Type II, but not type I, cGMP-dependent protein kinase reverses bFGF-induced proliferation and migration of U251 human glioma cells

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Abstract. Previous data have shown that the type II cGMP-dependent protein kinase (PKG II) inhibits the EGF-induced MAPK signaling pathway. In order to thoroughly investigate PKG, it is necessary to elucidate the function of another type of PKG, PKG I. The aim of this study was to investigate the possible inhibitory effect of PKG II and PKG I activity on the basic fibroblast growth factor (bFGF)-induced proliferation and migration of U251 human glioma cells and the possible underlying mechanisms. U251 cells were infected with adenoviral constructs encoding cDNA of PKG I (Ad-PKG I) or PKG II (Ad-PKG II) to increase the expression levels of PKG I or PKG II and then treated with 8-Br-cGMP and 8-pCPT-cGMP, respectively, to activate the enzyme. An MTT assay was used to detect the proliferation of the U251 cells. The migration of the U251 cells was analyzed using a Transwell migration assay. Western blot analysis was used to detect the phosphorylation/activation of the fibroblast growth factor receptor (FGFR), MEK and ERK and the nuclear distribution of p-ERK. The results showed that bFGF treatment increased the proliferation and migration of U251 cells, accompanied by increased phosphorylation of FGFR, MEK and ERK. Furthermore, the nuclear distribution of p-ERK increased following bFGF treatment. Increasing the activity of PKG II through infection with Ad-PKG II and stimulation with 8-pCPT-cGMP significantly attenuated the aforementioned effects of the bFGF treatment, while increased PKG I activity did not inhibit the effects of bFGF treatment. These data suggest that increased PKG II activity attenuates bFGF-induced proliferation and migration by inhibiting the MAPK/ERK signaling pathway, whereas PKG I does not.

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Introduction

Basic fibroblast growth factor (bFGF) is a multifunctional growth factor involved in tumor development, including cell differentiation, cell growth, migration, angiogenesis and tumor formation (1-4). Its biological effects have been reported to be exerted mainly through interaction with its high-affinity receptor, fibroblast growth factor receptor 1 (FGFR1) (5-8). Narong and Leelawat (9) reported that bFGF enhances the migration of cholangiocarcinoma cells by the phosphorylation of MEK1/2. Results from previous studies have shown that bFGF signaling plays a key role in the development of cancer, including gastric, lung and endometrial cancer (10-12).

The cGMP-dependent protein kinases (PKGs) are serine/threonine kinases and include two types of PKGs, PKG I and PKG II (13,14). PKG I is widely distributed within the body and its expression levels are lower in various tumor tissues. PKG II is more tissue-restricted and is characterized by reduced expression levels in many types of tumor cells (15). PKG I leads to decreased tumor growth and invasiveness in many types of cells, including cardiomyocytes, mesangial cells and neutrophils (16-19). PKG I has been identified to be a tumor suppressor (20). Previous studies suggest that PKG II has a role in the regulation of cell proliferation and apoptosis (21-24). Swartling *et al* (25) reported that PKG II inhibits the proliferation of human neuroglioma cells and that the inhibition was related to reductions in transcription factor Sox9 expression levels and Akt phosphorylation. We have prevously observed that the expression and activity of PKG II in human gastric cancer cells were significantly lower compared with those in normal cells (26). Additionally, another study conducted in our laboratory demonstrated an inhibitory effect of PKG II on the proliferation of gastric cancer cells (27).

Previous studies have demonstrated the inhibitory effect of PKG on cell proliferation and the stimulatory effect of bFGF on cell proliferation and migration. However, whether PKG is able to attenuate the bFGF-induced effects on U251

cells remains to be elucidated. The aim of this study was to determine the relationship between PKG and bFGF, and to investigate how PKG exerts its inhibitory effects.

Materials and methods

Cell line. The human glioma cell line U251 was provided by the Institute of Cell Biology (Shanghai, China).

Reagents. Antibodies against MEK and p-MEK (Ser217/221) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against ERK, p-ERK1/2 and actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against p-ERK (Thr202/Tyr204), p-FGFR (Y154), FGFR and β-actin were from Bioworld Technology Co., Ltd. (St. Louis Park, MN, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). The cellular permeable cGMP analog 8-pCPT-cGMP and 8-Br-cGMP were from Calbiochem (San Diego, CA, USA). Electrochemiluminescence (ECL) reagent was from Millipore (Billerica, MA, USA). Dulbecco's modified Eagle's medium (DMEM) and newborn calf serum (NBCS) were from Gibco (Grand Island, NY, USA).

MTT assay. U251 cells (0.5-1x10^3) were plated on 96-well plates in 150 μl medium. The cells were infected with Ad-Lacz, Ad-PKG I or Ad-PKG II for 24 h to establish Ad-Lacz+bFGF, Ad-PKG I+bFGF and Ad-PKG II+bFGF groups. In the Ad-PKG I+bFGF and Ad-PKG II+bFGF groups, 250 μM 8-Br-cGMP and 250 μM 8-pCPT-cGMP were added to activate PKG I and PKG II, respectively. Then, the cells were incubated with bFGF (100 ng/ml) for 12 h. The cultured cells were washed with phosphate-buffered saline (PBS), treated with 20 μl MTT (0.5 mg/ml) and then incubated at 37˚C for 1 h. The medium was removed and 100 μl dimethylsulfoxide (DMSO) was added to each well. The absorbance was determined at 570 nm using a microplate reader. All the experiments were performed in triplicate.

Cell migration assay. The migration of the U251 human glioma cells was investigated using a chamber with 8-μm pore filters (Transwell, 24-well cell culture; Coster, Boston, MA, USA). U251 cells were infected with Ad-Lacz, Ad-PKG I or Ad-PKG II for 48 h to establish Ad-Lacz+bFGF, Ad-PKG I+bFGF and Ad-PKG II+bFGF groups. The cells were serum starved overnight and, in the Ad-PKG I+bFGF and Ad-PKG II+bFGF groups, 250 μM 8-Br-cGMP and 250 μM 8-pCPT-cGMP were added to activate PKG I and PKG II, respectively. The cells were then incubated with bFGF (100 ng/ml) for 12 h at 37˚C. Following incubation, the filters were fixed and stained with hematoxylin and the cells were counted in five random high-power fields under a light microscope.

Nuclear protein preparation. According to the method described by Chen *et al* (28), cells growing on 100-mm plates were harvested in HEM buffer (10 mM HEPES pH 7.5, 2 mM EDTA, 1 mM $MgCl_2$) and homogenized with an ultrasonic homogenizer. The homogenate was centrifuged at 500 x g at 4˚C for 5 min to obtain the nuclei of the cells. Pre-heated SDS-PAGE loading buffer was added to the pellet and boiled for 5 min to obtain the nuclear proteins.

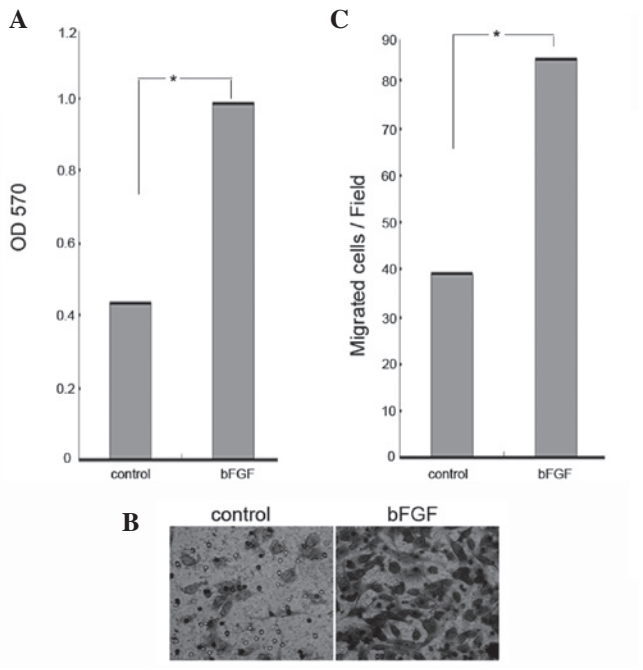

Figure 1. bFGF enhances the proliferation and migration of U251 human glioma cells. (A) An MTT assay was used to detect the proliferation of U251 cells. Compared with the control, cell growth was markedly increased following treatment with 100 ng/ml bFGF for 48 h. A Transwell migration assay was used to measure the migration of U251 cells. (B and C) Representative figures of cell migration in control and bFGF-treated U251 cells. Compared with the control, cell migration was markedly increased following treatment with 100 ng/ml bFGF for 12 h. The means of five independent experiments ± standard error are shown. *P<0.05. bFGF, basic fibroblast growth factor.

Western blot analysis. Sample proteins were separated on SDS-PAGE gels and blotted onto polyvinyl difluoride (PVDF) membranes. The PVDF membranes were blocked with 3% (w/v) bovine serum albumin (BSA) in TBS-T for 1 h at room temperature. Incubation with the primary antibody was conducted at 4˚C overnight, and incubation with the secondary antibody was conducted at room temperature for 1 h, with three washes following each incubation. ECL reagents were used to show the positive bands on the membrane. The bands were detected using Typhoon 9400 (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis. Values are expressed as the means ± SE (n=5; *P<0.05). The Student's t-test was used for comparisons of two sample means. A P-value of <0.05 (P<0.05) was considered to indicate a statistically significant difference.

Results

bFGF promotes the proliferation and migration of U251 human glioma cells. bFGF has been observed to stimulate cancer cell proliferation (29). In the present study, an MTT assay was used to determine whether bFGF had any effect on the proliferation of U251 human glioma cells. The U251 cells were treated with bFGF at a concentration of 100 ng/ml for

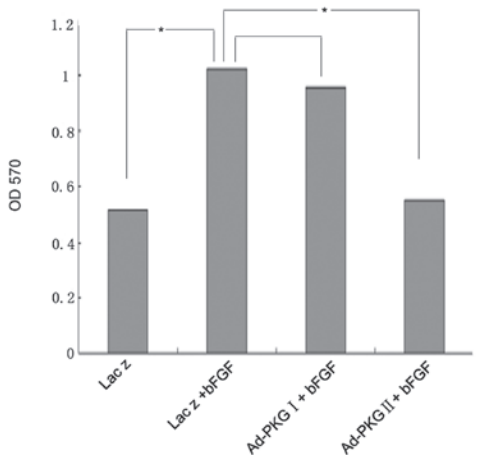

Figure 2. PKG II, but not PKG I, reverses bFGF-induced proliferation of U251 human glioma cells. An MTT assay was used to detect the proliferation of U251 cells. U251 cells were infected with Ad-Lacz, Ad-PKG I or Ad-PKG II for 24 h to establish Ad-Lacz+bFGF, Ad-PKG I+bFGF and Ad-PKG II+bFGF groups. The cells were serum starved overnight and, in the Ad-PKG I+bFGF and Ad-PKG II+bFGF groups, 250 μM 8-Br-cGMP and 250 μM 8-pCPT-cGMP were added to activate PKG I and PKG II, respectively. The cells were then incubated with bFGF (100 ng/ml) for 48 h. The means of five independent experiments ± standard error are shown. *P<0.05. PKG, cGMP-dependent protein kinase; bFGF, basic fibroblast growth factor.

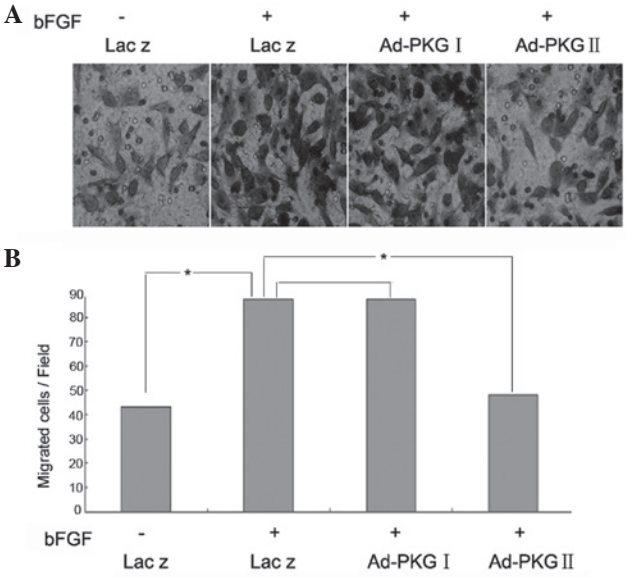

Figure 3. PKG II, but not PKG I, prevents bFGF-induced migration of U251 human glioma cells. (A and B) A Transwell migration assay was used to investigate the migration of U251 cells. U251 cells were infected with Ad-Lacz, Ad-PKG I or Ad-PKG II for 48 h to establish Ad-Lacz+bFGF, Ad-PKG I+bFGF and Ad-PKG II+bFGF groups. The cells were serum starved overnight and, in the Ad-PKG I+bFGF and Ad-PKG II+bFGF groups, 250 μM 8-Br-cGMP and 250 μM 8-pCPT-cGMP were added to activate PKG I and PKG II, respectively. Then, the cells were incubated with bFGF (100 ng/ml) for 12 h. The means of five independent experiments ± standard error are shown. *P<0.05. PKG, cGMP-dependent protein kinase; bFGF, basic fibroblast growth factor.

48 h. The results showed that there was a significant increase in the proliferation of cells treated with bFGF (Fig. 1A). Recent findings have shown that bFGF stimulates cancer cell migration (30). In order to determine the effects of bFGF on the migration of U251 cells, the cells were treated with bFGF

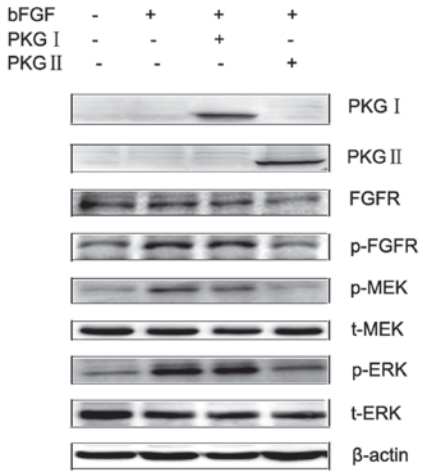

Figure 4. PKG II, but not PKG I, attenuated the bFGF-induced activation of the MAPK/ERK pathway in U251 human glioma cells. U251 cells were infected with Ad-Lacz, Ad-PKG I or Ad-PKG II for 48 h to establish Ad-Lacz+bFGF, Ad-PKG I+bFGF and Ad-PKG II+bFGF groups. The cells were serum starved overnight and, in the Ad-PKG I+bFGF and Ad-PKG II+bFGF groups, 250 μM 8-Br-cGMP and 250 μM 8-pCPT-cGMP were added to activate PKG I and PKG II, respectively. Then, the cells were incubated with bFGF (100 ng/ml) for 15 min. Whole cells were harvested and lysed as described in Materials and methods and cell lysates were subjected to western blot analysis. Results showed that infection with Ad-PKG I and Ad-PKG II caused a marked increase of PKG I and PKG II expression levels, respectively. bFGF treatment induced a significant increase of FGFR, MEK and ERK phosphorylation. Infection with Ad-PKG II and stimulation with 8-pCPT-cGMP, but not Ad-PKG I+8-Br-cGMP treatment, efficiently inhibited the bFGF-induced phosphorylation of FGFR, MEK and ERK. The means of five independent experiments ± standard error are shown. PKG, cGMP-dependent protein kinase; bFGF, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor.

at a concentration of 100 ng/ml for 12 h and then examined using a cell migration assay. Compared with the control, the percentage of U251 cell migration was significantly increased when the cells were treated with 100 ng/ml of bFGF (P<0.0051) (Fig. 1B). This demonstrates that bFGF increases both the proliferation and migration of U251 human glioma cells.

PKG II, but not PKG I, prevents the bFGF-induced proliferation of U251 human glioma cells. In the present study, we demonstrated that bFGF stimulates the proliferation of U251 human glioma cells. Since our previous study demonstrated that PKG II inhibits the proliferation of gastric cancer cells, the aim of the current study was to investigate whether PKG II and PKG I are able to attenuate the bFGF-induced proliferation of U251 cells. Compared with U251 cells treated with bFGF at a concentration of 100 ng/ml alone, cells infected with Ad-PKG II and stimulated with 8-pCPT-cGMP prior to treatment with bFGF, showed a reduction in proliferation, while there was no obvious change when the cells were infected with Ad-PKG I and stimulated with 8-Br-cGMP (Fig. 2). This indicates that PKG II, but not PKG I, inhibits the bFGF-induced proliferation of U251 cells.

PKG II, but not PKG I, prevents the bFGF-induced migration of U251 human glioma cells. In the present study, it was demonstrated that bFGF enhances the migration of U251 human glioma cells. There has been no data demonstrating

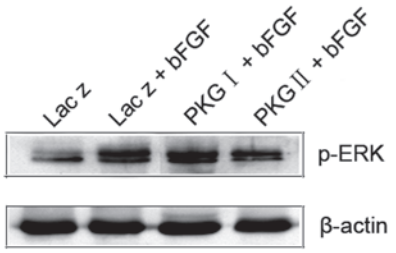

Figure 5. PKG II, but not PKG I, reverses the bFGF-triggered nuclear distribution of p-ERK in U251 human glioma cells. U251 cells were infected with Ad-Lacz, Ad-PKG I or Ad-PKG II for 48 h to establish Ad-Lacz+bFGF, Ad-PKG I+bFGF and Ad-PKG II+bFGF groups. The cells were serum starved overnight and, in the Ad-PKG I+bFGF and Ad-PKG II+bFGF groups, 250 μM 8-Br-cGMP and 250 μM 8-pCPT-cGMP were added to activate PKG I and PKG II, respectively. The cells were then incubated with bFGF (100 ng/ml) for 30 min. Nuclear cell lysate was prepared as described in Materials and methods and subjected to western blot analysis. The results indicated that bFGF treatment induced a significant increase in the expression of p-ERK in the nucleus. Infection with Ad-PKG II and stimulation with 8-pCPT-cGMP, but not Ad-PKG I+8-Br-cGMP treatment, efficiently inhibited the bFGF-induced nuclear distribution of p-ERK. The means of five independent experiments ± standard error are shown. PKG, cGMP-dependent protein kinase; bFGF, basic fibroblast growth factor.

the effect of PKG on the migration of cancer cells to date. In the present study, we investigated whether PKG was able to prevent the bFGF-induced migration of U251 cells. Compared with U251 cells treated with bFGF at a concentration of 100 ng/ml alone, cells infected with Ad-PKG II and stimulated with 8-pCPT-cGMP prior to treatment with bFGF, showed a decreased migratory activity, while there was no clear change of the cells infected with Ad-PKG I and stimulated with 8-Br-cGMP (Fig. 3). This indicates that PKG II, but not PKG I, inhibits the bFGF-induced migration of U251 cells.

PKG II, but not PKG I, prevents the bFGF-induced activation of the MAPK/ERK signaling pathway in U251 human glioma cells. FGF receptors activate several intracellular signaling pathways, including the MAP kinase pathway (31-33). Western blot analysis was used to detect FGFR phosphorylation. MEK1 and MEK2 are members of the dual specificity protein kinase family, which act as MAPK or ERK kinases. Phosphorylation at both Thr202/Tyr204 residues of ERK1 and Thr185/Tyr187 residues of ERK2 is required for full enzymatic activation. Western blot analysis was used to detect MEK and ERK phosphorylation. The results indicated that treatment with bFGF alone at a concentration of 100 ng/ml, increased the phosphorylation levels of FGFR, MEK and ERK. The increased phosphorylation was inhibited by pre-infecting the cells with Ad-PKG II and stimulating the enzyme with 8-pCPT-cGMP, while no significant inhibitory effect was achieved by pre-infecting the cells with Ad-PKG I and stimulating the enzyme with 8-Br-cGMP. These results demonstrate that increased PKG II activity prevents the bFGF-induced phosphorylation of FGFR, MEK and ERK in U251 human glioma cells but increased PKG I activity does not (Fig. 4). Furthermore, we investigated the effect of PKG on the bFGF-induced nuclear translocation of p-ERK. The results showed that bFGF stimulated the nuclear distribution of p-ERK, and that the stimulatory effect was inhibited by pre-infecting the cells with Ad-PKG II and stimulating the enzyme with 8-pCPT-cGMP, while pre-infecting the cells with Ad-PKG I and stimulating the enzyme with 8-Br-cGMP had no inhibitory effect (Fig. 5). The results indicate that increased PKG II activity attenuated the bFGF-triggered p-ERK nuclear distribution whereas increased PKG I activity did not.

Discussion

The growth of solid tumors depends on the occurrence of neovascularization. bFGF is an important angiogenic factor, widely distributed in neoplastic tissues (34). Numerous angiogenic peptides have been identified and their effects on tumor vascularity have also been identified (35-38). FGF receptors activate several intracellular signaling pathways, including MAP kinase pathways. MAP kinase pathways have been identified as the ERK/MAP kinase pathway, the JNK/SAPK pathway and the p38 pathway (39,40). These three pathways may be activated by different growth factors and mediate several cellular events, including cell differentiation, stress responses and growth. However, the activation of each type of MAP kinase mainly depends on the type of the stimulus and the cells.

PKG plays important regulatory roles in diverse processes in many cell types (15,41,42). Its expression is differently regulated in tumors and in normal tissue (14,43,44). In mammalian cells, two different genes encode type I and II PKGs (45). PKG I includes two isoforms, PKG Iα and PKG Iβ, which differ in the first ~100 amino acids (46). PKG I has been recognized as a tumor suppressor. PKG II is membrane-anchored and is present at low levels in several types of human cancer cells (47). Previous data have indicated that PKG II is related to cell proliferation and apoptosis (21,22). We have also found that PKG II attenuates the EGF-induced proliferation and apoptosis of gastric cancer cells (48,49). There has been no data showing the relationship between PKG and migration. In the present study, the exact stimulative effects of bFGF on the proliferation and migration of U251 human glioma cells was confirmed. Consequently, we performed further experiments to investigate whether PKG I or PKG II exerted inhibitory effects on the bFGF-induced proliferation and migration of human glioma cells, and the possible underlying mechanism.

In the present study, the PKG I-selective cGMP analog 8-Br-cGMP and the PKG II-selective cGMP analog 8-pCPT-cGMP were applied to increase PKG I or PKG II activity when cells were infected with Ad-PKG I or Ad-PKG II, respectively. After confirming the effects of bFGF on the proliferation and migration of U251 human glioma cells, we analyzed the effects of PKG I and PKG II on bFGF-stimulated cell proliferation and migration. Compared with treatment with bFGF alone, increased PKG II activity clearly attenuated bFGF-induced proliferation and migration, while increased PKG I activity had no effect. Then, we investigated the inhibitory effects of PKG I and PKG II on the bFGF-induced phosphorylation of FGFR, MEK and ERK. It was found that increased PKG II, but not PKG I, activity was able to attenuate bFGF-induced phosphorylation. Furthermore, the inhibitory effects of PKG I and PKG II on the bFGF-induced nuclear distribution of p-ERK were detected. The results obtained showed that increased PKG II, but not PKG I, activity was able to attenuate bFGF-induced p-ERK nuclear distribution.

In this study it was shown that increased PKG II, but not PKG I, activity inhibits bFGF-stimulated cell proliferation and migration, bFGF-induced FGFR, MEK and ERK phosphorylation and bFGF-induced p-ERK nuclear distribution in U251 human glioma cells. In conclusion, the inhibitory effects of PKG II on bFGF-induced cell proliferation and migration were mainly exerted by blocking the MAPK/ERK signaling pathway.

Acknowledgements

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