor H7 (50 μM). Subsequently, the cells were incubated overnight in the presence or absence of RA (1 μM), and luciferase activity was measured. Data are expressed as fold increase in response to RA treatment over control untreated samples for each condition. The mean ± S.E. values of three independent experiments for each panel are shown. **B.** MCF-7 cells were transfected with an RARE-luciferase construct. Forty-eight hours after transfection, the cells were pre-incubated for 60 min in the presence or absence of rottlerin, G06976, LY379196, or a PKC-ζ pseudosubstrate. Subsequently, the cells were incubated overnight in the presence or absence of RA (1 μM), and luciferase activity was measured. Data are expressed as fold increase in response to RA treatment over control untreated samples for each condition. The mean ± S.E. values of three independent experiments for each panel are shown.

**Figure 4:** PKC-δ is required for RA-dependent induction of Stat1-protein expression. **A.** NB-4 cells were incubated with RA (1 μM) for the indicated times, in the presence or absence of the PKC-δ inhibitor rottlerin, as indicated. Equal amounts of total cell lysates (100 μg/lane) were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of Stat1 on serine 727 (anti-Ser-727Stat1). **B.** NB-4 cells were treated with RA, in the presence or absence of the PKC-δ inhibitor rottlerin, for the indicated times in hours. Equal
amounts of total cell lysates (100µg/lane) were analyzed by SDS-PAGE and immunoblotted with an antibody against Stat1.

**Figure 5:** Enhancement of RA-dependent gene transcription via RARE elements by over-expression of wild-type PKC-δ or a constitutively active form of PKC-δ.  
A. MCF-7 cells were transfected with an RARE-luciferase construct, and either the empty pCDNA3 vector or a pCDNA3-PKC-δ wt construct. Forty-eight hours after transfection, cells were treated for 60 min in the presence or absence of rottlerin. The cells were then incubated overnight with or without RA (1µM), in the continuous presence or absence of rottlerin, and luciferase activity was measured. The data are expressed as relative luciferase units, normalized for β-galactosidase activity. The mean ± S.E. values of two independent experiments in each panel are shown.  
B. MCF-7 cells were transfected with an RARE-luciferase construct, and either the empty pCDNA3 vector or a pCDNA3-PKC-δ CAT construct. Forty-eight hours after transfection, the cells were treated with RA as indicated, and luciferase activity was measured. The data are expressed as relative luciferase units, normalized for β-galactosidase activity. The mean ± S.E. values of two independent experiments for each panel are shown.

**Figure 6:** Pharmacological inhibition of PKC-δ abrogates the synergistic effects of RA and IFNα on gene transcription via GAS elements. MCF-7 cells were transfected with an 8X-GAS-luciferase construct. Forty-eight hours after transfection, the cells were pre-incubated for 60 min in the presence or absence of rottlerin, as indicated. Subsequently, the cells were incubated for 12 hours in the presence or absence of RA, with or without an additional treatment with IFNα (5000 U/ml) for 6 hours, as indicated. The cells were subsequently lysed and luciferase...
activity was measured. The data are expressed as relative luciferase units, normalized for β-galactosidase activity. Means ± S.E. of two independent experiments are shown.

**Figure 7:** All-trans-retinoic acid induces formation of PKC-δ-containing DNA-binding complexes. A. Nuclear extracts from untreated or RA-treated NB-4 cells were incubated with 32P-radiolabeled RARE synthetic oligonucleotide, in the presence or absence of different amounts of the cold-oligonucleotide, as indicated. Protein-DNA complexes were resolved by native gel electrophoresis and visualized by autoradiography. For supershift experiments, protein extracts were incubated with 2 μg of an anti-PKC-δ antibody, or control rabbit immunoglobulin (RIgG), as indicated. B. NB-4 cells were incubated in the presence or absence of RA for the indicated times in hours. Nuclear cell extracts were incubated with 32P-radiolabeled RARE synthetic oligonucleotide. Protein-DNA complexes were resolved by native gel electrophoresis and visualized by autoradiography. For supershift experiments, 10 μg of a polyclonal antibody to RARα or control rabbit immunoglobulin (RIgG) were incubated with nuclear extracts.

**Figure 8:** PKC-δ associates with PML-RARα in a RA-dependent manner. NB-4 cells were incubated in the presence or absence of RA for the indicated times in hours. Nuclear extracts were subsequently obtained and immunoprecipitated with an antibody against PKC-δ or control RIgG, as indicated. The immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotted with an antibody against RARα (A). The blot shown in A was stripped and reprobed with an antibody against PKC-δ, to control for loading (B).
Figure 9: ChIP analysis of PKC-δ and RARα in NB-4 cells. NB-4 cells were incubated in the presence or absence of RA for 4 hours, as indicated. Chromatin immunoprecipitations were performed with either anti-PKC-δ or anti-RARα antibodies, or control non-immune rabbit immunoglobulin (RIgG), as indicated. The precipitated chromatin was analyzed using primers specific for a DR5-RARE sequence.

Figure 10: Inhibition of PKC-δ activation reverses RA-dependent differentiation of NB-4 cells. NB-4 cells were incubated with RA for 5 days in the presence or absence of rottlerin, as indicated. The cells were subsequently stained with a fluorescein isothiocyanate conjugated anti-CD11b antibody and analyzed by flow cytometry. Solid lines indicate cells labeled with isotype control. Dotted lines indicate cells labeled with anti-CD11b. The percentage of cells positive for CD11b is indicated for each condition.

Figure 11: Pharmacological inhibition of PKC-δ, but not other PKC-isoforms, reverses the growth inhibitory effects of RA on NB-4 cells. Cells were incubated with or without RA for 5 days, in the presence or absence of the PKC-δ inhibitor rottlerin (1μM), or the PKC-α inhibitor Go6076 (2.5nM), or the PKC-β inhibitor LY3791196 (50nM). Cell proliferation was assessed using an MTT assay.
Figure 1
Figure 2
Figure 3
Figure 4

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Blot: anti-phospho-ser727-Stat1

Blot: anti-Stat1b
Figure 5

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Figure 6
### Figure 7

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Gel shift assay
Figure 8
Figure 9
Figure 10
Figure 11
Activation of protein kinase C-delta (PKC-delta) by All-trans-retinoic acid
Suman Kambhampati, Yongzhong Li, Amit Verma, Antonella Sassano, Beata Majchrzak, Dilip K. Deb, Simrit Parmar, Nick Giafis, Dhananjaya V. Kalvakolanu, Arshad Rahman, Shahab Uddin, Saverio Minucci, Martin S. Tallman, Eleanor N. Fish and Leonidas C. Platanias

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