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Interruption of RNA processing machinery by a small compound, 1-[(4-chlorophenyl)methyl]-1*H*-indole-3-carboxaldehyde (oncrasin-1)

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Abstract

Protein kinase Cι (PKCι) is activated by oncogenic Ras proteins and is required for K-Ras–induced transformation and colonic carcinogenesis *in vivo*. However, the role of PKCι in signal transduction and oncogenesis is not clear. We recently identified a small molecule, designated 1-[(4-chlorophenyl)methyl]-1*H*-indole-3-carboxaldehyde (oncrasin-1), that can selectively kill K-Ras mutant cancer cells and induce abnormal nuclear aggregation of PKCι in sensitive cells but not in resistant cells. To determine the causes and biological consequences of PKCι aggregates in the nucleus, we analyzed the effect of oncrasin-1 on proteins involved in DNA repair and RNA processing. Our results showed that oncrasin-1 treatment led to coaggregation of PKCι and splicing factors into megaspliceosomes but had no obvious effects on the DNA repair molecule Rad51. Moreover, oncrasin-1 treatment suppressed the phosphorylation of the largest subunit of RNA polymerase II and the expression of intronless reporter genes in sensitive cells but not in resistant cells, suggesting that suppression of RNA transcription is a major effect of oncrasin-1 treatment. Studies with cultured cells or with recombinant proteins showed that oncrasin-1 can disrupt the interaction of PKCι and cyclin-dependent protein kinase 9/cyclin T1 complex, which is known to phosphorylate the largest subunit of RNA polymerase II and is required for RNA transcription. Together, our results suggest that oncrasin-1 suppresses the function of RNA processing machinery and that PKCι might be involved in the biological function of RNA processing complexes. [Mol Cancer Ther 2009;8(2):441–8]

Introduction

Protein kinase C (PKC) is a family of serine/threonine kinases that are activated by many extracellular signals, including hormonal, neuronal, and growth factor stimuli (1). At least 10 known isoforms have been identified and these are classified into three groups on the basis of their structure and activation signaling: (*a*) conventional PKCs (α, β1, β2, and γ), which are activated by phosphatidylserine and diacylglycerol and are Ca^{2+} dependent; (*b*) novel PKCs (δ, ε, η, and θ), which are activated by phosphatidylserine and diacylglycerol but are Ca^{2+} independent; and (*c*) atypical PKCs (ζ and ι/λ), which are not regulated by phosphatidylserine, diacylglycerol, or Ca^{2+} but are activated by 3-phosphoinositides, phosphoinositide-dependent kinase 1, and specific protein-protein interactions, including direct interaction with Ras protein (2). Phosphorylation of atypical PKCs at Thr^{410} (Thr^{403}) by phosphoinositide-dependent kinase 1 is phosphoinositide 3-kinase dependent and serves as a direct on/off switch (3).

Evidence shows that atypical PKCs play roles in signal transduction, cell proliferation, cell polarity, inflammation, and oncogenesis. Increased expression of PKCζ was observed in pancreatic cancer stromal cells and liver and prostate cancer tissues (4). Similarly, gene amplification and overexpression of PKCι was reported in human ovarian cancer and non–small cell lung carcinoma (5, 6) and this increased expression was associated with poor survival. Moreover, PKCι is required for K-Ras–induced transformation and colonic carcinogenesis *in vivo*, suggesting that it is a critical downstream effector of oncogenic Ras (7). Transgenic mice expressing constitutively active PKCι in the colon are highly susceptible to carcinogen-induced colon carcinogenesis, whereas mice expressing kinase-deficient PKCι are resistant to both carcinogenic and oncogenic Ras-mediated carcinogenesis (7). Nevertheless, like in other PKC isoforms, the downstream signaling effectors of atypical PKC are not well characterized, although direct interaction between atypical PKC and Phox-Bem1 domain proteins (8), such as Zip/62, Par6, and mitogen-activated protein kinase 5, has been reported (9–11). In addition, nucleolin, a major constituent of nucleoli in exponentially growing cells, has been identified as a substrate of PKCζ (12). However, evidence also suggests functional divergence between

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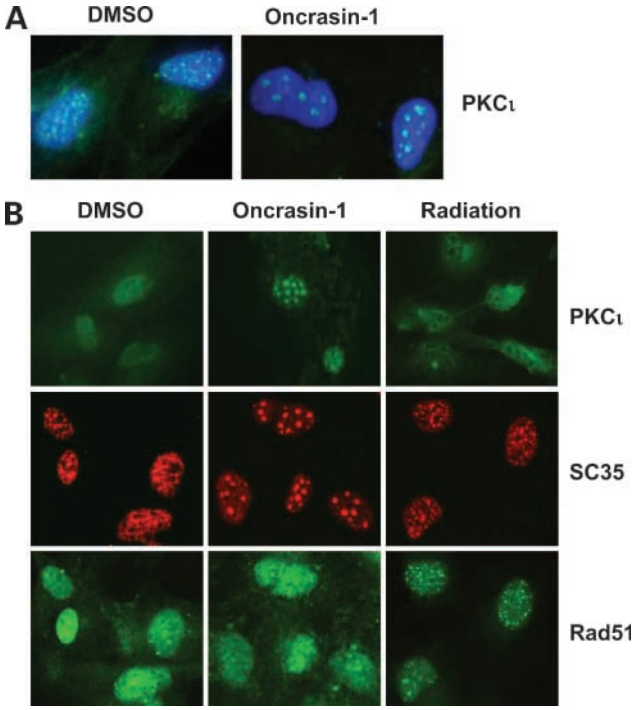

Figure 1. Effects of oncrasin-1 on PKCι, SC35, and Rad51. **A,** aggregation of PKCι in the nucleus of T29Kt1 cells 12 h after 10 μmol/L oncrasin-1 treatment. **B,** immunofluorescent staining of PKCι, SC35, and RAD51. T29Kt1 cells were treated with DMSO, 10 μmol/L oncrasin-1, or 10-Gy γ-irradiation for 12 h, and immunofluorescent staining was done to determine the intracellular localization of PKCι, SC35, and Rad51. Treatment with oncrasin-1 induced dramatic morphologic changes in PKCι and SC35 but not in Rad51. In contrast, no obvious changes were observed in PKCι and SC35 after radiation treatment.

PKCι and PKCζ. Mice with knockout of PKCζ are viable but have impaired nuclear factor-κB signaling and immune response (13), whereas mice with knockout of PKCλ, a mouse homologue of PKCι, do not survive past the embryonic stage (14).

We have recently identified a small molecule, 1-[(4-chlorophenyl)methyl]-1*H*-indole-3-carboxaldehyde (oncrasin-1), that can selectively kill K-Ras mutant cancer cells but has little effect on normal isogenic cells (15). Oncrasin-1 treatment resulted in aggregation of PKCι in the nucleus in sensitive cells but not in resistant cells. However, the causes and/or biological consequences of PKCι aggregation inside the nucleus are not clear; therefore, we tested the effect of oncrasin-1 on nuclear proteins involved in the processing of DNA or RNA, the two major molecules that are synthesized, repaired, or processed inside the nucleus. Oncrasin-1 treatment resulted in aggregation of RNA splicing factors in a similar pattern as that observed in PKCι but had no obvious effects on the DNA repair molecule Rad51. Moreover, oncrasin-1 treatment resulted in the aggregation of PKCι into megaspliceosomes and reduced phosphorylation of the largest subunit of RNA polymerase II and splicing factors. Thus, our results suggest that oncrasin-1 induces malfunction in the RNA processing machinery and that PKCι might be involved in the biological function of RNA processing complexes.

Materials and Methods

Cell Lines

Human non–small cell lung carcinoma H460 cells were propagated routinely in a monolayer culture in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 units/mL penicillin, and 100 mg/mL streptomycin. T29 and T29Kt1 cells were maintained in DMEM with the same supplements. All cells were maintained in the presence of 5% CO_2 at 37°C.

Chemicals and Antibodies

Oncrasin-1 was obtained from Chembridge Corporation. Active CDK9/cyclin T1, CDK7/cyclin H/MAT1, CDK2/cyclin A, CDK6/cyclin D3, histone H1, and CDK7/9 tide were purchased from Millipore. Recombinant PKCι were purchased from Calbiochem. Antibodies to the following proteins were used for Western blot analysis: PKCι, alternative splicing factor/splicing factor 2 (ASF/SF2), and Rad51 (Santa Cruz Biotechnology); β-actin (Sigma); antiphosphorylated SR proteins (Invitrogen); AKT, phosphatidylinositol 3-kinase, and phosphor-AKT (Cell Signaling); and purified mouse anti-SC35 (BD Pharmingen), H5 antibody (Covance) and cyclin T1 (Abcam). A 1:1,000 dilution was used for the Western blot analysis and a 1:200 dilution was applied to immunofluorescent slides. The specificity of PKCι antibody was verified by testing on Western blot analysis of 293 cells transfected with a control plasmid (pCMV-LacZ) or a PKCι-expressing plasmid pCMV-PKCι (Supplementary Fig. S1).[4]

Immunofluorescent Staining

Cells were seeded at a density of 1×10^5 per well in six-well plates. Each well contained a 1% gelatin-treated coverslide. Cells were allowed to grow overnight; they were then treated with different compounds or radiation, as indicated in the legends. After treatment, cells were washed twice with PBS, fixed with 2% paraformaldehyde for 20 min, permeabilized with 0.1% Triton-100 for 20 min, and blocked with 5% normal goat serum for 1 h. The slides were incubated with primary antibodies, followed by FITC or rhodamine-linked secondary antibodies. After being washed in PBS thrice, the slides were removed and mounted with Prolong Gold antifade reagent (Molecular Probes), containing 4′,6-diamidino-2-phenylindole. The slides were read under a Nikon Eclipse 50i fluorescence microscope or an Olympus IX71 fluorescence microscope using Fluoview version 4.3 software.

Western Blot Analysis

Western blot analysis was done as we have previously described (16). The immunoprecipitation experiments were done as follows: cells were lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate,

[4] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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and 0.1% SDS], passed through 21-gauge needles 10 times, and centrifuged at 10,000 rpm for 10 min. The supernatant was harvested and incubated with primary antibodies overnight under mild rotation. IgA/G beads (25 μL) were then added and the mixture was shaken for ~1 h. The beads were spun down and washed thrice with cold PBS. The beads were diluted with loading buffer and heated at 95°C for 10 min; SDS-PAGE and Western blot analysis were then performed.

Luciferase Assay

Cells (3×10^4/well) were seeded in 24-well plates overnight. They were then grown in serum-free medium for 12 h, followed by transfection with 250 ng of different luciferase reporter plasmids. FuGENE 6 (Roche Molecular Biochemicals) was used for plasmid transfection. The cells were kept in normal medium for another 24 h and then treated with 1 μmol/L oncrasin-1 for different times. Cells were harvested for the luciferase activity assay, which was done using the luciferase assay system (Promega Life Science) as instructed by the manufacturer. Cells transfected with pCDNA3.1 were used as the control.

Kinase Activity Assay

The kinase reactions (20 μL) were done at 30°C for 15 min. The solution contained 1× reaction buffer, 5 mmol/L $MgCl_2$, 50 μmol/L cold ATP, 1 μL γ[P^{32}]ATP (3,000 Ci/mmol), 50 ng individual kinase, and their corresponding substrates. CDK7/9 tide (500 mmol/L) was used as the substrate for CDK9/cyclin T1, CDK7/cyclin H/MAT1, whereas 0.1 mg/mL histone H1 was used for CDK2/cyclin A and CDK6/cyclin D3 kinase assay. The mix was incubated with different doses of oncrasin-1. Free γ[P^{32}]ATP was washed away through P81 phosphocellulose filters. The samples were read under a scintillation counter.

In vitro Binding Assay

Recombinant PKCι and CDK9/cyclin T1 were mixed *in vitro* with or without 1 μmol/L oncrasin-1. The mixture was gently mixed at 4°C for 2 h and then precipitated with anti-PKCι antibody. Normal rabbit IgG was used as the antibody control. The CDK9/cyclin T1 complex was used as a positive control for cyclin T1.

Statistical Analysis

Differences between the treatment groups were assessed by ANOVA using statistical software STATISTIC 6.0 (StatSoft). P values of <0.05 were considered significant.

Results

Oncrasin-1 Induced Aggregation of SC35, Similar to PKCι

We recently found that oncrasin-1 induces apoptosis in the K-Ras–transformed tumorigenic human ovarian T29Kt1 cell line but not in its parental, immortalized normal ovarian epithelial T29 cell line. Characterization of molecular mechanisms by testing on the levels and/or phosphorylation status of several proteins that are involved in apoptosis and/or Ras signaling pathways did not produce elucidative information. The oncrasin-1–induced antitumor activity is not affected by the Raf inhibitor Bay 43-9006, MEK inhibitor U0126, phosphatidylinositol 3-kinase inhibitor LY294002, and AKT inhibitor X (Supplementary Fig. S2).[4] Nevertheless, oncrasin-1 treatment resulted in the aggregation of PKCι into a few large foci inside the nuclei (Fig. 1A). To determine the causes and biological consequences of this abnormal subcellular distribution of PKCι, we tested the effect of oncrasin-1 on Rad51, a homologous DNA recombinase involved in DNA repair (17), and on SC35, a protein required for RNA splicing and spliceosome assembly (18). These molecules were selected for testing because they are involved with DNA or RNA metabolisms, the major nuclei events.

In DMSO-treated cells, Rad51 was uniformly distributed inside the nucleus, whereas SC35 was localized in the

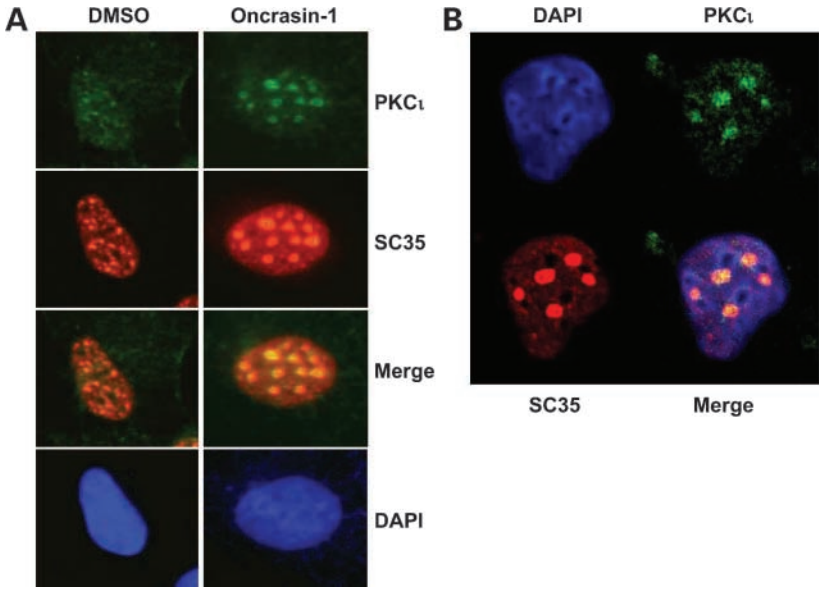

Figure 2. Colocalization of PKCι and SC35. **A,** fluorescence microscopy examination. **B,** confocal examination. T29Kt1 cells were treated with 10 μmol/L oncrasin-1 for 12 h and immunofluorescent costaining was done to determine the intracellular localization of PKCι and SC35.

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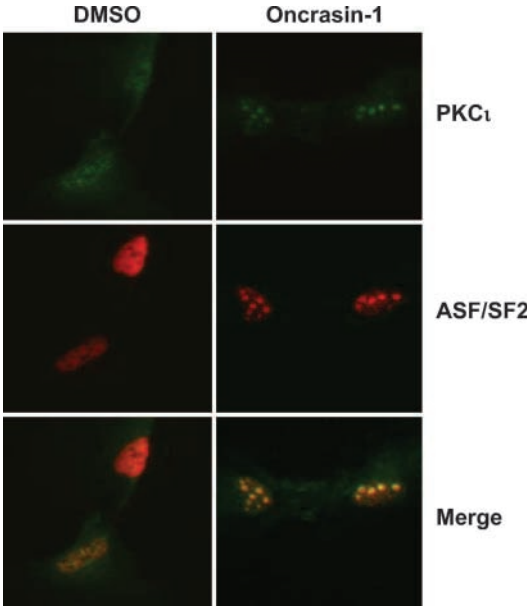

Figure 3. Colocalization of PKCι and ASF/SF2. Fluorescence microscopy examination. T29Kt1 cells were treated with 10 μmol/L oncrasin-1 for 12 h and immunofluorescent costaining was done to determine the intracellular localization of PKCι and ASF/SF2.

nucleus as small speckles, either diffusely distributed or concentrated as clusters of granules (19). Oncrasin-1 treatment had no obvious effect on Rad51 but resulted in aggregation of SC35 into several large foci in T29Kt1, a phenomenon similar to that seen in PKCι. On the other hand, treatment with 10-Gy radiations resulted in the formation of tiny foci of Rad51 in the nucleus, with no noticeable effect on SC35 (Fig. 1B). This result suggests that oncrasin-1 affects RNA processing machinery instead of inducing DNA damage.

PKCι Colocalized with Splicing Factors

Because oncrasin-1 induced similar nuclear distribution changes in PKCι and SC35, we determined whether they colocalized in the nucleus after oncrasin-1 treatment. T29Kt1 cells were treated with 10 μmol/L oncrasin-1 for 12 hours, costained with rabbit anti-PKCι and mouse anti-SC35 antibodies overnight, and incubated with FITC-labeled goat anti-rabbit immunoglobulin and rhodamine-labeled goat anti-mouse antibodies sequentially. After oncrasin-1 treatment, PKCι and SC35 were colocalized in megafoci in nuclei, possibly in megaspliceosomes (Fig. 2A). This result was further confirmed on examination of the slides under a confocal Olympus IX71 microscope with Fluoview version 4.3 software (Olympus; Fig. 2B).

We then tested whether oncrasin-1 treatment elicited similar effects on other proteins involved in RNA splicing. After oncrasin-1 treatment, T29Kt1 cells were costained with rabbit anti-PKCι and mouse anti-ASF/SF2 antibodies overnight and incubated with FITC-labeled goat anti-rabbit IgG and rhodamine-labeled goat anti-mouse antibodies sequentially. Similar to the effect seen in SC35, treatment with oncrasin-1 led to colocalization of PKCι and ASF/SF2 into megafoci in nuclei (Fig. 3). Together, these results suggest that PKCι is part of the RNA splicing machinery and aggregated into megaspliceosomes upon the treatment of oncrasin-1 in sensitive cells.

Oncrasin-1 Inhibited RNA Polymerase II and Serine-Arginine–Rich Protein Phosphorylation

Splicing factors are present in mammalian cells in nuclear compartments or speckles. As seen on electron microscopy, speckles consist of two distinct morphologic parts: the larger and more concentrated regions are called interchromatin granule clusters, which are transcriptionally inactive, and the more diffusely distributed splicing factors and regions at the periphery of interchromatin granule clusters correspond to perichromatin fibrils, which contain nascent transcripts (20–22). Speckles are highly dynamic structures that respond specifically to the activation of nearby genes. These dynamic events depend on RNA polymerase II transcription (19, 23). Upon inhibition of RNA polymerase II transcription (24, 25) or pre-mRNA splicing by oligonucleotides or antibodies (26), splicing factors redistribute and preferentially localize to interchromatin granule clusters, which become larger, inactive, and more uniform in shape. This aggregation of splicing factors into large foci suggests that oncrasin-1 affects RNA processing (transcription, splicing, or both). To determine whether oncrasin-1 has a biochemical effect on the proteins involved in RNA transcription or splicing, we evaluated the phosphorylation of the largest subunit of RNA polymerase II and splicing factors after oncrasin-1 treatment. T29, T29Ktl, and H460 cells were treated with oncrasin-1 at an optimal concentration (10 μmol/L for T29 and T29Kt1 and 1 μmol/L for H460). Cell lysates were collected 12 hours after treatment and subjected to Western blot analysis with antibodies specific for phosphorylated RNA polymerase II and serine-arginine–rich (SR) proteins. The results showed that oncrasin-1 treatment led to a dramatic suppression of phosphorylated RNA polymerase II and some SR proteins (Fig. 4A), suggesting that oncrasin-1 treatment affects biological functions of RNA processing factors. We also tested the time-dependent suppression of oncrasin-1 on RNA-processing factors in H460 cells. The phosphorylation of RNA polymerase II and SR proteins was reduced starting at 8 hours after oncrasin-1 treatment. This effect was more striking at 12 and 24 hours after treatment (Fig. 4B). Interestingly, oncrasin-1 did not induce obvious suppression of phosphorylated RNA polymerase II in H1299 cells that harbor an N-Ras mutation or in H322 cells that have wild-type Ras genes (Fig. 4C). Both H1299 and H322 cells are resistant to oncrasin-1 treatment.

Oncrasin-1 Inhibited the Transcription of Luciferase Reporter Gene Driven by Different Promoters

The dramatic effect on the phosphorylation status of the largest subunit of RNA polymerase II suggested that oncrasin-1 affects RNA transcription. To investigate this hypothesis, we transfected H460, T29Kt1, and T29 cells with pCMV-Luc plasmid expressing firefly luciferase

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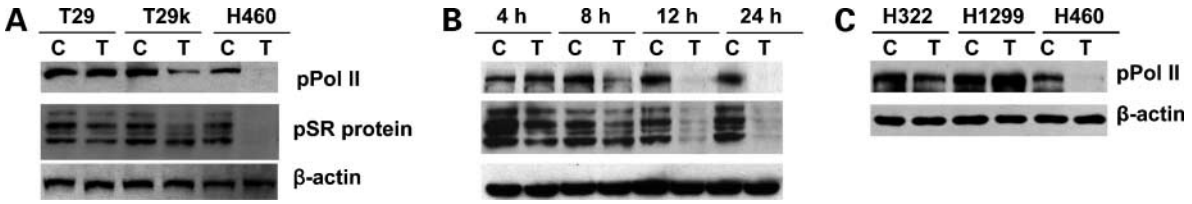

Figure 4. Effects of oncrasin-1 on the phosphorylation of RNA polymerase II and SR proteins. **A,** T29, T29Kt1, and H460 cells were treated with DMSO (*C*) or oncrasin-1 (*T*) (10 μmol/L for T29 and T29Kt1 and 1 μmol/L for H460) for 12 h. Cells were harvested for Western blot analysis with antibodies for the phosphorylated largest subunit of RNA polymerase II [H5 (*pPol II*)], phosphorylated SR proteins (*pSR*). Note the reduced levels of phosphorylation of RNA polymerase II and SR proteins. β-Actin was used as the loading control. **B,** time course of the effects of oncrasin-1 on the inhibition of RNA polymerase II and SR protein phosphorylation. H460 cells were treated with DMSO or 1 μmol/L oncrasin-1 for the indicated times and a Western blot analysis was done. **C,** effects on phosphorylation of CTD in lung cancer cell lines. H322 (with wild-type Ras genes), H1299 (with N-Ras mutation), and 460 (with K-Ras mutation) were treated with DMSO (*C*) or 1 μmol/L oncrasin-1 (*T*) for 12 h and a Western blot analysis was done with H5 pPol II antibody.

driven by cytomegalovirus promoter (Promega) or pRL-TK-Luc plasmid expressing renilla luciferase driven by herpes simplex virus thymidine kinase promoter (Promega); allowed the cells grow for 24 hours; and then treated them with 1 μmol/L oncrasin-1, DMSO, or 100 μmol/L of 5,6-dichloro-l-/3-D ribofuranosylbenzimidazole (DRB), which is a known transcription inhibitor (27, 28). Cells were harvested at different times after treatment and luciferase activity was determined. The data were then normalized to corresponding DMSO control groups. Compared with DMSO, oncrasin-1 markedly inhibited the expression of luciferase in H460 and T29Kt1 cells but has no effect or only mild effect in T29 cells (Fig. 5). The transcriptional inhibition effect of oncrasin-1 was stronger than that of DRB. Because the plasmids used in this experiment do not contain introns and do not require splicing for luciferase expression, these results suggest that treatment with oncrasin-1 led to inhibition of RNA transcriptional function.

Oncrasin-1 Induced the Dissociation between PKCι and the CDK9/Cyclin T1 Complex

Phosphorylation of the COOH-terminal domain (CTD) of the largest subunit of eukaryotic RNA polymerase II is required for efficient transcription elongation and recruitment of mRNA processing factors, including capping the enzyme and splicing factors required for efficient processing of RNA transcripts (28–31). Polymerase II enters the assembling transcription complex with its CTD unphosphorylated (IIa form). Phosphorylation of CTD by CDK7 and CDK9 converts polymerase II to its phosphorylated form, enabling efficient RNA elongation and processing (29, 30). To determine whether oncrasin-1 has a direct effect on CDK7, CDK9, or other cyclin-dependent kinases, we evaluated the kinase activity of recombinant CDK2, CDK6, CDK7, and CDK9 with or without oncrasin-1. The *in vitro* kinase assay results showed that oncrasin-1 inhibited the kinase activity of CDK6 and CDK9 in a dose-dependent manner (Supplementary Fig. S3).

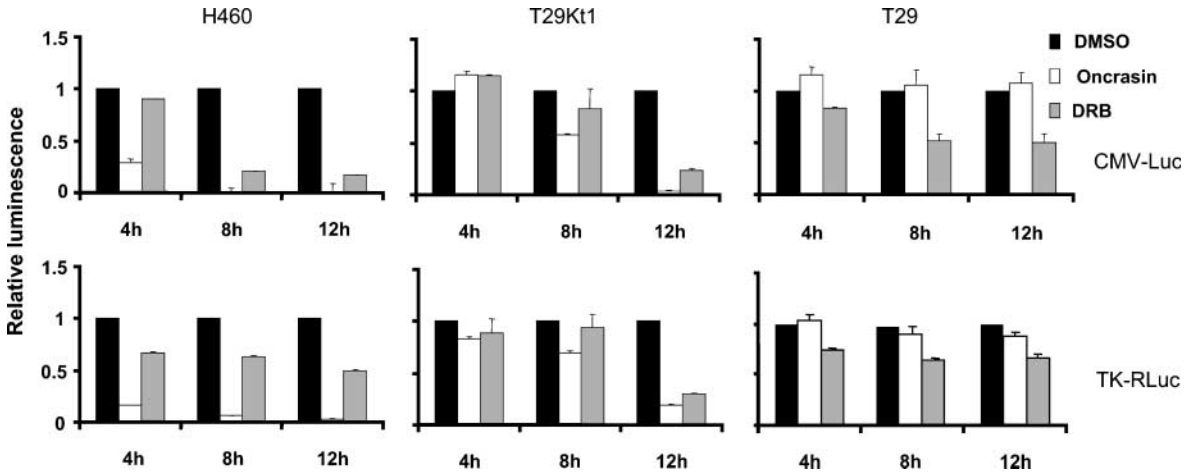

Figure 5. Oncrasin-1 inhibited transcription of the luciferase reporter gene driven by different promoters. H460, T29, and T29Kt1 were transfected with pCMV-Luc or pRL-TK-Luc plasmids. Twenty-four hours after transfection, cells were treated with oncrasin-1 (10 μmol/L for T29 and T29Kt1 and 1 μmol/L for H460), DMSO, or 100 μmol/L DRB for the indicated time periods. Cells were harvested at 4, 8, and 12 h after treatment and the luciferase activities were determined. The values of DMSO treatment groups served as controls. Their values were set up as 1. The values (relative luciferase activities) of oncrasin-1 and DRB treatment groups were normalized to the corresponding DMSO control groups. *Columns,* mean of three assays; *bars,* SD.

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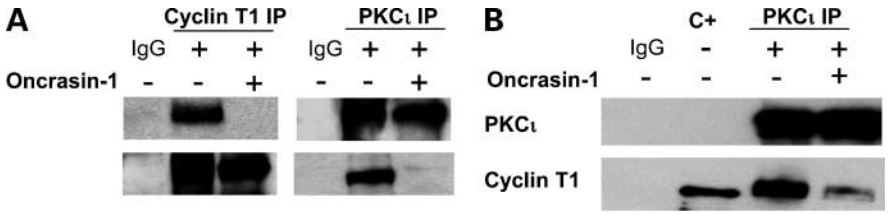

Figure 6. Effect on PKCι and cyclin T1 interaction. **A,** immunoprecipitation of PKCι and the CDK9/cyclin T1 complex. H460 cells were treated with DMSO or 1 μmol/L oncrasin-1 for 12 h. Cell lysates were harvested to perform the immunoprecipitation (*IP*) assay. Normal rabbit IgG was used as the control. **B,** recombinant PKCι and CDK9/cyclin T1 were mixed *in vitro* and then precipitated with anti-PKCι antibody. Normal rabbit IgG was used as the antibody control. CDK9/cyclin T1 complex was used as a positive control for cyclin T1.

However, at a high concentration, oncrasin-1 also inhibited CDK2 and CDK7. Nevertheless, the direct inhibitory effect of oncrasin-1 on those kinases was moderate and it required high concentrations of oncrasin-1 to suppress 50% of enzymatic activity (Supplementary Fig. S3).[4] The abnormal distribution of PKCι led us to perform an immunoprecipitation assay to detect possible interaction between PKCι and the CDK9/cyclin T1 complex. In DMSO-treated cells, PKCι can be coprecipitated with cyclin T1–specific antibodies. However, after oncrasin-1 treatment, PKCι was not detected in the CDK9/cyclin T1 complex (Fig. 6A). The disruption of the interaction between PKCι and cyclin T1 was also observed in an *in vitro* assay with recombinant PKCι and CDK9/cyclin T1 complex. PKCι-specific antibody but not a control antibody could effectively precipitate PKCι and cyclin T1. However, the presence of oncrasin-1 dramatically suppressed this immunocoprecipitation (Fig. 6B). These data indicate that PKCι is part of the CDK9/cyclin T1 complex in cells and that the interaction between PKCι and the CDK9/cyclin T1 complex was disrupted by oncrasin-1 treatment, which may lead to transcription dysfunction.

Discussion

Both SC35 and ASF/SF2 proteins are members of the SR protein family, which contains an NH_2-terminal RNA-binding domain that interacts with pre-mRNA and a COOH-terminal SR domain that functions as a protein interaction domain (32). SR proteins are essential for both the operation and regulation of RNA splicing. As part of the large nuclear protein complex called a spliceosome, these proteins are localized in the nucleus as highly dynamic structures known as speckles. The morphologic characteristics of speckles depend on the functions of RNA polymerase II and splicing factors. Under normal conditions, the nucleus of mammalian cells contains 20 to 40 tiny speckles (19). However, when the function of RNA polymerase II or pre-mRNA splicing factors is suppressed, spliceosomes aggregate into a few large foci, known as megaspliceosomes (24–26). The formation of megaspliceosomes is a hallmark of functional disruption of the RNA processing machinery. In this study, we found that oncrasin-1 treatment led to aggregation of both SC35 and ASF/SF2 into megaspliceosomes, suggesting that one biological effect induced by oncrasin-1 is disruption of the RNA processing machinery.

Because phosphorylation of the CTD domain of the largest subunit of RNA polymerase II is a critical step for both the transcriptional elongation and regulation of pre-mRNA splicing (33), we tested the phosphorylation status of CTD by Western blot analysis. Phosphorylation of the CTD domain and SR proteins were suppressed by oncrasin-1 treatment, suggesting that oncrasin-1 affects RNA elongation, RNA splicing, or both. The expression of intronless reporter genes was significantly suppressed by oncrasin-1, indicating that inhibition of RNA transcription is one of the major biological events induced by this compound. Because inhibition of RNA polymerase II will lead to inhibition of RNA splicing machinery, it is not clear whether malfunction of spliceosomes is an indirect effect of suppression of RNA polymerase II.

Messenger RNA production by RNA polymerase II is the first step in gene expression and is central to the life of cells. Evidence also indicates that transformed cells require continuous activity of RNA polymerase II to resist oncogene-induced apoptosis (28). Inhibition of polymerase II in untransformed cells resulted in growth arrest but not apoptosis. In contrast, transforming cells with c-Myc dramatically increased their sensitivity to DRB, an adenosine analogue that inhibits CDK7 and CDK9, indicating that apoptosis after the inhibition of RNA polymerase II function is greatly enhanced by oncogenic expression (28). The suppression of CTD phosphorylation by oncrasin-1 led us to evaluate the effects of oncrasin-1 on the kinase activities of recombinant CDK7 and CDK9. Oncrasin-1 had moderate inhibitory effect on the kinase activity of CDK9, CDK6, CDK7, and CDK2. An analysis of cultured cells revealed that oncrasin-1 treatment disrupted the interaction between cyclin T, a major regulatory subunit of CDK9, and PKCι, whose abnormal nuclear aggregation led us to perform the studies described here (34). The interaction between cyclin T and PKCι has not been previously reported, although it was reported by others that CDK7 was colocalized with and phosphorylated by PKCι (35). However, whether PKCι actively participates in the regulation of CDK7 or CDK9 activity is not yet clear. Although the oncrasin-1–induced abnormal subcellular

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distribution of PKCι led us to discover the effects of oncrasin-1 on RNA processing machineries, it needs further investigation to determine possible role of PKCι in regulating RNA transcription and splicing, including the interaction between PKCι and cyclin T1.

It is noteworthy that oncrasin-1 was originally identified through synthetic lethality screening in K-Ras mutant tumor cells. Whether active K-Ras facilitates oncrasin-1–mediated RNA polymerase II suppression is not yet clear. However, Ras proteins are involved in regulation of RNA polymerase II phosphorylation and function (36, 37). Interestingly, mutations compromising the function of CTD were reported to be synthetically lethal with alterations that led to elevated levels of Ras signaling pathway in yeast (38). It is possible that, similar to genetic disruption of CTD function observed in yeast, inhibition of CTD function by oncrasin-1 is synthetically lethal for elevated K-Ras activities. Nevertheless, we could not detect the obvious effect of oncrasin-1 on CTD phosphorylation in normal human ovarian epithelial cells T29 and in human lung cancer cell lines H1299 (with N-Ras mutation) and H322 (with wild-type Ras genes). Thus, it is also possible that K-Ras and oncrasin-interacting protein both regulate CTD phosphorylation. Functional change of either alone, such as elevated K-Ras activity or alteration of oncrasin-interacting protein, is not sufficient to alter CTD phosphorylation. However, if they occur together, the combination leads to CTD dephosphorylation and, as a result, the death of the cell.

Disclosure of Potential Conflicts of Interest

M.D. Anderson filed a patent for oncrasin-1; W. Guo, S. Wu, J. Liu, and B. Fang are the inventors. No other potential conflicts of interest were disclosed.

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