Activation of Protein Kinase G Increases the Expression of $p21^{CIP1}$, $p27^{KIP1}$, and Histidine Triad Protein 1 through Sp1

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Abstract

The anticancer role of cyclic guanosine 3′,5′-monophosphate (cGMP)–dependent protein kinase G (PKG) has become of considerable interest, but the underlying mechanisms are not fully established. In this study, we examined the effects of activation of PKG on the expression of three tumor suppressor proteins in human SW480 colon cancer cells. Our results revealed that treatment with cell permeable cGMP derivatives, or the cGMP phosphodiesterase inhibitor sulindac sulfone (exisulind, aptosyn, hereafter called exisulind) led to increased expression of the tumor suppressor proteins $p21^{CIP1}$, $p27^{KIP1}$, and Histidine triad protein 1 (HINT1), and their corresponding mRNAs. Overexpression of PKG Iβ also caused increased expression of the $p21^{CIP1}$, $p27^{KIP1}$, and HINT1 proteins. Both the $p21^{CIP1}$ and $p27^{KIP1}$ promoters contain Sp1 binding sites and they were activated by PKG in luciferase reporter assays. Specific Sp1 sites in the p21 and p27 promoters were sufficient to mediate PKG-induced luciferase reporter activity, suggesting an interaction between Sp1 and PKG. Indeed, we found that PKG can phosphorylate Sp1 on serine residue(s) and this resulted in transcriptional activation of Sp1. Knockdown of Sp1 expression with siRNA inhibited the increased expression of $p21^{CIP1}$, $p27^{KIP1}$, and HINT1 induced by the cGMP derivative 8-pCPT-cGMP in SW480 cells. These novel effects of PKG activation on the expression of three tumor suppressor genes may explain, at least in part, the anticancer effects of activation of PKG. They also provide a rationale for further developing activators of PKG for the prevention and treatment of cancer. [Cancer Res 2008;68(13):5355–62]

Introduction

Cyclic guanosine 3′,5′-monophosphate (cGMP) is an ubiquitous second messenger that mediates cellular responses to various exogenous and endogenous signaling molecules. cGMP regulates physiologic processes by activating protein kinase G (PKG), the cGMP-gated channels, and specific phosphodiesterases. PKG belongs to the family of serine/threonine kinases and they are present in a variety of eukaryotes (1, 2). Mammals have two PKG genes that encode PKG I and PKG II. There are two splice variants of PKG I, which are designated Iα and Iβ (3).

PKG I null mice seemed to have lost NO/cGMP-dependent smooth muscle relaxation leading to severe vascular and intestinal dysfunction with death at a young age (4). PKG I also regulates myocardial contractility, platelet aggregation, endothelial cell permeability, neuronal cell survival, and synaptic plasticity. PKG has important regulatory roles in diverse processes in many cell types such as cell growth and differentiation (for review see ref. 5). More recently, an antiproliferative/apoptotic effect of PKG has been reported in many types of tumor cells. PKG has been implicated in the mechanism underlying the induction of apoptosis in colon as well as many other tumor cells in response to the phosphodiesterase inhibitor exisulind (for references, see ref. 5). Indeed, in a previous study, we showed that activation of PKG is sufficient to induce apoptosis in human colon cancer cells (6). In colon cancer cell lines, exisulind can induce up-regulation of PKG expression in addition to enzyme activation (7). A recent study (5) found that PKG levels are decreased in most tumors compared with the normal tissue counterpart. Moreover, in the same study, antitumor properties of PKG were revealed by exogenous expression of this enzyme in a colon carcinoma cell line, which resulted in decreased growth of xenografts in nude mice. However, the underlying mechanisms by which PKG exerts antitumor effects have not been fully elucidated.

Both $p21^{CIP1}$ and $p27^{KIP1}$ (hereafter called p21 and p27) are members of the cip/kip family of cyclin-dependent kinase inhibitors. They are critical regulators of cell cycle progression and considered important targets for cancer therapeutics (8, 9). Although mutations in p21 are rare in human cancers, a variety of studies suggest a tumor suppressor function for p21 (10–13). Homozygous loss of p27 or silencing of the locus in human tumors is extremely rare. However, an inverse correlation between p27 protein levels and prognosis was noted in many cancers (see review ref. 9). Cellular levels of p21 are controlled transcriptionally by p53-dependent and p53-independent mechanisms (14) and post-transcriptionally by the proteasome-mediated proteolysis (15–20). Similarly, p27 protein levels are regulated both at the transcriptional level (21, 22) and also at the posttranscriptional level by proteasomal proteolysis (23–26). It is of interest that multiple Sp1 binding sites are present in the promoters of both the *p21* and *p27* genes. Indeed, Sp1 mediates induction of the *p21* and *p27* genes in response to various stimuli (14, 21).

The Histidine triad protein 1 (HINT1) is a member of the Hint1 branch of the evolutionary conserved histidine triad (HIT) protein family. These proteins contain a characteristic HIT motif, His-X-His-X-His-XX (X is a hydrophobic residue). All members show nucleotide hydrolase and/or transferase activity (27). HINT1 has emerged as a novel tumor suppressor (28, 29). It plays an important role in regulating the activities of several transcription factors, and its expression is inhibited by promoter methylation in several human cancer cell lines (30–33). However, it is not known how cells regulate the expression of HINT1 at the level of transcription.

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In the present study, we discovered that activation of PKG increases the expression of three tumor suppressor proteins, p21, p27, and HINT1 in SW480 human colon cancer cells and that this is associated with increased transcription of the corresponding genes. Luciferase reporter assays indicate that the transcription factor Sp1 is directly involved in this up-regulation. Furthermore, we obtained evidence that PKG can phosphorylate Sp1 on serine residues, thus, presumably, causing its activation. The ability of PKG to increase the expression of these three tumor suppressors may explain, at least in part, the anticancer effects of various activators of PKG.

Materials and Methods

Materials and cell lines. Exisulind and 8-bromoguanosine-3′,5′-cyclomonophosphate (8-Br-cGMP) were purchased from Sigma. The cGMP compound 8-para-chlorophenylthio-cGMP (8-pCPT-cGMP) was from BIOLOG. An anti-p21 antibody and an anti-$p15^{INK4B}$ antibody were from Cell Signaling, an anti-p27 antibody and an anti–β-Catenin antibody were from Transduction Laboratories, an anti–β-actin, an anti-phosphothreonine, and an anti-hemagglutinin (HA) antibody were from Sigma. An anti-phosphoserine antibody was from Zymed. An anti-Sp1 antibody was from Santa Cruz. An anti-PKG Iβ antibody was from Stressgen. An anti-HINT1 antibody was previously described (34). The construction and characteristics of plasmids that encode HA-COOH-terminal–tagged wild-type, constitutively active (SD), and dominant negative (KR) forms of PKG Iα and PKG Iβ were described in our previous publication (35). The Sp1 expression plasmid was kindly provided by Dr. Robert Tjian (University of California, Berkeley, Berkeley, CA). The p21 and p27 promoter luciferase reporters and their corresponding mutants were kindly provided by Dr. Toshiyuki Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). SW480 cells stably expressing PKG Iβ and a control cell line were described previously (6).

Western blot analysis. Cells were harvested and then sonicated in radioimmunoprecipitation assay buffer. The lysates were separated using SDS-PAGE and then electophoretically transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% dry milk and incubated with the indicated antibodies. After washing, the membrane was incubated with a horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences). Protein bands were visualized with the enhanced chemiluminescence Western blotting system (Amersham Biosciences).

Immunoprecipitation assay. For immunoprecipitation, cells from 6-cm plates were lysed in 400 μL of immunoprecipitation buffer [20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 0.5% Triton X-100, 10% glycerol, and 1 mmol/L EDTA]. The lysates were rotated for 30 min at 4°C followed by a 10-s sonication and cleared by centrifugation at 13,000 × *g* for 10 min at 4°C. Immunoprecipitation was done using EZview Red Protein A Affinity gel (Sigma) and anti-Sp1 antibody (Santa Cruz). The beads were washed three times with immunoprecipitation buffer, and the proteins were resolved on SDS-PAGE.

Luciferase reporter assays. SW480 cells were plated at 1×10^5 cells per well of 6-well 35-mm-diameter plates, and 24 h later, they were transfected with pWWP or p27PF reporter plasmids or their corresponding mutants, and as indicated in the figure legends, they were cotransfected with the PKG IβSD expression plasmid, the PKG IβKR expression plasmid, or the Sp1 expression plasmid and the Renilla Luciferase reporter plasmid (as internal control), using the Lipofectin reagent (Invitrogen). Twenty four hours after transfection, the cells were incubated in fresh growth medium for additional 16 h and the cell extracts were analyzed using Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activities were normalized to the Renilla luciferase activities to compensate for variability in transfection efficiencies. The relative luciferase activity measured in the vector control cells was assigned the value of 100%. All experiments were performed with duplicate plates for each point and repeated at least three times.

Reverse transcription-PCR analysis. Total RNA was isolated from SW480 cells using Trizol reagent as recommended by the manufacturer (Invitrogen). cDNA was amplified from 10 ng (for β-actin) or 1 μg (for p21, p27, and HINT1) of total RNA using a Superscript One-Step reverse transcription-PCR (RT-PCR) with the platinum Taq DNA polymerase system (Invitrogen). Primer sets used were as follows: p21forward, 5′-GCGACTGTGATGCGCTAAT-3′; p21reverse, 5′-TAGGGTTCCTCTTGGAGAA-3′; p27 forward, 5′-TGCAACCGACGATTCTTCTACTCAA-3′; p27reverse, 5′-CAAGCAGTGATGTATCTGATAAACAAGGA-3′; Hint1forward, 5′-CGAGATGGCAGATGAGATTG-3′; Hint1reverse, 5′-CCTTATTCAGGCCCAGATCA-3′. As an internal control, we used β-actin, forward, 5′-GACCTGACTGACTACCTC-3′, reverse, 5′-GACAGCGAGGCCAGGATG-3′. After 30 cycles, PCR products were analyzed by agarose gel electrophoresis.

RNA interference. On-TargetPlus SMARTpool siRNA for Sp1 or On-TargetPlus siControl pool were purchased from Dharmacon, and 10 nmol/L of each pool were transfected into SW480 cells using HiPerfect transfection reagent (Qiagen). Treatment of the cells with 8-pCPT-cGMP was conducted 60 h after transfection.

Statistical and densitometry analysis. All assays were repeated at least three times. The results of quantitative studies are reported as mean ± SD. Differences were analyzed by Student's *t* test. A P value of <0.05 was regarded as significant, and such differences are indicated in the figures by an asterisk. Densitometry was determined with NIH ImageJ v1.38 software with normalization to the corresponding β-actin control.

Results

Activation of PKG leads to increased p21, p27, and HINT1 protein expression. To examine the effects of PKG activation on the expression of the above three tumor suppressor proteins, we treated the human colon cancer cell line SW480 with two PKG activators, exisulind and 8-Br-cGMP. Exisulind is an inhibitor of cGMP-phosphodiesterases and, thereby, increases cellular level of cGMP. 8-Br-cGMP is a cell membrane permeable cGMP derivative. Exisulind induced the up-regulation of p21 and HINT1 proteins starting from 4 hours after the compound was added to the medium, and this effect lasted until 16 h, the longest time point we tested (Fig. 1*A, left*). 8-Br-cGMP caused a similar effect on p21 and HINT1 protein expression with a slight difference: the up-regulation of p21 and HINT1 could be seen at an earlier time point, 2 hours (Fig. 1*A, right*), presumably because 8-Br-cGMP can bind PKG directly, whereas exisulind causes an increase of intracellular levels of cGMP indirectly by inhibiting phosphodiesterases that hydrolyze cGMP. Another potent cell-permeable cGMP analogue, 8-pCPT-cGMP, also caused increased expression of the p21, p27, and HINT1 proteins (Fig. 1*B*). These findings were confirmed by results obtained with a SW480 cell line that stably overexpresses PKG Iβ (Fig. 1*C*). Compared with the control cell line, the latter cells express markedly more p21, p27, and HINT1 proteins, whereas the level of another cyclin-dependent kinase inhibitor, $p15^{INK4B}$, was largely unchanged. Interestingly, overexpression of PKG Iβ also caused decreased expression of the β-catenin protein (Fig. 1*C*), a previously reported finding (6). Furthermore, transient overexpression of a dominant-negative form of PKG Iβ, PKG IβKR, significantly inhibited 8-pCPT-cGMP–induced up-regulation of p21 and p27 (~50% inhibition for both genes) in SW480 cells (Fig. 1*D*).

Activation of PKG leads to increased expression of p21, p27, and HINT1 mRNA. To examine if activation of PKG alters the transcription of p21, p27, and HINT1, we again treated SW480 cells with exisulind or 8-pCPT-cGMP. Using RT-PCR, we found that the mRNA levels of p21, p27, and HINT1 increased, although with slightly different kinetics after drug treatments (Fig. 2). This indicates that activation of PKG can activate the transcription of these three genes.

PKG activates the promoter of p21 through Sp1 binding sites. To explore how PKG increases the mRNA levels of p21, we

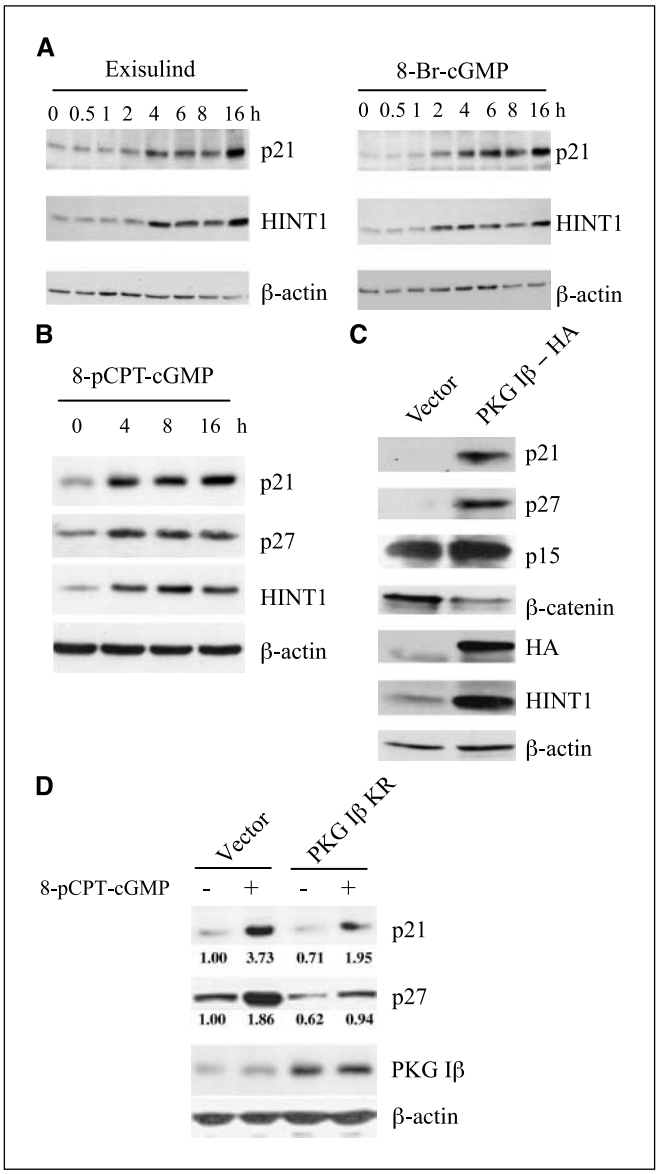

Figure 1. PKG increases p21, p27, and HINT1 expression in SW480 cells. SW480 cells were treated with 600 μmol/L exisulind (*A, left*), or 1 mmol/L 8-Br-cGMP (*A, right*), or 250 μmol/L 8-pCPT-cGMP (*B*) and harvested at the indicated time points. Cell lysates were then subject to Western Blot analysis with the indicated antibodies. All experiments were repeated at least three times and representative results are shown. *C,* SW480 cells stably transfected with the empty vector or the PKG Iβ expression construct were harvested, and cell lysates were subject to Western blot analysis with the indicated antibodies. *D,* SW480 cells were transiently transfected with the empty vector or the PKG IβKR expression construct. Forty hours after transfection, cells were treated with 250 μmol/L 8-pCPT-cGMP for 8 h and harvested, and cell lysates were subject to Western blot analysis with the indicated antibodies. Relative fold expression was analyzed with NIH ImageJ v1.38 software with normalization to the corresponding β-actin control. Similar results were obtained in three independent experiments, and a representative one is shown.

cotransfected a constitutively active mutant of PKG Iβ, PKG IβSD (35), with a full-length p21 promoter linked to a luciferase reporter (pWWP; Fig. 3*A*). A dose-dependent activation of pWWP was obtained in the presence of PKG IβSD. A dominant-negative kinase inactive mutant of PKG Iβ, PKG IβKR, served as a negative control (Fig. 3*B, top*). Results similar to those obtained with PKG IβSD were obtained with a constitutively active mutant of PKG Iα, PKG IαSD (data not shown). Indeed, PKG IαSD gave similar results to those obtained with PKG IβSD in all of the luciferase assays in this study (data not shown). For consistency, hereafter, we only show the results obtained with PKG IβSD. Treatment of cells with 8-pCPT-cGMP also caused statistically significant activation of pWWP (Fig. 3*B, bottom*) demonstrating that activation of PKG has a positive role on the transcriptional activity of the p21 promoter.

Because a striking feature of the p21 promoter is that it contains six potential Sp1 binding sites (14), we tested the possible role of Sp1 binding sites in PKG-induced p21 promoter activation by using two truncation mutants of pWWP, pWWP124 and pWWP101. Although pWWP124 has only five Sp1 sites and one AP2 site and pWWP101 contains only four Sp1 sites (Fig. 3*A*), both of these promoters could still be activated by PKG IβSD, and this activation was similar to that obtained with the pWWP except that the basal expression of both mutant promoters was slightly decreased (Fig. 3*C*). These findings suggest that Sp1 mediates PKG-induced activation of the p21 promoter.

Because PKG seems to activate the p21 promoter through Sp1 binding sites, we examined the combined effects of PKG and Sp1 on activation of the p21 promoter. SW480 cells were cotransfected with pWWP and a control vector, PKG IβSD, Sp1, or the combination of PKG IβSD and Sp1. Both PKG IβSD alone and Sp1 alone increased pWWP activity, and stronger activation was obtained when PKG IβSD and Sp1 were cotransfected (Fig. 3*D*).

PKG activates the promoter of p27 through Sp1 binding sites. A series of similar experiments were done to examine activities of the p27 promoter. The full-length p27 promoter was linked to a luciferase reporter (p27PF; Fig. 4*A*). The p27 promoter contains two Sp1 sites (21). This promoter was activated by PKG IβSD because we obtained a dose-dependent up-regulation of p27PF activity by transfection of SW480 cells with increasing amounts of the PKG IβSD expression plasmid (Fig. 4*B*). On the other hand, PKG IβKR did not activate p27PF (Fig. 4*B*). To determine the site of action of PKG on the p27 promoter, we also tested a series of mutants of p27PF. The truncation mutant p27No.2 reporter that only contains two Sp1 sites and one CTF site (CCAAT box) was still activated by PKG IβSD (Fig. 4*C*). Therefore, we then focused on the two Sp1 sites. We found that mutation of the Sp1 site, Sp1-1, caused a loss of PKG IβSD activation. Interestingly, mutation of the second Sp1 site, Sp1-2, did not result

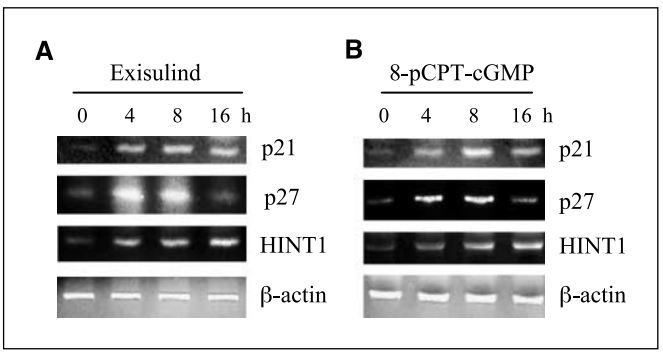

Figure 2. Treatment of SW480 cells with PKG activators led to increased p21, p27, and Hint1 mRNA expression. SW480 cells were treated with (*A*) 600 μmol/L exisulind or (*B*) 250 μmol/L 8-pCPT-cGMP and harvested at the indicated times. Total RNA was extracted using Trizol reagent, and a one-step RT-PCR kit (Invitrogen) was used to detect the mRNA expression. One microgram of total RNA was used for p21, p27, and HINT1, and 10 ng of total RNA were used for β-actin. All experiments were repeated at least three times, and representative results are shown.

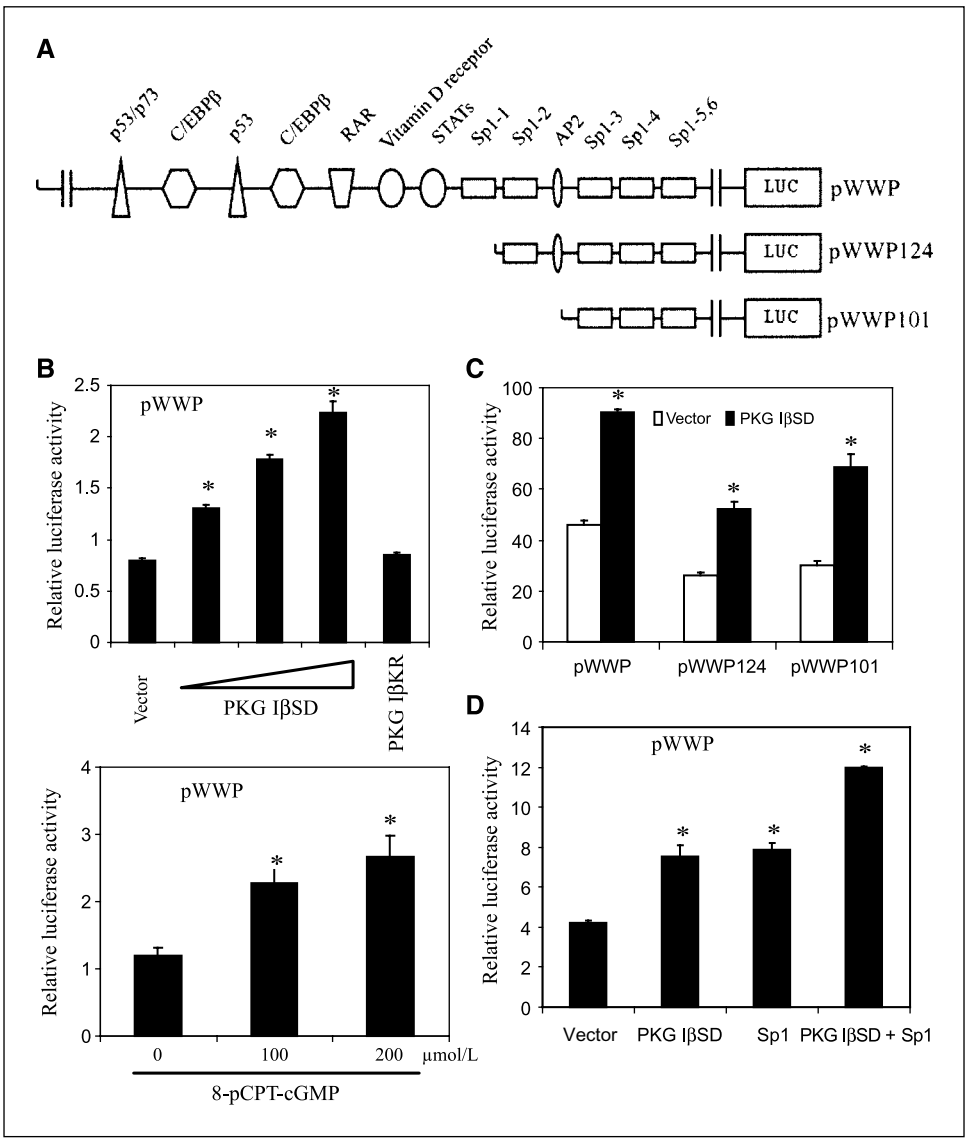

Figure 3. PKG increased p21 promoter luciferase reporter activity. *A,* schematic diagrams of p21 promoter luciferase reporter (pWWP) and its mutants. *B,* the pWWP (20 ng) and the pRL-SV40P (10 ng) plasmid (as an internal control) were cotransfected into SW480 cells together with the empty vector (0.5 μg) or increasing amounts of the PKG IβSD expression plasmid (0.5, 1, and 2 μg) or the PKG IβKR expression plasmid in 6-well plates (*top*). Or the pWWP (20 ng) and the pRL-SV40P (10 ng) plasmid were cotransfected into SW480 cells. Forty hours later, the cells were treated with indicated concentration of 8-pCPT-cGMP for additional 8 h (*bottom*). Luciferase activities were assayed 40 h after transfection and normalized to the Renilla luciferase activities carried by pRL-SV40P. All assays were conducted with duplicate plates at least three times. $P < 0.05$, versus controls. *C,* 20 ng of pWWP, pWWP124, and pWWP101 were each cotransfected with 10 ng of pRL-SV40P and 1 μg of PKG IβSD, as indicated. Luciferase assays were done as described in *A*. $P < 0.05$, versus controls. *D,* the pWWP reporter was cotransfected with the empty vector, PKG IβSD plasmid, and Sp1 plasmid as indicated. Luciferase assays were done as described above. $P < 0.05$, versus control.

in any loss of PKG IβSD activation (Fig. 4*C*). Therefore, it seems that Sp1 mediates the activation of the p27 promoter by PKG IβSD through the Sp1-1 site. A mutation in the CTF site of the p27 promoter did not have any effect on PKG IβSD activation (data not shown).

We also examined the combined effects of PKG and Sp1 on activation of the p27 promoter. SW480 cells were cotransfected with p27PF and a control vector, PKG IβSD, Sp1, or the combination of PKG IβSD and Sp1. Both PKG IβSD alone and Sp1 alone increased p27PF activity, and an additive effect was obtained when the cells were cotransfected with PKG IβSD and Sp1 (Fig. 4*D*).

Sp1 is required for PKG induced p21, p27, and HINT1 up-regulation. To further investigate the requirement of Sp1 for PKG induced up-regulation of p21, p27, and HINT1, we knocked down expression of endogenous Sp1 using RNA interference technology. The expression of Sp1 in SW480 cells was dramatically reduced by the siRNA of Sp1 compared with the control siRNA (Fig. 5, *top*). The activation of p21, p27, and HINT1 protein expression was clearly seen in control siRNA–transfected SW480 cells after treatment of the cells with 8-pCPT-cGMP. This activation was strongly inhibited by the knockdown of Sp1 expression (Fig. 5, *second, third,* and *fourth from the top*). The basal levels of all three proteins were also decreased in the latter cells (0 h; Fig. 5). Note that the level of expression of PKG Iβ was not affected by either Sp1 knockdown or 8-pCPT-cGMP treatment (Fig. 5, *fifth from the top*).

PKG phosphorylates Sp1 on serine residue(s). PKGs are serine/threonine kinases. Therefore, we examined the possibility that activated PKG can phosphorylate Sp1 on serine or threonine residues and thereby enhance its transcription activity. First, we treated SW480 cells with 8-pCPT-cGMP for the indicated times (Fig. 6*A*). Using an anti–phospho-serine antibody, at time 0, we detected a basal level of serine-phosphorylation of endogenous Sp1. We then observed increased phosphorylation of Sp1 at the 2- and 4-hour time points. This phosphorylation of Sp1 returned to the basal level at the 8-h time point (Fig. 6*A, top*). When we reprobed the same membrane with an anti–phospho-threonine antibody, we did not see a difference in the level of Sp1 threonine-phosphorylation between the different time points (Fig. 6*A, middle*). In these studies, the amount of endogenous Sp1 was equally immunoprecipitated at all time points (Fig. 6*A, bottom*).

To further establish the role of PKG in Sp1 phosphorylation, we transfected two constitutively active mutants of PKG I, PKG IαSD, and PKG IβSD (35) into SW480 cells. Both mutants caused increased serine-phosphorylation of endogenous Sp1 when compared with the control cells transfected with an empty vector (Fig. 6*B, top*). Neither mutant caused increased threonine-phosphorylation of Sp1 (Fig. 6*B, middle*).

Taken together, these studies indicate that activation of PKG can lead to serine-phosphorylation of the transcription factor Sp1.

Discussion

The present studies provide the first detailed evidence that activation of PKG can lead to increased expression of three tumor suppressor genes *p21, p27*, and *Hint1*, as well as evidence that this occurs via PKG activation of the transcription factor Sp1. Our study used a combination of pharmacologic and genetic approaches in SW480 colon cancer cells. Thus, the induced expression of these three genes was seen with the cGMP-phosphodiesterase inhibitor exisulind, two cell-permeable cGMP derivatives 8-Br-cGMP and 8-pCPT-cGMP, and the expression of constitutively activated mutants of PKG Iα or PKG Iβ. Although there is some controversy about the mechanism of action of exisulind (36), the results we obtained with the other approaches still provide strong support for this conclusion. Furthermore, it is unlikely that our results are an artifact due to overexpression of activated forms of PKG because of the similar results obtained with the two cell-permeable cGMP derivatives. More importantly, expression of dominant-negative form of PKG Iβ, PKG IβKR, inhibited cGMP induced up-regulation of p21 and p27 (Fig. 1*D*). This is a transient transfection experiment so that only ~60% of cells were transfected with PKG IβKR expression construct (estimation based on green fluorescent protein observation). However, we still obtained ~50% inhibition (Fig. 1*D*). In addition, we have previously reported that expression of activated forms of PKG Iα and PKG Iβ cause several effects that mimic those of exisulind and permeable forms of cGMP, including inhibition of cell proliferation, apoptosis, inhibition of β-catenin and cyclin D1 expression, and activation of 15-Lox-1 expression (6, 37). Previous studies in our laboratory showed that increased expression of PKG Iβ increases expression of the p21 protein (6). In the present study, we found that the up-regulation of p21 by activation of PKG occurs at the transcriptional level (Figs. 2 and 3). Induction of p21 expression by OSI-461, a potent derivative of exisulind that inhibits cGMP-phosphodiesterases, thereby increasing cellular levels of cGMP and activating endogenous PKG (7), has also been described in chronic lymphocytic leukemia cells but was not explored in detail (38). Our results are also consistent with the finding of Gu and colleagues (39) that treatment of adventitial fibroblast cells with nitric oxide, which activates guanylyl cyclase, and thereby PKG, caused increased expression of p21 via a cGMP-dependent pathway. We also found that activation of PKG causes up-regulation of the expression of p27 and

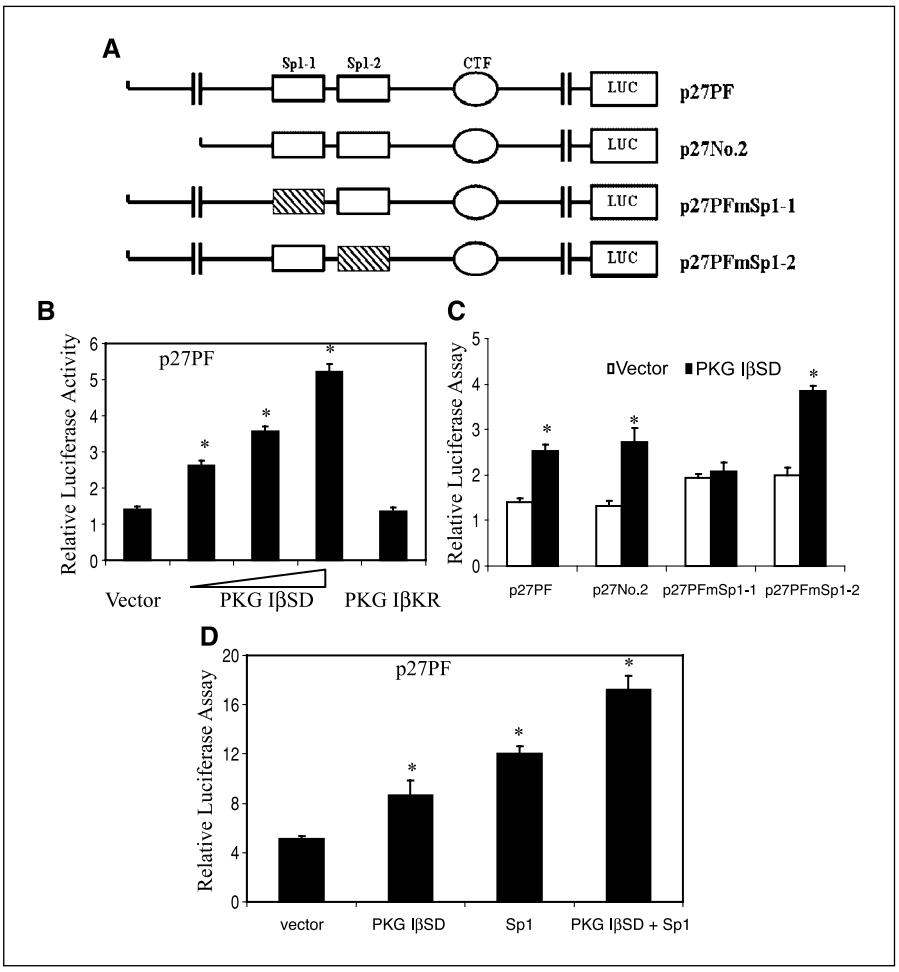

Figure 4. PKG increased p27 promoter luciferase reporter activity. *A*, schematic diagrams of p27 promoter luciferase reporter (p27PF) and its mutants. *B,* luciferase assays were conducted as described in Fig. 3*B* except that p27PF was used as the reporter instead of pWWP. $P < 0.05$, versus control. *C,* luciferase assays were conducted as described in Fig. 3*C* except that p27PF, p27No.2, p27PFmSp1-1, and p27PFmSp1-2 were used to replace pWWP, pWWP124, and pWWP101. $P < 0.05$, versus controls. *D,* the p27PF reporter was cotransfected with the empty vector, PKG IβSD plasmid, and Sp1 plasmid, as indicated. Luciferase assays were done as described in Fig. 3*D*. $P < 0.05$, versus control.

HINT1 through transcriptional activation of these two genes (Figs. 1, 2, and 4). Previous studies from our laboratory indicated that the cyclooxygenase (COX) inhibitor sulindac sulfide also increases cellular levels of p21 and p27 (40). Therefore, although exisulind does not inhibit COX1 or COX2 (41, 42), certain nonsteroidal anti-inflammatory drugs (NSAID) that do inhibit COX1 or 2 may exert anticancer effects by a COX-independent mechanism, involving PKG activation. Indeed, in a recent study (43), we found that when tested at concentrations that inhibit the growth of SW480 cells, the NSAIDs celecoxib, indomethacin, and meclofenamic acid caused, within 1 hour, a ~2-fold increase in cellular levels of cGMP, which persisted for at least 24 hours, and *in vitro* all three compounds inhibited cGMP phosphodiesterase activity. Therefore, in future studies, it will be of interest to determine if these NSAIDs also induce the expression of p21, p27, and HINT1 via PKG activation.

It is well-established that the transcription of p21 is regulated by both p53-dependent and p53-independent mechanisms (44). The promoter of the *p21* gene contains two conserved p53-binding sites, and at least one of these is required for p53 responsiveness after DNA damage (45). In addition, a variety of transcription factors that are induced by a number of different signaling pathways can activate p21 transcription by a p53-independent mechanism, including Sp1, Sp3, STATs, CAAT/enhancer binding protein (C/EBP), C/EBPβ, and Smad3 (for references see ref. 6). Because the SW480 cells that we used carry a mutant p53, the induction of p21 by PKG seems to be through a p53-independent pathway. Indeed, we found that Sp1 is sufficient to mediate transcriptional activation of the p21 promoter by PKG in the absence of p53 binding sites (Fig. 3).

Sp1 also plays an important role in transcriptional regulation of the p27 promoter, which contains two Sp1 binding sites (21, 22). We obtained evidence that activation of PKG increases the transcription of p27 through one of these Sp1 binding sites (Fig. 4*C*),

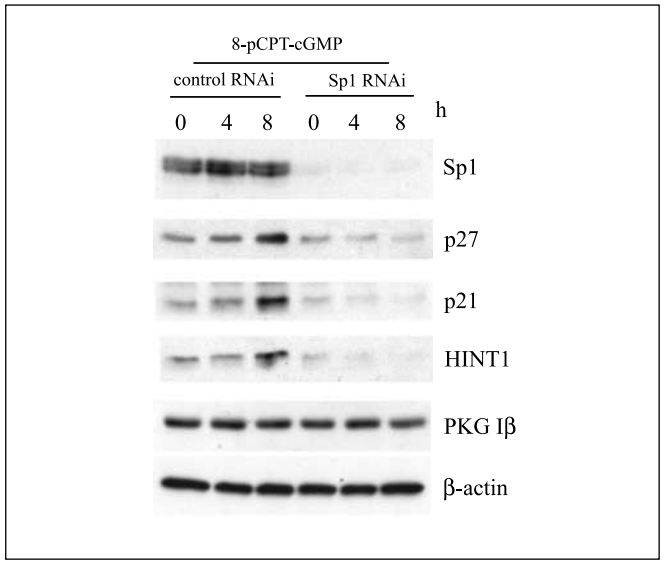

Figure 5. Sp1 is required for 8-pCPT-cGMP induced up-regulation of p21, p27, and HINT1. SW480 cells were transfected with a control siRNA pool or a Sp1 siRNA pool for 60 h and then the cells were treated with 250 μmol/L 8-pCPT-cGMP for the indicated times. Cell lystes were subject to Western blot analysis with antibodies specific for Sp1, p21, p27, HINT1, PKG Iβ, and β-actin. The same experiment was repeated three times and representative results are shown in this figure.

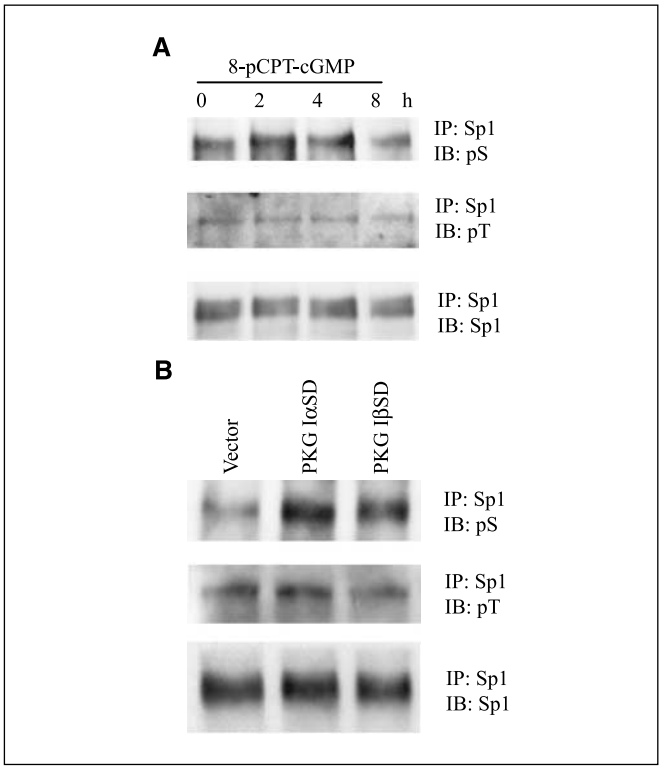

Figure 6. PKG phosphorylates Sp1 at serine site(s). *A,* SW480 cells were treated with 250 μmol/L 8-pCPT-cGMP for the indicated times and cell lystes were used for immunoprecipitation assays with an anti-Sp1 antibody. Phospho-serine (*pS*) bands were visualized by using an anti–phospho-serine antibody. The same membrane was stripped and reprobed with an anti–phospho-threonine (*pT*) antibody and an anti-Sp1 antibody, consecutively. *B,* SW480 cells were transfected with the empty vector, PKG IαSD, and PKG IβSD plasmids, as indicated. Thirty hours after transfection, cell lysates were used for immunoprecipitation assays and subsequent Westen blot analysis, as described in *A*. All experiments were repeated three times and representative results are shown.

thus resembling the mechanism by which PKG regulates p21 transcription. PKG may use a similar mechanism to modulate HINT1 expression because we found that activation of PKG increases expression of the HINT1 protein and mRNA (Figs. 1 and 2). Although the promoter of HINT1 has not been isolated, using computer-based analysis[3], we found potential Sp1 binding sites in the Hint1 promoter. The requirement of Sp1 for the up-regulation of p21, p27, and HINT1 by PKG is clearly shown in the Sp1 knockdown SW480 cells. When compared with the control RNAi SW480 cells, the PKG activator 8-pCPT-cGMP could not increase the expression of p21, p27, or HINT1 in Sp1 RNAi SW480 cells (Fig. 5).

Because PKG seems to up-regulate the expression of p21, p27, and HINT1 through activation of Sp1 (Figs. 3–5), we sought to examine if PKG can phosphorylate Sp1 *in vivo*. Indeed, we found that there is increased phosphorylation of endogenous Sp1 at serine sites after the treatment of SW480 cells with the above PKG activator (Fig. 6*A*) or overexpression of constitutively activated forms of PKG (Fig. 6). Posttranslational modifications of Sp1 such as phosphorylation were shown to stimulate or inhibit its transcriptional activity or DNA binding affinity in different contexts

[3] Unpublished observations.

(reviewed in ref. 46). The present study provides the first evidence that PKG can also phosphorylate Sp1. Sp1 phosphorylation by PKG enhances its transcriptional activities toward the promoters of p21, p27, and HINT1 because the transcription of these genes is all up-regulated by PKG. The phosphorylation and activation of Sp1 by PKG is consistent with previous reports that both PKG and Sp1 overexpression are sufficient to induce apoptosis (6, 47). We must emphasize, however, that we have not shown that PKG can directly phosphorylate and activate Sp1 *in vitro*, although our results (Figs. 5 and 6) strongly support this mechanism.

Several potential Sp1 binding sites have been detected in the promoter of the human PKG I gene (48). Interestingly, Sp1 was found to mediate the suppression of PKG Iα promoter activity induced by nitric oxide and cyclic nucleotides, including cGMP, in vascular smooth muscle cells. It also seems that PKA instead of PKG mediates the effect of cyclic nucleotides on PKG promoter activity (49). In the current study, we did not detect altered expression of PKG Iβ after treating SW480 cells with 8-pCPT-cGMP (Fig. 5). Therefore, the specificity of PKG for specific Sp1 binding sites requires further study. Indeed, among the prominent properties of Sp1 is its potential to rapidly regulate the expression of a wide array of genes (46). Multigene regulation can result in higher level physiologic outcomes. A previous study from our group demonstrating that constitutively activated mutants of PKG I induce apoptosis in SW480 colon cancer cells (6), plus the ability of PKG to activate the transcription of multiple tumor suppressor genes, of p21, p27, and HINT1, through phosphorylation of Sp1, provides another example of multigene regulation. Previous evidence that activation of PKG can decrease cellular levels of β-catenin, cyclin D1, 15-LOX-1, and can cause phosphorylation of VASP (for review, see ref. 37) provided mechanisms by which PKG activators can exert anticancer effects. The effects of PKG activation on three tumor suppressor genes described in the present study may further contribute to the anticancer effects of PKG activators. Thus, the findings in the present study provide a further rationale for developing activators of PKG as a novel therapy for cancer prevention and therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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