Activation of Protein Kinase G Is Sufficient to Induce Apoptosis and Inhibit Cell Migration in Colon Cancer Cells

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ABSTRACT

The activation of protein kinase G (PKG) by cGMP has become of considerable interest as a novel molecular mechanism for the induction of apoptosis in cancer cells, because sulindac sulfone (exisulind, Aptosyn) and certain derivatives that inhibit cGMP-phosphodiesterases and thereby increase cellular levels of cGMP appear to induce apoptosis via this mechanism. However, other effects of these compounds have not been excluded, and the precise mechanism by which PKG activation induces apoptosis has not been elucidated in detail. To directly examine the effects of PKG on cell growth and apoptosis, we generated a series of mutants of PKG Iα: PKG IαS65D, a constitutively activated point mutant; PKG IαΔ, a constitutively activated N-terminal truncated mutant; and PKG IαK390R, a dominant-negative point mutant. A similar series of mutants of PKG Iβ were also constructed (Deguchi et al., Mol. Cancer Ther., 1: 803–809, 2002). The present study demonstrates that when transiently expressed in SW480 colon cancer, the constitutively activated mutants of PKG Iα, and to a lesser extent PKG Iα, inhibit colony formation and induce apoptosis. We were not able to obtain derivatives of SW480 cells that stably expressed these constitutively activated mutants, presumably because of toxicity. However, derivatives that stably overexpressed wild-type PKG Iβ displayed growth inhibition, whereas derivatives that stably expressed the dominant-negative mutant (KR) of PKG Iβ grew more rapidly and were more resistant to Aptsyn-induced growth inhibition than vector control cells. Stable overexpression of PKG Iβ was associated with decreased cellular levels of β-catenin and cyclin D1 and increased levels of p21CIP1. Reporter assays indicated that activation of PKG Iβ inhibits the transcriptional activity of the cyclin D1 promoter. We also found that transient expression of the constitutively activated mutants of PKG Iβ inhibited cell migration. Taken together, these results indicate that activation of PKG Iβ is sufficient to inhibit growth and cell migration and induce apoptosis in human colon cancer cells and that these effects are associated with inhibition of the transcription of cyclin D1 and an increase in the expression of p21CIP1.

INTRODUCTION

cGMP is an important second messenger that mediates the action of several hormones, neurotransmitters, and drugs (1) by regulating various physiological functions including neurotransmission, platelet aggregation, and smooth muscle tone (2). There is increasing evidence that it can play an important role in cellular proliferation, differentiation, and apoptosis (3–5). The intracellular level of cGMP is regulated through a dynamic balance between its rate of synthesis by guanylyl cyclases and degradation by specific phosphodiesterases (PDEs), especially PDEs 2 and 5 (6). cGMP has several intracellular targets. Thus, it can bind to specific PDEs, thereby stimulating or inhibiting their activities (7); it can bind to and activate cGMP-gated channels; it can bind to and activate protein kinase G (PKG); and, under certain conditions, it can bind to and activate protein kinase A (8).

Two major forms of PKG have been identified in mammalian cells, PKG I and PKG II. In addition, there are two splice variants of PKG I, which are designated Iα and Iβ (9). PKG I is expressed in platelets, vascular smooth muscle cells, fibroblasts, certain endothelial cells, the lung, the cerebellum, and the heart (10, 11). In endothelial cells, activation of PKG I by cGMP is associated with a reduction in thrombin-induced calcium transients and paracellular permeability (12, 13). Direct evidence for functional roles of PKG I in relaxation of smooth muscle, inhibition of Ca2+ transients, and inhibition of platelet adhesion and aggregation was obtained from studies of PKG I knockout mice (14, 15). Vasodilator-stimulated phosphoprotein (VASP) is known to be one of the substrates of PKG I, and its phosphorylation plays a role in inhibiting platelet aggregation and focal adhesion (16). PKG II is expressed mainly in the brush border of the intestinal mucosa and in specific regions of the brain and plays a role in transethelial Cl− and Na+ transport in the intestine (17, 18).

Expression of PDE 5 has been detected in the normal bladder, colon, pancreas, lung, placenta, prostate, small intestine, and stomach (19). It is of interest that metastatic breast cancers, colon adenocarcinoma, bladder squamous carcinoma, and lung cancers often express increased cellular levels of PDE 2 or 5 when compared with adjacent normal tissues (20–25). In addition, the endogenous polypeptide guanylyl cyclase activators guanylin and uroguanylin are expressed at reduced levels in colon cancer cells (26, 27). Taken together, these findings suggest that cGMP-mediated pathways are suppressed in colon cancer cells, presumably to inhibit downstream signaling pathways related to the activation of PKG. There is also supporting evidence that activation of PKG by cGMP in cardiomyocytes (3), pancreatic B cells (4), and cultured smooth muscle cells (28, 29) can cause growth inhibition and apoptosis. In addition, PKG activation negatively regulates interleukin-2 signaling in T cell lines (30). Furthermore, recent studies indicate that sulindac sulfone (Aptosyn), a metabolite of the nonsteroidal anti-inflammatory drug sulindac, and two potent derivatives of Aptosyn, OSI-248 and OSI-461, specifically inhibit the cGMP-specific PDEs 2 and 5. Evidence has also been obtained that the resulting increase in cellular levels of cGMP in human colon cancer cells leads to activation of PKG and thereby the induction of apoptosis (31–33). These novel effects of Aptosyn and related drugs may explain why these compounds exert anticaner effects in a variety of biological systems even though, in contrast to conventional nonsteroidal anti-inflammatory drugs, they do not inhibit cyclooxygenase activity (34). The precise pathway by which PKG activation leads to apoptosis is not known, although it appears to involve both a decrease in cellular levels of β-catenin and the activation of c-Jun NH2-terminal kinase 1 (31, 32, 35, 36).

Cell cycle progression is regulated by the orderly activation and inactivation of a series of cyclins and cyclin-dependent kinases and is coordinated by both internal and external signals that act at key checkpoints during cell cycle progression (37). Cyclin D1 is a major positive regulator of the Gl-S transition. It acts by binding to and
activating cyclin-dependent kinase 4 or cyclin-dependent kinase 6, which then phosphorylates and thereby inactivates the tumor suppressor protein pRB (38). These activities are negatively regulated by \( p16^{INK4A}, p21^{CIP1} \) (p21), and \( p27^{KIP1} \) (39). \( p21^{CIP1} \) was first cloned and characterized as a mediator of p53-induced growth arrest (40), but its expression can also be up-regulated by p53-independent mechanisms (41). Although the cyclin D1 gene is not amplified in human colon cancer, the expression of cyclin D1 is induced in about 30% of human adenocarcinoma and also in adenomatous polyps of the colon (42, 43). Furthermore, inhibition of cyclin D1 expression with an antisense cDNA causes growth inhibition in colon carcinoma cell lines (44). Colon cancers also frequently display elevated levels of cyclin D1 expression, which can be increased by \( p53 \)-independent mechanisms (45). This results in increased binding of \( p53 \)-catenin to the transcription element T-cell factor/lymphoid enhancer factor-1 and increased transcription of cyclin D1 and other genes that enhance growth (46, 47). Therefore, the high expression levels of cyclin D1 in colon cancer are at least in part due to up-regulation of \( p53 \)-catenin/T-cell factor transcriptional activity. Recent studies by Li et al. (48) provide evidence that activated PKG directly phosphorylates \( p53 \)-catenin in the COOH-terminal region of the molecule, in contrast to GSK3, which phosphorylates \( p53 \)-catenin at a characteristic site in the NH2 terminus, and that this phosphorylation by PKG results in proteolytic degradation of \( p53 \)-catenin.

Previous studies implicating PKG in growth inhibition and apoptosis in colon cancer cells are based on using pharmacological agents (31–33). In the present study, we obtained more direct evidence by exploring the effects of expressing a series of wild-type (WT), constitutively active, or dominant-negative mutants of PKG, previously developed in our laboratory (33), in the SW480 human colon cancer cell line.

MATERIALS AND METHODS

Materials and Cell Culture. The cell-permeable cGMP compound 8-para-chlorophenylthio-cGMP (8-pCPT-cGMP) and the anti-PKG Ig antibody were purchased from Calbiochem (San Diego, CA), an \( \beta \)-catenin antibody from Transduction Laboratories (San Diego, CA), a polyclonal antibody to cyclin D from Upstate USA, Inc. (Charlottesville, VA), an anti-\( \beta \)-catin monoclonal antibody from Sigma (St. Louis, MO), an anti-\( \beta \)-catenin antibody from Santa Cruz Biotechnology (Santa Cruz, CA), and an anti-hemagglutinin (HA) antibody from Covance (Richmond, CA). Aprotinin and OSI-461 were provided by OSI Pharmaceuticals, Inc. The construction and characteristics of plasmids that encode HA-COOH-terminal tagged WT, constitutively active (\( \Delta \) and SD), and dominant-negative (KR) forms of PKG I\( \alpha \) and PKG I\( \beta \) are described in our previous publication (33). Similar vector-only plasmids were used as controls. SW480 and 293T cells were cultured in DMEM with 10% fetal bovine serum.

Colony Formation Assay. SW480 cells were transfected with various constructs of PKG I\( \alpha \) and PKG I\( \beta \) as described previously (33). After 18 h, the cells were replated into 100-mm dishes with 1 \( \times \) 105 cells/dish, and the cells were cultured in the presence of 1 mg/ml G418 for 3 weeks. The colonies were fixed with formalin, stained with Giemsa, and counted. All assays were done in triplicate, and the results are expressed as \( \% \) of control, i.e., the number of colonies obtained compared with the vector control plasmid.

Production and Infection with Retrovirus Constructs. The above-described HA-tagged PKG constructs were subcloned into the expression vector pMIG upstream of the IRES-enhanced Green Fluorescent Protein (GFP) sequence. pMIG was provided by Dr. Jeremy Luban (Columbia University). 293T cells were cotransfected with pMIG, PMDG, and pCL-Eco as described previously (49, 50). After 16 h, cells were incubated with fresh DMEM/10% fetal bovine serum medium. The retrovirus containing medium was harvested after another 24 or 48 h. SW480 cells were infected with the indicated retroviruses encoding PKG for 6 h and then incubated in fresh DMEM/10% fetal bovine serum medium for 72 h.

RESULTS

Constitutively Activated Mutants of PKG I\( \alpha \) and PKG I\( \beta \) Inhibit Colony Formation in SW480 Cells. To directly examine the effects of PKG on growth and apoptosis, we constructed a series of expression vectors that encode WT-PKG I\( \alpha \) or the following mutants of PKG I\( \alpha \): PKG I\( \alpha \)S65D, a constitutively activated point mutant; PKG I\( \alpha \)Δ, a constitutively activated N-terminal truncated mutant; and PKG I\( \alpha \)K390R, a dominant-negative point mutant. A similar series of expression vectors of PKG I\( \beta \) were also constructed, i.e., PKG I\( \beta \), PKG I\( \beta \)S80D, PKG I\( \beta \)Δ, and PKG I\( \beta \)K405R (33). We transfected each of these vectors into SW480 cells, the cells were grown in

Determination of Apoptotic Index. SW480 cells were infected with the indicated PKG retrovirus constructs as described above. After 72 h, the cells were harvested and analyzed on a FACSCalibur instrument using CELLQuest software (Becton Dickinson, Mountain View, CA) with the FL-1 channel to detect GFP. This indicated that the infection efficiency in all samples was about 80%. Therefore, we used the entire cell population to determine the sub-G1 population. The extent of apoptosis was determined using the FL-2 channel after propidium iodide (Sigma) staining and expressed as a percentage, by calculating the number of cells that were in the sub-G1 population divided by the total number of cells examined. For the Annexin V staining assay, the infected SW480 cells were incubated for 72 h. The harvested cells were then stained with phycoerythrin-conjugated Annexin V (PharMingen, San Diego, CA) for 15 min in the dark, according to the manufacturer’s protocol. The stained cells were then analyzed on a FACSCalibur instrument using CELLQuest software (Becton Dickinson). The apoptotic index was expressed as a percentage, by calculating the number of cells that were positive for both phycoerythrin-conjugated Annexin V and GFP divided by the total number of GFP-positive cells examined.

Determination of Cell Doubling Time. Cells were plated at a density of 2 \( \times \) 105 in 15.6-mm diameter, 24-well dishes. The numbers of cells per well were counted daily for the next 7 days, using a Coulter counter. The doubling times were measured during the period of exponential growth.

Western Blot Analysis. SW480 cells were harvested and then sonicated in radioimmunoprecipitation assay (RIPA) buffer, and extracts were examined by Western blot analysis as described previously (33). The lysates were electrophoresed on a 10% polyacrylamide gel and then electrophoretically transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% dry milk and incubated with the indicated antibody. After washing, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ). Protein bands were visualized with the enhanced chemiluminescence Western blotting system (Amersham Biosciences). Fold expression was determined with NIH Image1.62 software.

Luciferase Reporter Assays. SW480 cells were plated at 1 \( \times \) 105 cells per 6-well 35-mm-diameter plates, and 24 h later, they were transfected with 500 ng of the cyclin D1-luc (51) reporter plasmid, the indicated PKG expression plasmids, and 10 ng of cytomegalovirus-\( \beta \)-galactosidase reporter plasmid (as internal control), using Lipofectin (Invitrogen, Carlsbad, CA). After 18 h of transfection, some of the cells were incubated in fresh growth medium in the presence or absence of 8-pCPT-cGMP for 24 h before harvesting. Luciferase activities were normalized to \( \beta \)-galactosidase activities, to correct for differences in transfection efficiency. The relative luciferase activity measured in the vector control cells was assigned the value of 100%. All assays were done in triplicate.

Cell Invasion Assays. SW480 cells were infected with the indicated retroviruses of PKG, as described above. After 18 h, the infected cells were replated into the upper well of transwell chambers (Matrigel; BD Biosciences, Bedford, MA). After 24 h incubation with or without 100 \( \mu \)M 8-pCPT-cGMP, the cells on the upper surface of the chamber were removed by scraping with a cotton swab. Cells that had migrated through the filter were stained with 1% crystal violet and counted by microscopy. Similar assays were done with cells that stably overexpressed PKG I\( \alpha \) or I\( \beta \) as described in Fig. 4.

Statistical Methods. Results of the experimental studies are reported as mean \( \pm \) SD. Differences were analyzed by Student’s \( t \) test. A value of \( P < 0.05 \) was regarded as significant.
selection medium containing G418, and afterward, the colonies were stained with Giemsa solution and counted. The results are representative of triplicate experiments. Assays were done in triplicate, and the data are plotted as mean values with SDs (error bars). *, significant inhibition when compared with the control (P < 0.05). For additional details, see "Materials and Methods."

Constitutively Activated Mutants of PKG I Induce Apoptosis in SW480 Cells. In view of the above results, it was of interest to examine whether activation of PKG I is itself sufficient to induce apoptosis in SW480 cells. To assure that our PKG I constructs were expressed with high efficiency, we incorporated the HA-tagged PKGs into the retrovirus vector pMIG. This vector also encodes eGFP so that we could monitor the infection efficiency (see "Materials and Methods"). Indeed, we found that the infection frequency was about 80% (data not shown). The extent of apoptosis was monitored by determining the sub-G1 fraction of cells by flow cytometry at 72 h after infection with the various retrovirus constructs (Fig. 2A). We found that when compared with the vector control-infected cells, the PKG Iα, IαKR, and IβKR vectors did not induce apoptosis. However, the IαΔ, IαSD, Iβ, and IβSD vectors caused about a 1.5-fold increase in apoptosis (P < 0.05, 0.001, 0.001, and 0.001, respectively). The strongest effect was seen with IβΔ vector because it caused about a 2-fold increase in apoptosis (P < 0.001). Similar effects were seen in a repeat experiment (data not shown). The relatively high apoptosis in the vector control cells is probably due to the infection procedure itself, which involved serum starvation. Significantly greater apoptotic effects were seen with the Iβ WT, Δ, and SD constructs than the corresponding Iα constructs. (P < 0.005, 0.01, and 0.001, respectively). To extend the results obtained with the PKG Iβ constructs, a similar experiment was done, but after infection, the extent of apoptosis was assayed by the Annexin V staining procedure (see "Materials and Methods"). In this study, WT-PKG Iβ caused a small increase in apoptosis (P < 0.05), but IβΔ and IβSD constructs produced strong increases in apoptosis (P < 0.001 and 0.01, respectively), and again, the IβKR construct did not induce apoptosis (Fig. 2B). Thus, constitutive activation of PKG I, especially PKG Iβ, is sufficient to induce apoptosis in SW480 cells. These results are consistent with the inhibitory effects of activated mutants of PKG Iα and Iβ on colony formation, described in Fig. 1.

Stable Overexpression of WT-PKG Iβ Inhibits Cell Proliferation in SW480 Cells. Because the above studies suggested that PKG Iβ appeared to have greater growth inhibitory activity than PKG Iα, we examined the effects of PKG Iβ in greater detail by establishing derivatives of SW480 cells that stably overexpress this enzyme. We could not develop derivatives that stably overexpressed the constitutively activated mutants PKG IαΔ or IαSD, presumably because of their cytotoxicity. We were however, successful in developing pools of cells that stably overexpressed WT-PKG Iβ or the KR (dominant-negative) mutant of PKG Iβ, as demonstrated by Western blot analysis (Fig. 3A). The cells that overexpressed the mutant KR were more condensed and more adherent than the vector control cells. Conversely, the cells that overexpressed WT-PKG Iβ were less adherent (data not shown). We then compared the growth rates of these two types of derivatives with that of a pool of cells derived from SW480 cells that had been transfected with only the control vector. Whereas,
Thus, stable overexpression of PKG I increases in the cellular level of p21 CIP1 in the inhibitor cells and the IKR overexpressor cells were 29.4 ± 0.4 and 16.8 ± 0.7 h, respectively. These experiments were repeated at least twice with similar results.

The exponential doubling time for the vector control cells was 21.4 ± 1 h, the corresponding values for the WT-PKG I overexpressor cells and the I#KR overexpressor cells were 29.4 ± 0.4 and 16.8 ± 0.7 h, respectively (P < 0.0001 and < 0.05, respectively). Thus, stable overexpression of PKG I inhibits the proliferation of SW480 cells. Because the parental SW480 cells express detectable levels of endogenous PKG I (data not shown), the fact that the derivatives that stably express a dominant-negative PKG I grow more rapidly suggests that endogenous PKG I can itself exert a partial growth inhibitory effect in these cancer cells.

**Overexpression of PKG I Inhibits Expression of Cyclin D1 and β-Catenin but Increases Expression of p21 CIP1.** Because we found that stable overexpression of PKG I inhibited cell proliferation, we examined possible effects of PKG Iβ on expression of the cell cycle control proteins cyclin D1 and p21 CIP1 on and on the signaling molecule β-catenin whose function is often perturbed in colon cancer cells (45). In these studies, we used two clonal derivatives of SW480 cells that stably expressed WT-PKG Iβ (clones I#3 and I#6), two clonal derivatives that stably overexpressed PKG I#KR (I#KR#3 and I#KR#9), and a vector control clone (v#3). Cell extracts of these clones were obtained from exponentially growing cultures and examined by Western blot analysis. We found that the I#3 and I#6 cells displayed a marked decrease in the cellular level of cyclin D1 and some decrease in β-catenin, when compared with the v#3 cells. The I#KR#9 cells had higher levels of cyclin D1 than the v#3 cells, but no apparent change in the level of β-catenin. There was also a marked increase in the cellular level of p21 CIP1 in the I#3 and I#6 cells when compared with the v#3 cells (Fig. 4). The latter finding is consistent with our finding that treatment of SW480 cell with Aptsyn, OSI-461, or 8-pCPT-cGMP, agents that activate PKG, causes rapid induction of p21 CIP1 (data not shown). Induction of p21 CIP1 expression by OSI-461 has also been described in chronic lymphocytic leukemia cells (52).

Because the SW480 cells that stably overexpress PKG Iβ had decreased levels of the cyclin D1 protein (Fig. 4), we examined whether PKG Iβ affects the transcriptional activity of the promoter region of the cyclin D1 gene by carrying out transient transfection reporter assays using a cyclin D1 promoter-luciferase reporter. Co-transfection of the cells with WT-PKG Iβ partially inhibited luciferase activity, and this was additionally enhanced by treating the transfected cells with a cell-permeable activator of PKG 8-pCPT-cGMP (Fig. 5A), co-transfection with PKG IΔ or PKG IβSD caused marked inhibition, but co-transfection with PKG IβKR had no effect, on luciferase activity (Fig. 5A). To obtain additional evidence that activation of PKG inhibits the transcriptional activity of the cyclin D1 promoter, we examined the effects of treating parental SW480 cells with two compounds, Aptsyn and OSI-461, that inhibit cGMP PDEs 2 and 5, thereby activating endogenous PKG (31). It is apparent that both compounds inhibited luciferase activity in these reporter assays (Fig. 5B).

**Effects of PKG Iβ Expression on Sensitivity to Growth Inhibition by Aptsyn.** It was of interest to determine whether derivatives that stably overexpress WT-PKG Iβ or the dominant-negative mutant PKG Iβ differ in their sensitivity to growth inhibition by Aptsyn, because as described above, the growth inhibitory and apoptotic effects of this compound appear to be mediated, at least in part, through the activation of PKG (31–33). Cell viability assays using SW480 cells carried out with increasing concentrations of Aptsyn indicated that with the v#3 control cells, the IC50 for Aptsyn was 300
Migration through the phosphorylation of VASP, presumably because activation of PKG in human endothelial cells caused inhibition of cell migration in SW480 cells, employing Matrigel-coated transwell chambers. In one set of studies, SW480 cells were infected with the above-described series of retroviruses encoding various forms of PKG and the vector control cells was assigned the value of 100%. Error bars indicate SDs of triplicate assays. Similar results were obtained in three independent experiments.

On the other hand, the PKG Iβ3 clone that stably overexpresses wt-PKG Iβ displayed an IC_{50} of 250 μM (P > 0.05), but this difference was not statistically significant. However, 600 μM Aptosyn, which is twice the IC_{50} for the parental SW480s and v#3 cells, caused a significantly greater inhibition of the Iβ3 cells than that obtained with the v#3 cells (P < 0.01). On the other hand, the PKG IJKR#9 cells were more resistant to Aptosyn than the v#3 cells because the IC_{50} with the former cells was greater than 600 μM (P < 0.001; Fig. 6). Similar results were obtained in repeat studies (data not shown). These results provide additional evidence that PKG plays a role in mediating the growth inhibitory effects of Aptosyn and related compounds, although it is possible that our findings also reflect a more general effect of PKG on cell viability. The possible clinical relevance of these results is mentioned under “Discussion.”

Activation of PKG Inhibits Cell Migration in SW480 Cells. We previously reported that treatment of SW480 cells with Aptosyn and other compounds that lead to activation of PKG or the overexpression of constitutively activated mutants of PKG results in increased phosphorylation of the protein VASP (33). Smolenski et al. (16) found that activation of PKG in human endothelial cells caused inhibition of cell migration through the phosphorylation of VASP, presumably because VASP plays a role in regulating focal adhesion complexes. Therefore, it was of interest to examine the effects of our PKG constructs on cell migration in SW480 cells, employing Matrigel-coated transwell chambers. In one set of studies, SW480 cells were infected with the above-described series of retroviruses encoding various forms of PKG I and then assayed 24 h later for cell migration (Fig. 7A). We found that the WT, Δ, and SD constructs of both PKG Iα and PKG Iβ, but not the KR constructs, caused significant inhibition of cell migration. These effects were strongest with the PKG Iβ constructs, especially the Δ and SD constitutively activated mutants. This inhibition is not simply because of loss of cell viability due to induction of apoptosis, because although some of these vectors can induce apoptosis at 72 h after infection (see Fig. 2), no increase in apoptosis was seen at 24 h (data not shown). Nevertheless, it was important to confirm these effects by also examining the derivatives of SW480 cells that stably overexpress PKG Iα or PKG Iβ and yet remain viable. These studies were done with and without the addition of 8-pCPT-cGMP to the medium to enhance the activation of PKG. Fig. 7B indicates that when the vector control derivative was treated with 8-pCPT-cGMP, there was significant inhibition of cell migration. Even in the absence of treatment with this cGMP compound, the PKG Iα#1 clone and the PKG Iβ3 clone displayed significant decreases in cell migration when compared with the untreated vector control cells (P < 0.001). This inhibition was additionally increased when these cells were treated with the cGMP compound (P < 0.001). As in our previous studies (Fig. 1; Fig. 2), these effects were greater in the PKG Iβ than in the PKG Iα cells. Thus, in addition to causing growth inhibition and activation of apoptosis, activation of PKG can inhibit the migration of colon cancer cells. This effect may be mediated, at least in part, through the phosphorylation of VASP, because VASP phosphorylation can cause disruption of focal adhesions (16) and possibly other changes in the cellular cytoskeleton.

DISCUSSION

The present studies provide the first direct evidence that activation of PKG is sufficient to induce growth inhibition and apoptosis and also inhibit cell migration in human cancer cells. Our findings are consistent with previous evidence that activation of PKG in vascular smooth muscle cells can have similar effects (28, 29) and that overexpression of a constitutively activated PKG mutant also inhibits growth and cell migration in the porcine vascular system (29). It is of interest that in the latter study, vascular surgery decreased the expression of PKG, and this was associated with restenosis. Previous studies indicate that various chemical agents that cause an increase in cellular

Fig. 6. Effects of expression of PKG Iβ on sensitivity to Aptosyn-induced growth inhibition in SW480 cells. Control v#3 cells, PKG Iβ#3 cells that stably overexpress WT-PKGβI (Iβ3), and PKG IJKR#9 cells that stably overexpress a dominant-negative mutant of PKG Iβ (IJKR#9; see Fig. 4) were treated with Aptosyn (Exsulind) at the indicated concentrations. The numbers of cells were counted after 72 h using a Coulter counter and expressed as the percentage of the respective untreated cells. Error bars indicate means of triplicate assays. The inhibitory effects of 600 μM Aptosyn were significantly greater in the PKG Iβ3 cells (*, P < 0.01) and were significantly less in the PKG IJKR#9 cells (***, P < 0.001) than in the v#3 control cells. Similar results were obtained in three independent experiments.
levels of cGMP lead to induction of apoptosis in human colon cancer cells (31–33, 35), but these findings did not provide direct evidence that activation of PKG was sufficient to produce this effect.

The present study indicates that activation of PKG, specifically PKG Iβ, is also sufficient to cause a decrease in cellular levels of cyclin D1 and an increase in cellular levels of the cell cycle inhibitory protein p21\(^{\text{CIP1}}\) in SW480 colon cancer cells (Fig. 4). Transient transfection reporter assays provide evidence that these effects are due to inhibition of the transcriptional activity of the cyclin D1 promoter (Fig. 5). Our results with cyclin D1 differ from those obtained by Hanada et al. (53), who found that overexpression of PKG Iβ in murine mesangial cells that were also treated with 8-bromo-cGMP inhibited the promoter activity of cyclin E but not cyclin D1. This difference may reflect differences between their cell system and ours or the fact that they used a reporter plasmid encoding the −944 to +139 region of the cyclin D1 promoter, whereas we used a full-length cyclin D1 promoter (−1745CD1; Ref. 51). Therefore, it is possible that the region of the cyclin D1 promoter between −1745 and −944 is required for PKG to exert its inhibitory effect. β-Catenin can enhance the transcription of cyclin D1 by binding to the T-cell factor/lymphoid enhancer factor-1 site of the cyclin D1 promoter (45, 46). We found that stable overexpression of WT-PKG Iβ decreased the cellular level of β-catenin in SW480 cells (Fig. 4). In addition, there is evidence that β-catenin is a substrate of PKG Iβ and that this phosphorylation can contribute to the degradation of β-catenin through a GSK3β-independent pathway (47). This mechanism may explain, at least in part, why activation of PKG causes decreased expression of cyclin D1, but possible effects on other transcription factors may also play a role.

It is known that the expression of p21\(^{\text{CIP1}}\) is regulated largely at the level of transcription by both p53-dependent and -independent mechanisms (40). The promoter of the p21\(^{\text{CIP1}}\) gene contains two conserved p53-binding sites, and at least one of these is required for p53 responsiveness after DNA damage (54). In addition, a variety of transcriptional factors that are induced by a number of different signal pathways can activate p21\(^{\text{CIP1}}\) transcription by a p53-independent mechanism, including SP1, SP3, STAT3, C/EBPa, C/EBPβ, and Smad3 (55–57). p21\(^{\text{CIP1}}\) expression is also regulated posttranscriptionally by both ubiquitin-dependent and -independent proteasome-mediated degradation (58, 59). Because the SW480 cells that we used carry a mutant p53, the induction of p21\(^{\text{CIP1}}\) by PKG appears to be through a p53-independent pathway. The precise mechanism by which PKG leads to increased expression of p21\(^{\text{CIP1}}\) remains to be determined. Our results are consistent with the finding of Gu et al. (60) that treatment of cells with nitric oxide, which activates guanylyl cyclase, caused increased expression of p21\(^{\text{CIP1}}\) via a cGMP-dependent pathway. In addition, we found that treatment of SW480 cells with Aptosyn or OSI-461, agents that increase cellular levels of cGMP (33), causes a rapid increase in the expression of both p21\(^{\text{CIP1}}\) mRNA and protein.\(^4\)

The decreased expression of β-catenin and cyclin D1 and increased expression of p21\(^{\text{CIP1}}\) may contribute to the growth inhibitory and apoptotic effects of PKG activation in SW480 cells. However, the role of p21\(^{\text{CIP1}}\) in growth inhibition and induction of apoptosis can vary in different cell systems (61). Thus, p21\(^{\text{CIP1}}\) null mice display an increase in tumor incidence (62), and overexpression of p21\(^{\text{CIP1}}\) enhances growth inhibition and apoptosis in glioma (63) and ovarian carcinoma (64) cell lines. On the other hand, up-regulation of p21\(^{\text{CIP1}}\) inhibits transforming growth factor β-induced apoptosis in retinal endothelial cells (65). In addition, other effects of PKG activation appear to play an important role in the induction of apoptosis by Aptosyn and related compounds, including activation of the MEKK1-SEK1-c-Jun NH2-terminal kinase 1 pathway (36). Furthermore, Shureiqi et al. (66, 67) have recently obtained evidence that increased expression of 15-lipoxygenase-1 can play an important role in the induction of apoptosis in human colon cancer cell lines by Aptosyn, and we have recently obtained evidence that PKG activation may also play a role in the induction of 15-lipoxygenase-1.\(^5\) Thus, the growth inhibition and apoptosis caused by activation of PKG is probably mediated by a complex series of events.

It is of interest that SW480 cells that stably express the dominant negative (KR) mutant of PKG Iβ were more resistant to the growth inhibitory effects of Aptosyn than vector control cells, whereas cells that overexpressed WT-PKG Iβ were more sensitive to this drug (Fig. 6). These results suggest that the sensitivity of different cancer cells to the growth inhibitory effects of this class of compounds may depend, at least in part, on their cellular levels of PKG, a finding that may be relevant to the clinical use of these compounds in cancer chemoprevention and treatment. We should, however, emphasize that the present results do not exclude the possibility that the growth inhibitory and apoptotic effects of these compounds are also mediated via pathways that do not involve the action of PKG.

Our finding that activation of PKG in SW480 cells inhibited their migration (Fig. 7) may also be of clinical relevance because it suggests that Aptosyn and related compounds may exert antitumor effects not only by inhibiting cell proliferation but also by inhibiting invasion and metastasis. We previously reported that activation of PKG in

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\(^4\) Unpublished observations.

\(^5\) Unpublished observations.
SW480 cells results in rapid and persistent phosphorylation of the focal adhesion-associated protein VASP (33). Because Smolenski et al. (16) have found that in endothelial cells phosphorylation of VASP by PKG inhibits the attachment of focal adhesions and thus inhibits cell migration, we suspect that a similar mechanism explains the inhibitory effect of PKG activation on cell migration in SW480 cells. In summary, we believe that the present studies provide evidence that PKG can play a direct role in inducing growth inhibition and apoptosis and inhibiting cell migration in cancer cells. These findings provide a rationale for targeting PKG and related pathways as a novel approach to cancer chemoprevention and therapy. Additional studies are required to determine the precise mechanisms by which PKG activation can inhibit growth and induce apoptosis in colon cancer cells.

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