Interferon-α-induced Expression of Phospholipid Scramblase 1 through STAT1 Requires the Sequential Activation of Protein Kinase Cδ and JNK*

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Ke-Wen Zhao1,2, Dong Li1, Qian Zhao1, Ying Huang1, Robert H. Silverman4, Peter J. Sims1, and Guo-Qiang Chen1,2

From the 1Department of Pathophysiology, Key Laboratory of Cell Differentiation and Apoptosis, Chinese Ministry of Education, Rui-Jin Hospital, Shanghai Jiaotong University School of Medicine (formerly Shanghai Second Medical University), Shanghai 200025, China, and 2Department of Cancer Biology, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195, and the 1Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037.

Phospholipid scramblase 1 (PLSCR1), a calcium-binding protein that either inserts into the plasma membrane or binds to genomic DNA in the nucleus, has been shown to contribute to the cell proliferation, differentiation, and apoptosis as well as antiviral activity of interferon (IFN). The expression of PLSCR1 protein is also known to be markedly increased in response to IFN and to some differentiation inducing agents such as all-trans retinoic acid, but the precise mechanisms of this response remain to be investigated.

In this study, we show that the protein kinase Cδ (PKCδ)-specific inhibitor rottlerin and the dominant negative mutant of PKCδ significantly antagonized IFN-induced PLSCR1 expression. The influence of PKCδ on IFN-mediated induction of PLSCR1 was dependent upon the phosphorylation of STAT1 at Ser-727. Furthermore, PKCδ-mediated activation of STAT1 required the activation of JNK, as the inhibition of JNK activity by its specific inhibitor or the suppression of its dominant negative mutant suppressed both serum phosphorylation of STAT1 and PLSCR1 expression but not the activation of PKCδ. In conclusion, our results suggest that the induction of PLSCR1 transcription through STAT1 depends upon sequential activation of PKCδ and JNK.

Human phospholipid scramblase 1 (PLSCR1) is a multiply palmitoylated endofacial plasma membrane protein with a proline-rich cytoplasmic domain containing several Src homology 3 and WW domain binding motifs (1, 2). PLSCR1 protein is also phosphorylated by several tyrosine kinases such as c-Abl and c-Src, which participate in multiple growth factor receptor signaling pathways (3–5). As an example, tyrosine phosphorylation of PLSCR1 by c-Src occurs in response to epidermal growth factor, resulting in the association of phosphorylated PLSCR1 with Shc and the activated epidermal growth factor receptor complex (6). PLSCR1 was also reported to be a substrate of protein kinase Cδ (PKCδ), although this could not be confirmed in our recent experiments with recombinant PKCδ and apoptosis induction by Fas ligation in Jurkat cells (7). When it fails to be palmitoylated, PLSCR1 can be imported into the nucleus where it binds to genomic DNA, suggesting a potential role for this protein in gene transcription (8, 9).

These biochemical studies strongly indicate that PLSCR1 exerts wide biological effects. Whereas the role of PLSCR1 in remodeling plasma membrane phospholipids and in cell surface exposure of phosphatidylinerine, which was originally identified (10, 11), remains a topic of great controversy, there is increasing evidence to suggest a direct role of this protein in regulating cell proliferation and terminal differentiation. As an example, proliferation and terminal differentiation of myeloid precursor cells in response to selective growth factors are impaired in PLSCR1−/− mice (12). Furthermore, the suppression of PLSCR1 expression by small interfering RNA and antisense RNA inhibits the all-trans-retinoic acid (ATRA) and/or phorbol 12-myristate 13-acetate (PMA)-induced leukemic cell differentiation (7, 13).

However, the mechanisms of regulation of PLSCR1 expression remain largely unknown thus far. Recently, we reported that PKCδ mediates ATRA and PMA-induced PLSCR1 expression (7). PLSCR1 has also been shown to be among the most potently activated of the interferon (IFNs)-stimulated genes, suggesting that it possibly participates in cellular response to this important cytokine (14, 15). As recently reported, gene deletion of PLSCR1 or suppression of PLSCR1 expression by small interfering RNA in wild type cells was found to markedly attenuate the antiviral activity of IFNs, which appeared to be related to diminished expression of a subset of IFN-stimulated genes with known antiviral activities in cells failing to up-regulate PLSCR1 (16). However, the mechanism by which IFN induces PLSCR1 expression also remains unresolved. Because IFNs was also shown to activate PKCδ (17), in the present study we investigated whether and how activated PKCδ contributes to the transcriptional activation of PLSCR1 by IFN.

MATERIALS AND METHODS

Reagents and Cell Lines—IFNα2a was purchased from Roche Applied Science. PMA and all specific inhibitors, including Gö6976, SB203580, U0126, and SP600125, were from Calbiochem, except for rottlerin (from Biomol, Plymouth, PA). Rabbit polyclonal antibodies for p38, ERK, c-Jun, JNK, and their phosphorylated forms as well as phos--
pho-PKCδ (Ser-643), phospho-STAT1 (Ser-727 and Tyr-701), and horseradish peroxidase-linked secondary antibodies were purchased from Cell Signaling (Beverly, MA), except for anti-phospho STAT1 (Ser-727) used in the experiments shown in Fig. 7 (Upstate Biotechnology, Inc., Waltham, MA). Rabbit polyclonal anti-PKCδ antibody and mouse monoclonal anti-STAT1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), but anti-STAT1 antibody to detect trans-activation of the dominant negative (DN) fragment of PKCδ carrying the dominant negative (DN) fragment of PKCδ and its empty vector pcDNA3 were generous gifts from Dr. Young-Mi Ham (Seoul National University, Seoul, Korea) (20). pcDNA3-DN-JNK1 was kindly provided by Dr. James Darnell (Rockefeller University, New York) (21). These plasmids were transfected into H1080 and U3A cells using Polyfect
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FIGURE 2. Dominant negative PKCδ (DNδ) blocks IFN induction of PLSCR1. A, HT1080 cells were transiently transfected with either HPPP-luc or pGL3 control vector together with pRL-SV40 vector. Twenty-four hours later, IFNα2a with or without rottlerin was added for an additional 18 h. Then, the cells were harvested for measurement of luciferase activities. The bar graph shows the ratio of luciferase to Renilla activities (ordinate). Error bars denote mean ± S.D. of triplicates in an independent experiment. Each experiment was repeated at least three times with similar results. *, p < 0.024, compared with column 2; #, p < 0.021, compared with column 4; & , p < 0.239, compared with column 2. B, HT1080 cells were transiently transfected with either pcDNA3 empty vector or DNδ DNA plasmid. Twenty-four hours after transfection, IFNα2a was added for an additional 18 h. PLSCR1 mRNA and protein were detected by real-time RT-PCR (top) and Western blot (bottom), respectively. The -fold change (means ± S.D.) for PLSCR1 mRNA in three independent samples with the same treatment and for PLSCR1 protein in three experiments is expressed. *, p < 0.05, compared with column 1; #, p < 0.05, compared with column 3; & , p > 0.05, compared with column 1. C, HT1080 cells were transiently transfected with either HPPP-luc luciferase plasmids or pGL3 control vector together with pRL-SV40 vector and DNδ mutant plasmids. After 24 h of transfection, cells were treated with IFNα2a for an additional 18 h. Cells were harvested for measurement of luciferase activities. The bar graph shows the ratio of relative luciferase activities. Error bars denote mean ± S.D. of triplicate assays in independent experiments, which were repeated at least three times and for which similar results were obtained. *, p = 0.004, compared with column 2; #, p = 0.008, compared with column 4; & , p = 0.068, compared with column 2. All experiments were repeated three times with the same results.

FIGURE 3. STAT1 is required for IFN-induced PLSCR1 transcription. STAT1-negative U3A cells (A) or STAT1-positive HT1080 cells (B) were treated with IFNα2a at 3000 IU/ml for different times as indicated. PLSCR1 mRNA and proteins (PLSCR1, STAT1 Ser-727, total STAT1, and β-actin) were detected by real-time RT-PCR (top) and Western blot (bottom). The Δ symbol represents the non-specific band.

Transfection reagent (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Expression of transfected cDNAs was confirmed by Western blotting.

Real-time Quantitative RT-PCR for PLSCR1 mRNA—Total RNA was isolated by Trizol kit (Invitrogen) and treated with DNase (Promega, Madison, WI). Complementary DNA was synthesized by using the cDNA synthesis kit according to manufacturer’s instructions (Applied Biosystems, Foster City, CA), and fluorescence real-time RT-PCR was performed as we described previously (7) with specific primers for PLSCR1 (sense strand, 5'-CTGACTTCTGAGAAGGTTG-3'; antisense strand, 5'-GAATGCTGTCGGTGGATACTG-3') and for β-actin (sense strand, 5'-CATCTCCAAGCTGGAAGTGACC-3'; antisense strand, 5'-AGCCCTGTAGACAGCTGATG-3'). The -fold change is shown as means ± S.D. in three independent samples with the same treatment.

Luciferase Reporter Assays —pRL-SV40 vector and human PLSCR1 promoter-primed (HPPP)-luciferase plasmid, which was a pGL3-basic-luciferase reporter vector (Promega), cloned with a 4.18-kb DNA fragment consisting of the 5′ flanking region (−1 to −4120) and the first 60 bp of the first exon of the human PLSCR1 gene (GenBankTM AF153715)
as described previously (14), were transfected into HT1080 cells with or without dominant negative JNK1 (DN-JNK1) or dominant negative PKCδ (DN-δ) using the Polyfect reagent (Qiagen) following the manufacturer’s protocol. Twenty-four hours after transfection, triplicate cultures were treated with IFNa2a in the presence or absence of rottlerin (10 μM) or G66976 (5 μM) for 18 h. Luciferase activity was measured using a Dual-Luciferase reporter assay system (Promega) following the manufacturer’s protocol. The measured luciferase activity was normalized against pRL-SV40 Renilla luciferase activity for each sample, and luciferase activity was expressed as -fold over empty pGL3 vector luciferase activity normalized by pRL-SV40 Renilla activity.

PKCδ Activity Assays—U937 cells were lysed in phosphorylation lysis buffer (1% Triton X-100, 150 mM NaCl, 150 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM EDTA, 50 mM HEPES, 1.5 mM magnesium chloride, 10% glycerol, and protease inhibitor mixture). Cell lysates were immunoprecipitated with anti-PKCδ 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, 1.5 mM EDTA, 20 μg of phosphatidylserine, and 20 μM ATP) and resuspended in 30 μl of kinase buffer containing 5 μg of histone H1 as an exogenous substrate, to which 1–2 μCi of [γ-32P]ATP was added. The reaction was incubated for 15–30 min at room temperature and terminated by the addition of 2× SDS sample buffer. Proteins were analyzed by SDS-PAGE, and phosphorylated histone H1 was detected by autoradiography (22).

Western Blot—Cells were harvested and lysed with ice-cold phosphorylation lysis buffer as described above and an equal volume of 2× SDS sample buffer. Cell lysates were loaded on 10% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane (Amersham Biosciences). After being blocked with 5% nonfat milk in Tris-buffered saline, the membranes were incubated with monoclonal anti-human PLSCR1 4D2 antibody (14) and other indicated antibodies followed by horseradish peroxidase-linked secondary antibodies. Detection was performed by chemiluminescence phototope-horseradish peroxidase kit according to the manufacturer’s instructions (Cell Signaling). Blots were stripped and reprobed with mouse monoclonal anti-β-actin antibody to ascertain equal loading of protein. As necessary, the signal intensities of proteins tested were normalized against the indicated internal control using a densitometer (SmartView, version 5.0, software from Furi, Shanghai, China), and the -fold change was expressed compared with untreated cells.

Statistical Analysis—Student’s t test was used to compare the difference between two different groups. A value of p < 0.05 was considered to be statistically significant.

RESULTS

As reported previously (14, 15), IFNα2a highly induced PLSCR1 mRNA and protein in leukemic U937 cells as determined by real-time quantitative RT-PCR and Western blotting, respectively (Fig. 1A). Prior to PLSCR1 expression, IFNα2a treatment rapidly and time-dependently resulted in an increase in PKCδ phosphorylation in intact cells. As shown in Fig. 1B, increased phosphorylated PKCδ began to appear at 5 min after IFN treatment and then became more significant. To determine the possible involvement of PKCδ in IFN induction of PLSCR1, leukemic U937 cells were treated with IFN-α2a in the absence and presence of the PKCδ inhibitor, rottlerin. Pretreatment with rottlerin for 1 h effectively inhibited IFN activation of PKCδ as evidenced by PKCδ phosphorylation (Fig. 1C) and the ability of immunoprecipitated PKCδ to phosphorylate histone H1 (Fig. 1D). It was noteworthy that in addition to PKCδ several other kinases, including p38, ERK, and Cdc2/Cdk1, could not be detected in such immunoprecipitates (Fig. 1E), indicating the specificity for in vitro kinase assays of PKCδ. More intriguingly, pretreatment with rottlerin prevented IFN induction of PLSCR1.

FIGURE 6. Kinetics of the phosphorylation of ERK, p38, JNK, and compared with the induction of PLSCR1 in response to IFN. U937 cells were incubated with IFNα2a at 3000 IU/ml for different times as indicated. Total cell lysates were analyzed by Western blot with specific antibodies as shown. Levels of total ERK, p38, JNK, and c-Jun are shown beneath the phosphorylated forms of these proteins.
mRNA and protein (Fig. 1F, left panel). In contrast, Go6976, an inhibitor of conventional PKCs, was ineffective to IFN-induced PLSCR1 expression (Fig. 1F, right panel).

As reported previously, rottlerin directly uncouples mitochondrial respiration from oxidative phosphorylation and thus reduces cellular ATP levels that could block any number of ATP-dependent processes (23). On the other hand, rottlerin has also been shown to inhibit other kinases (24, 25). Therefore, the data gathered from using rottlerin should be evaluated cautiously. Considering this, we also tested the effect of a dominant negative mutant of PKCα (DNα), which selectively inhibited activation of PKCα (19). For the convenience of transfection, the human fibrosarcoma cell line HT1080 was used. First, a plasmid containing the PLSCR1 promoter linked to luciferase cDNA (HPPP-luc) or the empty vector was transfected into HT1080 cells. As seen in Fig. 2A, IFN treatment resulted in an increase in the promoter activity, which was significantly suppressed by rottlerin but not by Go6976 (data not shown). In agreement with the effects of rottlerin, transient transfection of DNα inhibited IFN induction of PLSCR1 mRNA/protein (Fig. 2B) and the PLSCR1 promoter–reporter construct (Fig. 2C). All of these findings indicate that IFN activation of PKCα precedes to and contributes to the subsequent induction of PLSCR1. Additionally, rottlerin and DNα also slightly abrogated the basal level of PLSCR protein and its promoter activity, suggesting that basal activity of PKCα possibly contributes to the constitutive expression of PLSCR1.

Because PKCα is known to phosphorylate STAT1 on Ser-727 in response to IFN, STAT1, Ser-727 phosphorylation, and PLSCR1 induction were monitored in HT1080 in comparison with its derivative, the STAT1-null U3A cell line (Fig. 3A) (18). In response to IFN, STAT1 Ser-727 phosphorylation increased at 30 min of treatment (lower, Fig. 3B), which appeared after activation of PKCα (Fig. 3B), whereas the induction of PLSCR1 mRNA and protein was not seen until after 3 and 6 h of treatment, respectively (Fig. 3B). Similar results were obtained in U937 cells (data not shown). Consistent with our previous report (14), however, PLSCR1 was not induced by IFN in the STAT1-deficient U3A cells (Fig. 3A).

We further determined the role of STAT1 Ser-727 phosphorylation in IFN-induced PLSCR1 expression. For this purpose, wild type STAT1 and mutant STAT1-S727A were transfected into U3A cells. Western blots showed the effectiveness of transfection of wild type STAT1 and mutant STAT1-S727A, the former but not the latter being phosphorylated on Ser-727 (Fig. 4). The results showed that ectopic expression of STAT1 but not STAT1-S727A increased PLSCR1 expression in U3A cells (Fig. 4).

In the next phase of analysis, we extended our investigation to the potential relationship between PKCα and STAT1 in the induction of PLSCR1. Toward this end, HT1080 cells were treated with IFNα2a in combination with either rottlerin or DNα transfection (Fig. 5). The results showed that both rottlerin and DNα transfection effectively inhibited IFNα2a-stimulated phosphorylation on STAT1 at Ser-727, suggesting that PKCα contributes, directly or indirectly, to IFNα2a-induced STAT1 phosphorylation.

Previous studies (26, 27) showed that IFN treatment leads to activation of several serine-threonine kinases including mitogen-activated protein kinases (MAPKs) besides PKCα, although the mechanism(s) is incompletely understood. Indeed, several members of the MAPK family including ERK1/2, p38 and JNK, as well as the JNK substrate transcription factor c-Jun, were rapidly but transiently phosphorylated upon IFNα2a treatment (Fig. 6). Thus, we sought to determine whether any of these MAPKs contribute to PLSCR1 expression in response to IFNα2a treatment. U937 cells were pretreated for 1 h with MAPK inhibitors U0126 (28), SB203580 (29), and SP600125 (30). The results showed that SP600125, which inhibited phosphorylation of c-Jun by JNK, blocked IFNα2a stimulation of STAT1 Ser-727 phosphorylation and PLSCR1 expression (Fig. 7A), but U0126 and SB203580 failed to do so. These two inhibitors diminished phosphorylation of ERK1/2 and p38, respectively, after IFNα2a treatment (Fig. 7B and C). These results indicate that JNK, but not ERK1/2 or p38, contributes to STAT1 Ser-727 phosphorylation and PLSCR1 expression induced by IFNα2a. Consistent with this fact, DN-JNK, a dominant negative mutant JNK, also reduced phosphorylation of STAT1 Ser-727 induced by IFNα2a and blocked IFN-
a2a-induced expression of both the PLSCR1 promoter construct and endogenous PLSCR1 in HT1080 cells (Fig. 8).

Finally, we sought to determine whether the effects of PKCδ and JNK on STAT1 Ser-727 phosphorylation are independent or in the same signaling pathway. Here we found that inhibition of PKCδ by rottlerin reduced IFNαa2-induced activation of JNK in U937 cells, as determined by reduced phosphorylation of c-Jun. However, inhibition of JNK with SP600125 had no effect on the IFN stimulation of PKCδ activation (Fig. 9). In agreement with the effects of these inhibitors, transfection of DN-δ could inhibit the phosphorylation of JNK and c-Jun, whereas DN-JNK failed to affect the activation of PKCδ in HT1080 cells (Fig. 10). These results indicate that PKCδ is upstream of and required for JNK activation and that both kinases are involved in IFN signaling to the PLSCR1 gene.

FIGURE 8. Dominant negative JNK inhibits IFNαa2-induced phosphorylation of Ser-727 STAT1 and PLSCR1 transcription. A, HT1080 cells were transfected with the dominant negative mutant of JNK (DN-JNK) or empty vector. Twenty-four hours later, IFNαa2 was added for an additional 18 h. Total cell lysates were used in Western blots for phosphorylated 727 STAT1, total STAT1, PLSCR1, and β-actin. The Δ symbol shows the nonspecific band. The altered fold of Ser-STAT1/STAT1 or PLSCR1/β-actin against untreated cells is shown as means ± S.D. from three independent experiments. *p < 0.05, compared with column 1; #p < 0.05, compared with column 3; &p > 0.05, compared with column 1. B, HT1080 cells were transfected with either HPPP-luc luciferase plasmids or pGL3 control vector together with pRL-SV40 vector and DN-JNK plasmids. After 24 h, IFNαa2 (3000 IU/ml) was added for an additional 18 h. The cells were harvested for the measurement of luciferase activities (see “Materials and Methods”). The values are expressed as mean ± S.D. of relative luciferase activities of triplicates from an independent experiment. The experiments were repeated at least three times, and similar results were obtained each time.*p = 0.014, compared with column 2; #p = 0.032, compared with column 4; &p = 0.025, compared with column 2.

FIGURE 9. JNK activity is not required for IFN activation of PKCδ. After preincubation for 1 h in the presence or absence of rottlerin or SP600125, U937 cells were treated with IFNαa2 for 4 h. A, phospho-c-Jun or phospho-PKCδ was measured in a Western blot. The altered fold of phospho-c-Jun/c-Jun or phospho-PKCδ/PKCδ against untreated cells is shown as means ± S.D. from three independent experiments. Left panel: *p = 0.045, compared with column 1; #p = 0.039, compared with column 3; &p = 0.062, compared with column 1; right panel: *p = 0.045, compared with column 1; 5p = 0.459, compared with column 3; [caret]p = 0.141, compared with column 1. B, in vitro PKCδ kinase assay was performed as described under “Materials and Methods” with histone H1 as a substrate. U937 cell extract with PMA treatment for 1 h was used as the positive control.

DISCUSSION

Recently we reported that the activation of PKCδ by phosphorylation mediates ATRA and PMA-induced PLSCR1 expression in leukemic cells (7). In this work, we continued to show that the inhibition of PKCδ activation by the chemical inhibitor rottlerin and transfection of the dominant negative mutant of PKCδ significantly inhibited IFNαa2-induced expression of PLSCR1 mRNA and protein, whereas such inhibition was not observed for G66976, an inhibitor of the conventional PKC. These data, combined with our previous report demonstrating that transfection of the catalytic fragment of PKCδ directly up-regulates PLSCR1 expression (7), strongly suggest that PKCδ is a common mediator for PLSCR1 expression induced by IFNαa2, ATRA, or PMA. More recently, PLSCR1 expression has been shown to mediate the antiviral activity of IFNs (16). Our previous studies also showed that induced expression of PLSCR1 contributes to ATRA/PLA2-induced leukemic cell differentiation (7). Furthermore, IFNαa2 and ATRA act synergistically in the induction of cell differentiation and growth inhibition (22). Therefore, it is of interest that IFNαa2 and ATRA both up-regulate PLSCR1 expression through PKCδ.

It is well known that IFN-α/β binding to the type I IFN receptor initiates a tyrosine phosphorylation cascade involving JAK kinases (JAK1 and TYK2), resulting in the heterodimerization of tyrosine-phosphorylated STAT1 and 2. These phosphorylated STAT proteins are transported to the nucleus and, in a complex with IRF9, bind to IFN-stimulated response elements and activate the transcription of many
different genes (31–33). However, an additional phosphorylation event on STAT1 Ser-727 is required for a full IFN-stimulated transcriptional response (34). Previously, IFN-α on STAT1 Ser-727 is required for a full IFN-stimulated transcriptional response as monitored by expression of PLSCR1. Our results indicated that JNK contributes to both STAT1 Ser-727 phosphorylation and an IFN transcriptional response as monitored by expression of PLSCR1. However, inhibition of JNK did not suppress IFNα2a-induced phosphorylation and activation of PKCδ. Therefore, JNK activation is downstream of PKCδ, whereas both of these kinases are required for maximal STAT1 Ser-727 phosphorylation. In summary, PKCδ inhibition suppressed IFN-α-initiated JNK activation, STAT1 Ser-727 phosphorylation, and expression of PLSCR1. Transcriptional induction of PLSCR1 through STAT1 that is induced by IFNα2a is dependent upon sequential activation of both PKCδ and JNK kinase.

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