Protein Kinase G Type Iα Activity in Human Ovarian Cancer Cells Significantly Contributes to Enhanced Src Activation and DNA Synthesis/Cell Proliferation

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Abstract

Previously, we showed that basal activity of nitric oxide (NO)/cyclic GMP (cGMP)/protein kinase G (PKG) signaling pathway protects against spontaneous apoptosis and confers resistance to cisplatin-induced apoptosis in human ovarian cancer cells. The present study determines whether basal PKG kinase activity regulates Src family kinase (SFK) activity and proliferation in these cells. PKG-Iα was identified as predominant isoform in both OV2008 (cisplatin-sensitive, wild-type p53) and A2780cp (cisplatin-resistant, mutated p53) ovarian cancer cells. In both cell lines, ODQ (inhibitor of endogenous NO-induced cGMP biosynthesis), DT-2 (highly specific inhibitor of PKG-Iα kinase activity), and PKG-Iα knockdown (using small interfering RNA) caused concentration-dependent inhibition of DNA synthesis (assessed by bromodeoxyuridine incorporation), indicating an important role of basal cGMP/PKG-Iα kinase activity in promoting cell proliferation. DNA synthesis in OV2008 cells was dependent on SFK activity, determined using highly selective SFK inhibitor, 4-((4′-phenoxyanilino)-6,7-dimethoxyquinazoline (SKI-1). Studies using DT-2 and PKG-Iα small interfering RNA revealed that SFK activity was dependent on PKG-Iα kinase activity. Furthermore, SFK activity contributed to endogenous tyrosine phosphorylation of PKG-Iα in OV2008 and A2780cp cells. In vitro coinoculation of recombinant human c-Src and PKG-Iα resulted in c-Src-mediated tyrosine phosphorylation of PKG-Iα and enhanced c-Src autophosphorylation/activation, suggesting that human c-Src directly tyrosine phosphorylates PKG-Iα and the c-Src/PKG-Iα interaction enhances Src kinase activity. Epidermal growth factor–induced stimulation of SFK activity in OV2008 cells increased PKG-Iα kinase activity (indicated by Ser239 phosphorylation of the PKG-Iα catalytic domain) and inhibited DNA synthesis, suggesting an important role of Src/PKG-Iα interaction in promoting cell proliferation through the DNA synthesis/cell proliferation pathway.

Introduction

Cyclic GMP (cGMP)–dependent protein kinase [protein kinase G (PKG)], a widely expressed serine/threonine kinase in mammalian cells, is recognized as the key downstream protein kinase mediating many of the biological effects of nitric oxide (NO) and natriuretic peptides in the cardiovascular system (1–4). Early studies using isolated arteries had shown that endogenous kinase activity of PKG in vascular smooth muscle cells was significantly stimulated by basal endogenously generated NO, synthesized by nearby endothelial cells, as well as by exogenous NO generated by certain cardiovascular therapeutic agents, such as sodium nitroprusside (5). Furthermore, PKG activity in vascular smooth muscle cells was found to be stimulated by atrial natriuretic peptide (also called atriopeptin), a heart-derived hormone important for regulating blood pressure and kidney function (6).

More recent studies from our laboratory have shown the cGMP/PKG signaling pathway plays another important biological role as an antiapoptotic mechanism that promotes cell survival in many types of mammalian cells, including hippocampal neurons (7), PC12 pheochromocytoma cells (8), neuroblastoma and neuroblastoma-glioma hybrid cells (9–11), immortalized uterine epithelial cells (12), and...
ovarian cancer cells (13, 14). We identified PKG-Iα as the predominant PKG isoform expressed in the neuroblastoma and neuroblastoma-glioma hybrid cells, suggesting that PKG-Iα represents the key isoform of PKG mediating the cGMP-stimulated antiapoptotic effects in these cells (10-12).

In human ovarian cancer cells, we have found that the basal activity of the cGMP/PKG pathway is essential for preventing spontaneous apoptosis (13) and that endogenously produced NO, generated by two NO synthases (NOS), endothelial NOS (also called NOS-3) and neuronal NOS (also called NOS-1), contribute significantly to the cisplatin resistance phenotype in chemoresistant ovarian cancer cells (14). Interestingly, the chemoresistant ovarian cancer cells could be dramatically sensitized to cisplatin by inhibiting endogenous NO synthesis. These data suggest that the NO/cGMP/PKG signaling pathway plays a key role in protecting human ovarian cancer cells against both spontaneous and cisplatin-induced apoptosis. However, it was not clear whether the NO/cGMP/PKG pathway also regulates other biological functions, such as cell proliferation, in human ovarian cancer cells.

In vascular smooth muscle cells, our laboratory has shown that basal kinase activity of PKG (likely involving selectively the PKG-Iα isoform) plays a critically important role in promoting the DNA synthesis and cell proliferation that occurs under typical culturing conditions (i.e., cells stimulated by the mitogenic effects of fetal serum growth factors; ref. 15). This growth-promoting effect of PKG is in stark contrast to the previously proposed antiproliferative effect of PKG, which was based on earlier studies that had shown a growth-inhibitory role of PKG in various types of cells, including vascular smooth muscle cells (16), cardiac fibroblasts (17), T-cell lymphocytes (18), and colon cancer cells (19). However, in all of these cells, the growth-inhibitory responses were initiated by either overexpression of PKG (especially the PKG-Iβ isoform) or the elevation of PKG activity (not by basal PKG activity) and seemed, in all cases, to involve the PKG-Iβ isoform. Thus, there is growing evidence that the different isoforms of PKG may be regulating cell proliferation in very different ways, with PKG-Iα kinase activity (especially at basal levels) promoting cell proliferation, whereas PKG-Iβ kinase activity (when hyperactivated or when PKG-Iβ is overexpressed) inhibiting cell proliferation (15). The present study determines if and how the basal kinase activity of PKG (specifically PKG-Iα) regulates DNA synthesis/cell proliferation in human ovarian cancer cells.

Src (or c-Src) and other members of the Src family kinases (SFK) of nonreceptor tyrosine kinases are thought to play an important role in growth-factor–induced DNA synthesis and cell proliferation in both normal and transformed mammalian cells, including the mitogenic responses stimulated by epidermal growth factor (EGF; refs. 20-22). Src is overexpressed and/or aberrantly activated in a variety of cancer cells, including human ovarian cancer cells (22). Src overexpression is especially noticeable in late-stage human ovarian cancer, suggesting that Src kinase activity may contribute to ovarian cancer progression (23). In lung cancer cells, Src, via its tyrosine kinase activity, is known to catalyze the tyrosine phosphorylation of the EGF receptor (EGFR), thereby enhancing EGFR downstream mitotic signaling, including mitogen-activated protein kinase signaling, resulting in enhanced cell proliferation (21, 24).

In the present study, we have determined if basal PKG kinase activity regulates DNA synthesis in human ovarian cancer cells and if an interaction between PKG and Src plays a role in this response. To assess endogenous PKG activity in the ovarian cancer cells, phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at Ser239 was used. VASP, an actin-binding protein involved in focal adhesion, is a PKG downstream substrate that is directly phosphorylated at Ser239 by PKG in mammalian cells, providing a useful indicator of endogenous PKG kinase activity (25-27). The present study shows that OV2008 (chemosensitive, with wild-type p53) and A2780cp (chemoresistant, with mutated p53) human ovarian cancer cells express predominantly the PKG-Iα isoform and that PKG-Iα kinase activity contributes significantly to promoting DNA synthesis in both cell lines. Our findings also suggest that PKG-Iα interacts with Src (or related SFK) in a way that enhances both SFK activity (assessed by autophosphorylation) and PKG-Iα kinase activity (assessed by VASP phosphorylation at Ser239) and that both contribute to enhanced DNA synthesis in ovarian cancer cells. We also found that EGF-induced stimulation of SFK activity and DNA synthesis is dependent on PKG-Iα kinase activity in these cells. Thus, the data suggest that PKG-Iα plays an important role in promoting SFK activity and DNA synthesis in human ovarian cancer cells.

Materials and Methods

Culture of Ovarian Cancer Cells

Two established human epithelial ovarian cancer cell lines, OV2008 and A2780cp, were used in this study (provided by the laboratory of Dr. Benjamin Tsang). OV2008 cells are cisplatin sensitive and A2780cp cells are cisplatin resistant (13, 14, 28). Both cell lines were derived from ovarian cystoadenocarcinoma patients without prior chemotherapy; the OV2008 cells have wild-type TP53, whereas the A2780cp cells harbor a TP53 mutation, as determined by direct sequencing (28). The cell lines were cultured at 37°C in an atmosphere of 5% CO2/95% air. OV2008 cells were maintained in RPMI 1640, whereas A2780cp cells were maintained in DMEM. All media were supplemented with fetal bovine serum (10%), streptomycin (50 μg/mL), penicillin (50 units/mL), and nonessential amino acids (1%; Life Technologies). Cells were plated for 16 to 18 h before experimental treatments at a density of 5 × 10³ cells/cm² in six-well plates and kept at <85% confluent at the time of treatment with human recombinant EGF (Invitrogen) and/or the pharmacologic inhibitors.

Pharmacologic Inhibitors and Small Interfering RNA Gene Knockdown

ODQ (1H-[1,2,4]oxadiazolo[4,3-α]quinazolin-1-one) and Src kinase inhibitor 1 (SKI-1; 4-(4′-phenoxyanilino)-6,7-dimethoxyquinazoline) were purchased from...
Calbiochem. DT-2 was purchased from Biollog. SU6656 was purchased from Sigma.

For small interfering RNA (siRNA)–mediated silencing of gene expression, cells were transfected with 50 and 100 nmoL of Stealth siRNA (siRNA, 5′-GAGGAAGACUUUGCCAGAUUCUA-3′) for specifically targeting the expression of PKG-Iα (Invitrogen). Transfection of the human ovarian cancer cells was conducted using RNAiMAX (Invitrogen). Nonsilencing siRNA (Invitrogen) was used as the negative control. At 72 h after transfection, the culture medium was changed and fresh medium was supplied. The cells were used in experiments 16 h later.

**Determination of Cell Proliferation Rates**

DNA synthesis/cell proliferation rates were determined by measuring the rate of bromodeoxyuridine incorporation into DNA over a 3-h incubation using an ELISA kit (Roche). Most experiments were conducted using 10% fetal bovine serum to stimulate basal cell proliferation. For the EGF stimulation experiments, cells were first starved in serum-free medium for 24 h. The extraction and assay procedures were as recommended by the manufacturer.

**Determination of Cellular SFK Activity**

Relative levels of SFK phosphorylation at the autophosphorylation/activation site (equivalent to Tyr416 phosphorylation in v-Src) were determined by ELISA (Roche) and Western blot analysis. In both types of analysis, the antibody that was used recognizes the phosphorylated form of Tyr416 of activated v-Src as well as the equivalent autophosphorylation/activation sites in activated c-Src and other activated SFKs. The ELISA technique used a cell-based ELISA that directly measures protein phosphorylation in cultured cells. The kits included anti-Src and anti–phospho-Tyr416-Src antibodies for colorimetric quantification, and the amount of phospho-Src protein was normalized against the amount of total Src protein. Western blot analysis of Src Tyr416 phosphorylation is described below. In some experiments, human EGF (Invitrogen) at 10 and 50 ng/mL was added to the cells for 30 min to stimulate autophosphorylation of Src/SFKs.

**Protein Extraction and Western Blotting Using IR Imaging**

Cells were lysed in 85°C hot 1× SDS lysis buffer [50 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10 mmol/L DTT, and 10% glycerol]. The supernatant fractions were collected by centrifugation at 15,000 × g; 10 min. The total amount of protein in the lysates was calculated from the fluorescence-based protein quantitation kit EZQ (Molecular Probes). The amount of total protein loaded on the gels for PKG-Iα/PKG-β determination was 20 μg for mouse aorta and MSTD-211H mesothelioma cells and 80 μg for ovarian cancer cells. For the determination of other proteins, 50 μg of total protein were used. Proteins were separated on 4% to 12% polyacrylamide NuPage gels (Invitrogen) and then transferred to nitrocellulose membranes. Membranes were blocked (room temperature, 1 h) with blocking buffer (Rockland Immunochecmicals) and then incubated at 4°C overnight with primary anti-bodies [PKG-Iα/β (1:1,000), phospho-Tyr416-Src (1:1,000), total Src (1:2,000), anti-tyrosine phosphorylation antibody (PY20; 1:1,000), phospho-Tyr416-EGFR (1:1,000), total EGFR (1:1,000), phospho-Se239-VASP (1:500), total VASP (1:1,000); all from Cell Signaling Technology], and glyceraldehyde-3-phosphate dehydrogenase (1:2,500; Santa Cruz Biotechnology) and subsequently with secondary antibodies labeled with IR dyes (1:25,000 in blocking buffer; room temperature for 1 h; LI-COR Biosciences). The membranes were scanned on the Odyssey IR imaging system (LI-COR Biosciences).

**Immunoprecipitation of PKG in Ovarian Cancer Cells**

Cells were lysed in 0.5 mL ice-cold radioimmunoprecipitation assay buffer [with freshly added protease and phosphatase inhibitors, phenylmethylsulfonyl fluoride (1 mmol/L), aprotonin (10 g/L), and Na3VO4 (1 mmol/L)] for 10 min on ice followed by centrifugation at 10,000 × g for 10 min. Supernatant fractions were transferred to new tubes. Primary anti–PKG-Iα/β antibody (1:100; Cell Signaling Technology) was added to the cell lysates with gentle shaking overnight at 4°C. Protein G–Sepharose (40 μL; Invitrogen) was then added and suspensions were incubated overnight at 4°C. Immunoprecipitates were collected by centrifugation at −1,000 × g for 5 min, and pellets were washed thrice with 0.5 mL radioimmunoprecipitation assay buffer. Pellets were resuspended in 1× SDS buffer and analyzed by Western blot using anti-tyrosine phosphorylation antibody (PY20) and anti–PKG-Iα/β antibody (Cell Signaling Technology).

**In vitro Phosphorylation Reaction of Recombinant Human PKG-Iα and Recombinant Human c-Src**

Interactions between recombinant human PKG-Iα and recombinant human c-Src (both from Calbiochem) were determined using in vitro experiments. The recombinant PKG-Iα (75 ng) was incubated with the recombinant c-Src (10 ng) in a kinase reaction buffer containing Tris buffer (50 mmol/L), MgCl2 (0.1 mol/L), bovine serum albumin (10 mg/mL), and ATP (1 mmol/L) at 30°C for 10 min. The recombinant PKG-Iα alone group and the recombinant c-Src alone group were done in the same way. To show dephosphorylation of PKG-Iα, protein tyrosine phosphatases (Millipore) were also included as a group. The samples were analyzed by Western blot analysis using the anti-tyrosine phosphorylation antibody (PY20) antibody (Cell Signaling Technology). A similar in vitro procedure was used for determining if the autophosphorylation/activation of recombinant human c-Src is affected by coinubcation with recombinant human PKG-Iα. The samples were analyzed by Western blot using anti–phospho–Tyr416-Src antibody and anti–total Src antibody (Cell Signaling Technology).

**Statistical Analysis**

Results are expressed as the mean ± SE of at least four independent experiments. Statistical analysis was done by one- or two-way ANOVA using GraphPad (Prism Software). Bartlett’s tests were used to establish the homogeneity of variance on the basis of the differences among SDs. Differences between experimental groups were determined by the
Bonferroni multiple comparison test. A value of \( P < 0.05 \) was considered to be significant.

**Results**

**Inhibition of cGMP Biosynthesis Using ODQ Decreases Both DNA Synthesis and Endogenous Kinase Activity of PKG (Phosphorylation of VASP at Ser\(^{239}\)) in Both Chemosensitive (OV2008) and Chemoresistant (A2780cp) Human Ovarian Cancer Cells**

Previous studies from our laboratory have shown that ODQ, an agent that selectively inhibits endogenous NO-induced activation of soluble guanylyl cyclase (sGC), significantly reduces the basal cGMP levels in human ovarian cancer cells to approximately two thirds, one third, and one forth of control levels when ODQ is used at 10, 50, and 100 \( \mu \)mol/L, respectively, and this results in concentration-dependent increases in both p53 protein levels and apoptosis (13). Coadministration of a cell-permeable analogue of cGMP (8-bromo-cGMP, a direct activator of PKG) prevented the ODQ-induced p53 elevation and apoptosis, suggesting that ODQ-induced elevation of p53 and apoptosis in ovarian cancer cells was a consequence of the decrease in basal PKG kinase activity. These findings highlighted an important biological role of basal PKG kinase activity in preventing apoptosis in ovarian cancer cells.

In Fig. 1 of the present study, we have determined if ODQ-induced inhibition of cGMP biosynthesis affects the endogenous PKG kinase activity (assessed by VASP phosphorylation at Ser\(^{239}\)) and the rate of DNA synthesis/cell proliferation in two ovarian cancer cell lines, OV2008 and A2780cp cells. OV2008 cells are sensitive to cisplatin-induced apoptosis and possess wild-type p53, whereas A2780cp cells are resistant to cisplatin-induced apoptosis and possess mutated p53 (13, 14, 28).

In Fig. 1A, we show that ODQ caused concentration-dependent reduction in the rate of DNA synthesis in OV2008 cells, with significant decreases noted at 50 and 100 \( \mu \)mol/L ODQ. Western blot analysis showed that VASP phosphorylation at Ser\(^{239}\) was also decreased in response to ODQ at 50 and 100 \( \mu \)mol/L, suggesting that there was substantial downregulation of the basal PKG kinase activity caused by treatment with ODQ. This inhibition of basal PKG kinase activity by ODQ corresponded, in a concentration-dependent manner, to the decreases in DNA synthesis. Figure 1B shows that inhibition of cGMP biosynthesis with ODQ resulted in similar responses in the p53-mutated, chemoresistant A2780cp cells, suggesting that the ability of ODQ to inhibit both PKG kinase activity and DNA synthesis in the ovarian cancer cells is independent of p53 mutation.

**OV2008 and A2780cp Human Ovarian Cancer Cells Express Predominantly the PKG-I\(\alpha\) Isoform and the Inhibition of the Endogenous PKG-I\(\alpha\) Kinase Activity Decreases DNA Synthesis**

Figure 2A shows the results of Western blot analysis determining the expression of the PKG-I\(\alpha\) isoforms in OV2008 and A2780cp cells. For positive controls, mouse aorta (expressing predominately PKG-I\(\alpha\)) and the MSTO-211H mesothelioma cell line (expressing both PKG-I\(\alpha\) and PKG-I\(\beta\)) were included. Recombinant human PKG-I\(\alpha\) and PKG-I\(\beta\) were also used as positive controls. The primary antibody recognized the COOH-terminal region of PKG-I, which is common for both of the PKG-I splice variants PKG-I\(\alpha\) and PKG-I\(\beta\). A single band, corresponding to PKG-I\(\alpha\), was observed for both ovarian cancer cell lines, whereas two bands, corresponding to PKG-I\(\alpha\) and PKG-I\(\beta\), were observed for MSTO-211H cells. To our knowledge,
this represents the first report of PKG expression in these cancer cell lines.

Figure 2B shows the effects of inhibiting PKG-Iα kinase activity (using DT-2) on the DNA synthesis in OV2008 cells. DT-2 is a peptide inhibitor of PKG-Iα kinase activity that is cell permeable in mammalian cells (because of the inclusion of a membrane translocation sequence), has exceptionally high specificity (1,300-fold specificity for PKG-Iα/β over a closely related protein kinase, protein kinase A), and has high potency (IC50 value of 12.5 nmol/L for inhibiting the kinase activity of purified PKG-Iα under in vitro conditions; refs. 29-31). However, when used in intact mammalian cells, DT-2 has a considerably higher IC50 value of 3.7 μmol/L for inhibiting endogenous (intracellular) PKG kinase activity; with the PKG kinase-inhibitory effects beginning at a threshold concentration of 2 μmol/L and reaching maximal inhibitory effects at 10 μmol/L DT-2 when used in vascular smooth muscle cells (31). In the present study, Fig. 2B shows that DT-2 caused a concentration-dependent inhibition of DNA synthesis and VASP Ser239 phosphorylation in OV2008 cells (with inhibitory effects occurring at concentrations of DT-2 similar to those found to be effective in vascular smooth muscle cells). The data suggest a key role of basal PKG-Iα kinase activity in promoting DNA synthesis in the chemosensitive ovarian cancer cell line.

Figure 3 shows that DT-2 also inhibits the DNA synthesis and endogenous VASP Ser239 phosphorylation in the chemoresistant, p53-mutated A2780cp cells. The data suggest that the effects of basal PKG-Iα kinase activity on promoting DNA synthesis in human ovarian cancer cells may be independent of p53 mutational status and whether the cells are resistant to cisplatin.

Both Basal and EGF-Induced Stimulation of SFK Activity Are Dependent on PKG-Iα Kinase Activity in OV2008 Cells

Figure 4A shows that inhibition of endogenous basal PKG-Iα kinase activity using DT-2 significantly lowers the SFK activity in OV2008 cells, assessed by the autophosphorylation...
of the activation site (the SFK equivalent of Tyr416 in v-Src) using both Western blot analysis and ELISA (graph). The data suggest that basal PKG-Iα kinase activity, under normal growing conditions of ovarian cancer cells, promotes SFK activity. Figure 4B further shows that the DT-2–induced inhibition of SFK activity corresponds to the inhibition of basal PKG-Iα kinase activity (assessed by phosphorylation of VASP at Ser239) and that EGF-induced stimulation of SFK activity is also dramatically inhibited by DT-2. Furthermore, EGF-induced stimulation of DNA synthesis in the OV2008 cells was completely inhibited by DT-2. The data suggest that the stimulatory effects of EGF on SFK activity and cell proliferation in ovarian cancer cells are dependent on PKG-Iα kinase activity.

**Gene Knockdown of PKG-Iα Expression Using siRNA Decreases Both SFK Activity and DNA Synthesis in Both OV2008 and A2780cp Human Ovarian Cancer Cells**

Figure 5 shows the effects of silencing the expression of PKG-Iα by transfecting the cells with PKG-Iα–specific siRNA in OV2008 cells (Fig. 4A) and A2780cp cells (Fig. 4B). Western blot analysis shows that PKG-Iα expression (at the protein level) was partially and almost completely knocked down when using PKG-Iα siRNA constructs at 50 and 100 nmol/L, respectively. The knockdown of PKG-Iα expression corresponded to decreases in both SFK activity (autophosphorylation of SFKs at the equivalent of Tyr416) and DNA synthesis rate in both ovarian cancer cell lines. Thus, these data, using a gene knockdown approach, confirm the data using pharmacologic inhibitors (ODQ and DT-2) shown above, indicating that basal PKG-Iα plays an important role in promoting SFK activity and DNA synthesis in human ovarian cancer cells.

**SKI-1 Inhibits Basal and EGF-Stimulated SFK Activity, PKG-Iα Kinase Activity (VASP Ser239 Phosphorylation), and DNA Synthesis in OV2008 Human Ovarian Cancer Cells**

EGF, via activation of EGFR, is thought to play an important role in stimulating ovarian cancer cell proliferation (32, 33). The activation of EGFR by EGF is known to stimulate the SFK signaling pathway, leading to enhanced cell proliferation in various types of cancer cells (21, 22). To assess the effects of EGF on SFK activity in the ovarian cancer cells of the present study, we determined the time course and concentration-response relationship of EGF-induced stimulation of SFK activity (autophosphorylation of SFKs at the equivalent of Tyr416) in OV2008 cells. Figure 6 shows the results of these experiments, confirming that EGF causes both time-dependent (Fig. 6A) and concentration-dependent (Fig. 6B) stimulation of SFK activity in these cells. Figure 6B also shows that EGF stimulates Ser239 phosphorylation of VASP, which indicates that EGF not only stimulates SFK activity but also...
enhances the endogenous kinase activity of PKG-\(\alpha\) in the ovarian cancer cells.

Figure 6C shows the effects of SU6656, a selective SFK inhibitor, on the EGF-induced stimulation of SFK and PKG-\(\alpha\) activities in OV2008 cells. SU6656, an ATP-competitive inhibitor of SFKs, has been shown to inhibit SFK activity in murine embryonic stem cells when used at 4 and 8 \(\mu\)mol/L (34). In the present study, preincubation with SU6656 at 1 and 5 \(\mu\)mol/L suppressed the stimulatory effects of EGF on SFK activity (autophosphorylation at site equivalent to Tyr\(^{416}\) and Tyr\(^{845}\) phosphorylation of EGFR (site phosphorylated by activated Src) in the OV2008 cells. Interestingly, SU6656 also prevented the EGF-induced stimulation of VASP phosphorylation at Ser\(^{239}\), indicating that the stimulation of PKG-\(\alpha\) activity by EGF in ovarian cancer cells requires the presence of SFK activity.

Figure 7 shows the effects of SKI-1, a recently developed highly selective SFK inhibitor, on basal and EGF-stimulated SFK activities and DNA synthesis in OV2008 human ovarian cancer cells. SKI-1 derives its superior selectivity by being able to block both the ATP-binding site and the peptide/protein substrate–binding site of Src and several closely related SFKs (35). The specificity of SKI-1 as an inhibitor of Src kinase activity in mammalian cells has been validated in experiments using specific Src siRNA gene knockdown (21). Conventional Src/SFK inhibitors, such as dasatinib and PP1, typically block only the ATP-binding site of Src/SFKs and consequently have nonspecific inhibitory effects, resulting in the inhibition of various other protein kinases, such as EGFR, platelet-derived growth factor receptor, Kit, and Abl (35, 36). The IC\(_{50}\) value of SKI-1 was reported to be 44 nmol/L under \textit{in vitro} condition using purified Src (35). However, when using SKI-1 to inhibit endogenous Src kinase activity in intact mammalian cells, considerably higher concentrations are needed. For example, the successful inhibition of endogenous (intracellular) Src kinase activity required using SKI-1 in a concentration

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**FIGURE 4.** Inhibition of endogenous basal PKG-\(\alpha\) activity using DT-2 in OV2008 ovarian cancer cells reduces the basal level of autophosphorylation of endogenous SFKs and completely inhibits EGF-induced stimulation of SFK autophosphorylation and DNA synthesis. A, the PKG-\(\alpha\) kinase inhibitor DT-2 dramatically lowered the basal SFK autophosphorylation level, assessed by both Western blot analysis determining autophosphorylation (the equivalent of Tyr\(^{416}\); top image) and ELISA measuring Tyr\(^{416}\) autophosphorylation versus total Src (graph). B, EGF (10 ng/mL) elevated VASP Ser\(^{239}\) phosphorylation and Src Tyr\(^{416}\) phosphorylation in OV2008 cells, assessed by Western blot analysis, and elevated DNA synthesis in OV2008 cells, assessed by bromodeoxyuridine incorporation. Pretreatment with DT-2 (2.5 \(\mu\)mol/L) for 2 h completely inhibited the EGF-stimulated increases in VASP phosphorylation, Src/SFK autophosphorylation, and DNA synthesis. The Western blots shown in A and B are representative of four experiments. Columns, mean of four experiments; bars, SEM. **, \(P < 0.01\); ***, \(P < 0.001\), compared with the control; ###, \(P < 0.001\), compared with EGF alone.
range of 0.5 to 2.5 μmol/L in lung cancer cells (21) and 5 to 20 μmol/L in murine embryonic stem cells (34).

Figure 7 shows that both basal and EGF-stimulated SFK activity (A and B) and DNA synthesis (C) in OV2008 cells are inhibited by SKI-1 at 0.5 and 1 μmol/L. In addition, Fig. 7A shows that SKI-1 inhibits the phosphorylation of EGFR at Tyr845 (site phosphorylated by Src), confirming that SKI-1, at these concentrations, inhibits endogenous Src kinase activity in the ovarian cancer cells. Like SU6656 (shown in Fig. 6C), SKI-1 also inhibited the EGF-stimulated phosphorylation of VASP at Ser239, further indicating that the increased PKG-Iα kinase activity stimulated by EGF (shown in Figs. 4 and 6) is dependent on SFK activity. Interestingly, in the case of the SKI-1–inhibitory experiments, even basal activity of PKG-Iα (i.e. basal phosphorylation of VASP at Ser 239) in the OV2008 ovarian cancer cells was highly dependent on SFK activity (Fig. 7A). Thus, Src or a closely related SFK seems to interact with PKG-Iα in both an upstream and downstream role, with SFK activity enhancing the serine/threonine kinase activity of PKG-Iα (Fig. 7) and PKG-Iα kinase activity enhancing the tyrosine kinase activity of Src/SFKs (Figs. 4 and 5).

In vitro and In vivo Cross talk of PKG-Iα and Src

A previous study, using in vitro phosphorylation experiments, had shown that the viral form of Src (v-Src) is capable of phosphorylating PKG-Iα on a tyrosine residue, which dramatically enhanced both basal activity and cGMP activatability of PKG-Iα (37). Because of the similarities between v-Src and the cellular form of Src (c-Src) found in mammalian cells, we had hypothesized that c-Src may interact with PKG-Iα in human ovarian cancer cells, resulting in c-Src–catalyzed tyrosine phosphorylation of PKG-Iα. Predictably, this would result in enhanced basal PKG kinase activity as well as enhanced cGMP-stimulated PKG kinase activity (i.e., intracellular cGMP at basal levels would become more effective as an endogenous activator of PKG-Iα).

To test whether human c-Src directly phosphorylates human PKG-Iα on a tyrosine residue in vitro, we used recombinant human PKG-Iα and recombinant human c-Src, which were incubated in a kinase reaction buffer containing the necessary ATP and Mg2+. Tyrosine phosphorylation of the proteins was determined by Western blot analysis using the anti-phosphotyrosine antibody PY20, which recognizes general tyrosine phosphorylation.

FIGURE 5. Gene knockdown using PKG-Iα–specific siRNA lowers basal Src Tyr416 autophosphorylation and DNA synthesis in both OV2008 cells (A) and A2780cp cells (B). The Western blots shown are representative of four experiments. Columns, mean from four experiments; bars, SEM. *** , P < 0.001, compared with the negative control.
Figure 8A shows the results of a representative in vitro experiment. Lane 4 contains a band at 60 kDa, representing the autophosphorylated form of human c-Src. When both human c-Src and PKG-Iα were present together (lane 1), a second band appeared at a molecular weight of ∼76 kDa (corresponding to PKG-Iα), indicating that recombinant human PKG-Iα was phosphorylated on a tyrosine residue by the recombinant human c-Src. Addition of protein tyrosine phosphatases to the reaction mixture reduced the tyrosine phosphorylation of PKG-Iα (lane 2). The data indicate that human c-Src, like v-Src, is capable of directly phosphorylating PKG-Iα on a tyrosine residue.

Figure 8B shows the results of an experiment using intact ovarian cancer cells, both OV2008 and A2780cp cells, determining whether endogenous SFK activity is capable of phosphorylating PKG-Iα on a tyrosine residue. The cells were exposed to SKI-1 to inhibit the endogenous SFK activity, and the levels of tyrosine phosphorylation of immunoprecipitated PKG-Iα were determined by Western blot analysis. The data show that SKI-1 substantially reduced the level of tyrosine phosphorylation of PKG-Iα in both ovarian cancer cell lines, suggesting that endogenous SFK activity contributes to the tyrosine phosphorylation of PKG-Iα in intact ovarian cancer cells.

The data shown in Figs. 2 to 5 above had suggested that Src (or a closely related SFK) may be serving not only as an upstream stimulator of PKG-Iα but also as a downstream target of PKG-Iα, which was indicated by the decrease in SFK activity caused by the pharmacologic inhibition of PKG-Iα activity and by the siRNA gene knockdown of PKG-Iα expression. To test the possibility that PKG-Iα may directly stimulate c-Src kinase activity, we conducted additional in vitro experiments using recombinant human c-Src and PKG-Iα. Figure 8C shows the results of these experiments. In this case, the Western blots were probed using an antibody that specifically recognized the tyrosine-phosphorylated form of the autophosphorylation/activation site of Src (and other SFKs; equivalent to Tyr416 site of c-Src).

**FIGURE 6.** EGF stimulates both Src/SFK autophosphorylation and PKG-Iα kinase activity (VASP Ser239 phosphorylation) in OV2008 ovarian cancer cells and these responses are inhibited by SU6656, a SFK-selective tyrosine kinase inhibitor. A, Western blot showing that EGF (10 and 50 ng/mL) increases Src/SFK autophosphorylation (equivalent of Tyr416) in OV2008 cells in a time-dependent manner, with the phosphorylation reaching a peak at 15 to 30 min. B, Western blot showing that EGF (10 and 50 ng/mL) increases both Src/SFK activity (autophosphorylation) and PKG-Iα kinase activity (assessed by downstream phosphorylation of the PKG-Iα substrate, VASP at Ser239) in OV2008 cells in a concentration-dependent manner. C, SU6656 (1 and 5 μmol/L) inhibited the EGF-induced stimulation of the EGFR phosphorylation at Tyr1068 (site phosphorylated by Src), the Ser239 phosphorylation of VASP (PKG-Iα substrate), and the autophosphorylation of Src/SFK. The Western blots shown are representative of four experiments.
v-Src). Figure 8C shows that the copresence of PKG-Iα does indeed enhance the autophosphorylation of c-Src, confirming that PKG-Iα interacts with c-Src in a way that elevates Src kinase activity. At present, it is unclear how PKG-Iα is able to enhance the Src kinase activity. However, the experiments shown in Fig. 4, which used DT-2 to inhibit the endogenous kinase activity of PKG-Iα in ovarian cancer cells, suggest that the stimulatory effect of PKG-Iα on Src/SFK activity may involve the serine/threonine kinase activity of PKG-Iα and potentially the phosphorylation of a serine or threonine residue in Src/SFK, which could potentially enhance Src/SFK autophosphorylation and activation. Further experiments, beyond the scope of the present study, will be needed to confirm this idea.

Figure 8D shows a model, based on the data of the present study, illustrating the proposed role of the NO/cGMP/PKG-Iα signaling pathway and its interaction with Src in promoting DNA synthesis and cell proliferation in ovarian cancer cells. Previous studies from our lab have shown that both endothelial NOS and neuronal NOS are expressed in
ovarian cancer cells and, via their ability to generate endogenous NO, play an important role in contributing to chemoresistance (14). Furthermore, our previous studies have shown that endogenous NO, via its ability to bind and activate the heme moiety of sGC, enhances the generation of cGMP in ovarian cancer cells, which in turn protects these cells against the development of spontaneous apoptosis (13). The present study, using both pharmacologic inhibitors (ODQ, DT-2, and SKI-1) and gene knockdown technique (siRNA), shows that PKG-Iα kinase activity and the interactions between PKG-Iα and Src play a key role in promoting DNA synthesis in ovarian cancer cells.

Discussion

The present study showed that basal PKG-Iα kinase activity plays an important role in enhancing the DNA synthesis rate in two human ovarian cancer cell lines: OV2008 cells (wild-type p53 and cisplatin sensitive) and A2780cp cells (mutated p53 and cisplatin resistant). Thus, the ability of PKG-Iα to promote cell proliferation in human ovarian cancer cells seems to be independent of the p53 mutation status and whether the cancer cells are sensitive to cisplatin. Inhibition of either the basal PKG-Iα serine/threonine kinase activity or the basal Src/SFK

![Figure 8](image-url)
tyrosine kinase activity decreases DNA synthesis in these cells. Both in vitro and in vivo experiments suggest that PKG-Iα and Src interact in a way that results in the enhancement of both PKG-Iα serine/threonine kinase activity and Src tyrosine kinase activity, both of which seem to be important for the enhanced DNA synthesis in human ovarian cancer cells.

Several previous studies have suggested that PKG can participate in either the inhibition of cell proliferation or the stimulation of cell proliferation, apparently depending on the types of cells studied and/or the conditions of the experimental procedures. For example, stimulation of endogenous PKG kinase activity in vascular smooth muscle cells (16), cardiac fibroblasts (17), T-cell lymphocytes (18), and human colon cancer cells (19) all resulted in a decrease in cell proliferation rate. In contrast, stimulation of endogenous PKG kinase activity in human umbilical vein endothelial cells (HUVEC) resulted in enhanced rate of cell proliferation (38). In the HUVECs, vascular endothelial growth factor, a proangiogenic factor important in tumor angiogenesis, was shown to stimulate the proliferation of these cells via a cellular mechanism involving vascular endothelial growth factor–induced/Akt-mediated phosphorylation (and stimulation) of endothelial NOS, enhanced production of endogenous NO, elevation of cGMP levels, and stimulation of PKG kinase activity (38).

The reason for the opposite effects of PKG on cell proliferation in the different cell types/experimental conditions is currently unclear. However, it is important to note that the previous reports showing PKG-mediated inhibition of cell proliferation in vascular smooth muscle cells, cardiac fibroblasts, T-cell lymphocytes, and human colon cancer cells were all linked to activation of the PKG-Iβ isoform of PKG (16–19), suggesting that the antiproliferative effects of PKG activation may be mediated selectively by the PKG-Iβ isoform. Although the PKG isoform mediating the growth-promoting effects in HUVECs was not identified in the previous report (38), recent studies in our laboratory have shown that PKG-Iα is the predominant isoform expressed in HUVECs and that PKG-Iα participates in promoting HUVEC cell proliferation. Likewise, we have recently shown that the basal kinase activity of endogenous PKG-Iα in vascular smooth muscle cells is critically important for promoting the cell proliferation of these cells when grown in culture (15). Thus, the different effects of PKG on cell proliferation may depend on which isoform of PKG-I is expressed and activated (i.e., PKG-Iα [at basal activity] mediating increased cell proliferation, whereas PKG-Iβ [when highly activated or overexpressed] mediating decreased cell proliferation). The data of the present study are consistent with this concept, showing that human ovarian cancer cells, which express predominantly the PKG-Iα isoform, exhibit enhanced cell proliferation promoted by the basal kinase activity of PKG-Iα. To our knowledge, this is the first report of the involvement of endogenous PKG Iα in the regulation of cell proliferation in human ovarian cancer cells.

Previous data from our laboratory had shown that basal activity of the NO/sGC/cGMP/PKG signaling pathway in chemosensitive human ovarian cancer cells results in a continuous suppression of p53 accumulation and protection against spontaneous onset of apoptosis (13). Because p53, in addition to regulating apoptosis, is also involved in regulating cell cycle progression (39), the present study determined if basal activity of the PKG pathway may also provide a continuous control over the proliferation rate of human ovarian cancer cells in a way that may be dependent on the mutational status of p53. Using two human ovarian cancer cell lines with different p53 status, we show in the present study that blocking the endogenous NO-induced activation of sGC (using ODQ) or the inhibition of endogenous PKG-Iα kinase activity (using DT-2–selective or PKG-Iα–selective siRNA) significantly reduces the rate of DNA synthesis in both OV2008 cells (with wild-type p53) and A2780cp cells (with mutated p53). Although the level of inhibition of DNA synthesis by DT-2 seemed to be somewhat smaller in the A2780 cells compared with the OV2008 cells (Figs. 2 and 3), the data obtained with PKG-Iα siRNA gene knockdown (Fig. 5) show that PKG-Iα contributes similarly to promoting cell proliferation in both cell lines. Thus, the data suggest that PKG-Iα kinase activity in human ovarian cancer cells plays an important role in promoting cell proliferation and that the mechanism seems to be independent of the p53 mutational status.

The present study also shows that both OV2008 and A2780cp ovarian cancer cells express PKG, predominately the PKG-Iα isoform. This observation is in contrast to a previous report showing that, although PKG was highly expressed in normal surface epithelial cells of the ovary, the expression of PKG seemed to be “absent” in various ovarian cancer cells (40). The difference may be related to the use of different ovarian cancer cell lines in the two studies or to differences in sensitivity for detecting PKG expression. Nevertheless, the present study shows that at least two human ovarian cancer cell lines do indeed express PKG-Iα and that the expression levels are sufficient to play an important role in promoting SFK activity and cell proliferation.

Src is overexpressed and/or aberrantly activated in late-stage human ovarian cancer cells (23). Via its tyrosine kinase activity, which phosphorylates EGFR and other growth-promoting proteins on tyrosine residues, and via its ability to inhibit the actions of the tumor suppressor p53, Src is known to promote cell proliferation in a variety of cells (20, 22). The present study shows that there is another potential site of action of Src within mammalian cells (i.e., Src-mediated tyrosine phosphorylation of PKG-Iα), which may result in enhanced serine/threonine kinase activity of PKG-Iα (indicated in the present study by the enhanced Ser239 phosphorylation of VASP). Previously, the
viral homologue of c-Src, v-Src, was shown to be capable of phosphorylating PKG-Iα on a tyrosine residue during in vitro coinubcation (37). The tyrosine phosphorylation of PKG-Iα leads to elevated basal activity as well as substantial enhancement in the ability of cGMP to stimulate PKG-Iα kinase activity, shifting the $K_{\text{cat}}$ for cGMP from 109 nmol/L (in PKG-Iα without tyrosine phosphorylation) to 39 nmol/L (in PKG-Iα with tyrosine phosphorylation). However, to our knowledge, the phosphorylating effect of a mammalian form of Src (mammalian c-Src) on PKG-Iα has not been reported previously. We predicted, based on the similarity between v-Src and c-Src, that endogenous c-Src in human ovarian cancer cells may phosphorylate endogenous PKG-Iα, greatly enhancing its ability to be stimulated by the basal cGMP levels within these cells. Because ovarian cancer cells often overexpress Src or posses a higher level of Src kinase activity, it is further hypothesized that PKG-Iα would be activated to a relatively high level (i.e., hyperactivated) in these cancer cells and this may contribute to enhanced proliferation rate.

The present study, using both in vitro and in vivo (intact cell) experiments, suggest that the human form of c-Src is indeed capable of tyrosine phosphorylating PKG-Iα (illustrated in Fig. 8). Using the highly selective Src/SFK tyrosine kinase inhibitor SKI-1, we showed that both OV2008 and A2780cp ovarian cancer cells have tyrosine phosphorylation of the endogenous PKG-Iα under normal culturing conditions that is dependent on endogenous Src/SFK tyrosine kinase activity (Fig. 8B). Thus, the data of the present study suggest that the overexpressed/activated Src within ovarian cancer cells may be continually phosphorylating PKG-Iα on a tyrosine residue (likely the same tyrosine residue phosphorylated by v-Src used in the previous in vitro study; ref. 37), resulting in high-level activation of PKG-Iα in ovarian cancer cells. The data presented in Figs. 1 to 3 of the present study further suggest that the endogenous PKG-Iα kinase activity (indicated by VASP Ser239 phosphorylation) is at a high basal level in both OV2008 and A2780cp cells, which is consistent with the idea that PKG-Iα kinase activity is continually enhanced by the high-level Src tyrosine kinase activity within these cells.

The mitogenic effect of EGF observed in the present study is relatively small, likely because basal SFK activity and EGFR kinase activity are already partially activated in the absence of exogenous EGF. This is suggested by the data in Figs. 6 and 7, which show that inhibition of SFK activity with SU6656 or SKI-1 causes substantial lowering of both SFK autophosphorylation and EGFR phosphorylation at Tyr845. Elevations of basal EGFR/Src kinase activity are frequently reported in cancer cells due to the overexpression of ligand and/or mutation(s) of EGFR (41). We do not rule out the possibilities that the ovarian cancer cells of the present study may harbor such kinds of abnormalities.

To our knowledge, the present study represents the first report showing direct cross talk between PKG and c-Src/SFKs in intact mammalian cells resulting in the regulation of cell proliferation. Two previous reports, showing that c-Src is involved in the antiapoptotic effects of NO in RINm5F insulin-producing cells, have proposed that Src and PKG may interact to mediate the NO-induced antiapoptotic effects; however, a direct interaction between PKG and c-Src was not established (42, 43). The data of the present study suggest that Src and possibly other SFKs serve both upstream and downstream of PKG-Iα in human ovarian cancer cells and that these interactions promote DNA synthesis. Src is a close associator with EGFR, and they mutually activate each other at Tyr845 of EGFR and Tyr416 of Src. PKG-Iα may exert its mitogenic effect in ovarian cancer cells via the enhanced activation of the EGFR/Src signaling pathway, which includes downstream effectors such as Akt, signal transducer and activator of transcription 3/5, and mitogen-activated protein kinase (24), as well as other promitogenic mechanism mediated by activated Src.

In conclusion, the present study provides the first report showing the direct cross talk between PKG-Iα and Src in human ovarian cancer cells. Both basal PKG-Iα serine/threonine kinase activity and Src/SFK tyrosine kinase activity were found to be important for promoting DNA synthesis in human ovarian cancer cells. Furthermore, EGF stimulation of SFK activity and the resulting enhanced DNA synthesis seemed to be dependent on PKG-Iα kinase activity. Overall, the present data suggest the presence of a novel signaling cross talk between Src and the NO/cGMP/PKG-Iα signaling pathway that is important for promoting DNA synthesis and cell proliferation in human ovarian cancer cells. Therapeutic agents that target the NO/cGMP/PKG-Iα signaling pathway or the novel Src/PKG-Iα cross talk may represent a new approach for effectively controlling the proliferation of human ovarian cancer cells in the treatment of ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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PKG-Iα Promotes Src Activation in Ovarian Cancer

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Protein Kinase G Type Iα Activity in Human Ovarian Cancer Cells Significantly Contributes to Enhanced Src Activation and DNA Synthesis/Cell Proliferation

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