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Specific Isoforms of Protein Kinase G Downregulate the Transcription of Cyclin D1 in NIH3T3

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To elucidate the role of PKG isoforms in transcriptional control of cyclin D1, we employed a series of expression vectors of PKG 1α and PKG 1β which encode HA-tagged wild type and constitutively active (SD and ΔN) mutants. Our present study demonstrates that both the constitutively active mutants of PKG 1β downregulate the transcription of cyclin D1 when transiently transfected in NIH3T3 cells, whereas PKG 1α mutants show weak inhibition. We further studied the transcriptional regulators of cyclin D1, such as, c-fos, NF-κB, and CRE by using the luciferase reporter assay. Constitutively active mutants of PKG 1β showed marked transcriptional downregulation of c-fos in NIH3T3 cells, whereas PKG 1α downregulated c-fos to a lesser extent. We also found that the constitutively active mutants of PKG negatively regulated the activation of NF-κB and CRE, suggesting their involvement in the regulation of cyclin D1.

Key Words : PKG, Cyclin D1, c-fos, NF-κB, CRE

Introduction

Cyclic guanosine monophosphate (cGMP), an important cytosolic second messenger, is synthesized by gunylate cyclase, which converts GTP to cGMP and degraded by specific phosphodiesterases (PDEs), especially 2 and 5.[1] The role of cGMP in relaxation of blood vessel, inhibition of cell proliferation, platelet aggregation, GI motility, neuronal function, apoptosis and bone formation have been established.[2-5] cGMP mediates these action through activation of protein kinase G (PKG), the cGMP-gated channels, and specific phosphodiesterases.

PKG, a cGMP dependent serine/threonine specific protein kinase, phosphorylates a number of biologically important targets and is implicated in the regulation of smooth muscle relaxation, platelet activation, sperm metabolism, cell division, and nucleic acid synthesis.[3,6-8] Two PKG genes, coding for PKG type I (PKG I) and type II (PKG II), have been identified in mammalian cells.[9-11] In addition, there are two isoforms of PKG I, designated as PKG Iα and PKG Iβ which are encoded by two alternatively spliced exons of N-terminus PKG I. PKG I and PKG II are homodimers of two identical subunits, a regulatory domain and a catalytic domain. PKG I, with an acetylated N-terminus, is usually associated with the cytoplasm, whereas PKG II, with a myristylated N-terminus, is generally associated with the plasma membrane.[12] PKG I has been detected at high concentrations in all type of smooth muscle cells (SMCs), including vascular SMCs and in platelets. Lower levels are present in vascular endothelium and cardiomyocytes. This is also expressed in fibroblasts, certain types of renal cells, the cerebellum and leukocytes.[10,13] Both isoforms of PKG I are expressed in smooth muscle, Iβ predominantly in platelets and neurons express either the PKG Iα or the PKG Iβ isoform.

Although cGMP decreases the expression of cyclin D1 in various cell types, it is still unknown whether it is mediated by the PKG dependent pathway.[14,15] Cyclin D1 controls the critical transition of G1 to S phase in mammalian cell cycle and any abnormalities in this step can enhance cell proliferation, genome instability and tumor progression. The induction of cyclin D1 in response to cell cycle progression is likely regulated at the transcription level and is one of the major targets for several growth stimulatory signaling pathways.[16,17] Therefore, in the present study we examined the roles of PKG isoforms in the transcriptional control of cyclin D1 in NIH3T3 cells to delineate the underlying molecular mechanism.

Previous studies have identified several *cis*-acting regulatory elements in the cyclin D1 promoter, including CRE/ATF2, NF-κB, AP1, TCF/LEF1, SP1 and Ets, that have been implicated as critical for cyclin D1 expression in both human and non-human cell lines.[16,18-24] To address the role of PKG isoforms in cyclin D1 regulation we used constitutively activated mutants of PKG Iα and PKG Iβ. We found that between the two isoforms of PKG, constitutively active mutants of PKG1β were more potent in inhibiting the cyclin D1 expression. Our study also provides evidence that PKG downregulate the transcription of cyclin D1 mainly by the regulation of c-fos, NF-κB, and CRE sites of cyclin D1 promoter.

Experimental

Plasmids. We generated a series of plasmids by using the expression vector pHACE that encode PKG Iα-WT, PKG

Abbreviations: PKG, protein kinase G; cGMP, cyclic guanosine monophosphate; SMC, smooth muscle cell; SRE, serum response element; SRF, serum response factor; TCF, ternary complex factor.

Iα-S65D and PKG α-ΔN mutants with a C terminal HA tag. We also generated a similar series of expression vectors of PKG Iβ (PKG Iβ-WT, PKG Iβ-S80D and PKG Iβ-ΔN). The details of these constructs have been described earlier.[25] The cyclin D1 promoter-luciferase plasmid was a gift from Dr. R. G. Pestell,[16] and the c-fos promoter-luciferase plasmid and SRE promoter-luciferase plasmid were gifts from Dr. R. Prywes.[26] The NF-κB promoter-luciferase plasmid and CRE promoter-luciferase were purchased from Stratagene.

Cell Cultures and Transfection. NIH3T3 mouse fibroblasts were routinely grown in Dulbecco's minimal essential medium containing 10% calf serum. For luciferase reporter assays, NIH3T3 cells were plated in 35 mm plates and twenty hours later, they were transfected using Lipofectin (Invitrogen) with 2 μg of the reporter plasmid, 1-5 μg of various expression vectors, and 1 μg of pCMV-β-gal. The pcDNA3 plasmid DNA was added to the transfections to achieve the same total amount of plasmid DNA per transfection.

Luciferase Reporter Assays. Twenty-four hours after transfection, cell extracts were prepared and luciferase assays were done using the Luciferase Assay System (Promega). Luciferase activities were normalized with respect to parallel β-galactosidase activities, to correct for differences in transfection efficiency. β-galactosidase assays were performed using the β-galactosidase Enzyme Assay System (Promega).

Results

PKG Downregulates the Transcription of Cyclin D1. cGMP, the upstream effector of PKG, can decrease expression of cell cycle promoting genes, such as cyclin D1 and E.[14,27] Hence, it was of interest to elucidate whether PKG can directly regulate the expression of cyclin D1. The effect of PKG on cyclin D1 promoter was studied by using a series of constitutively active mutants of both PKG Iα and PKG Iβ, as described in Figure 1. NIH3T3 mouse fibroblasts were transiently transfected with the control plasmid, PKG Iα-WT, PKG Iα-ΔN, PKG Iα-S65D, PKG Iβ-WT, PKG Iβ-ΔN or PKG Iβ-S80D constructs together with the luciferase reporter plasmid, which contains the full length cyclin D1

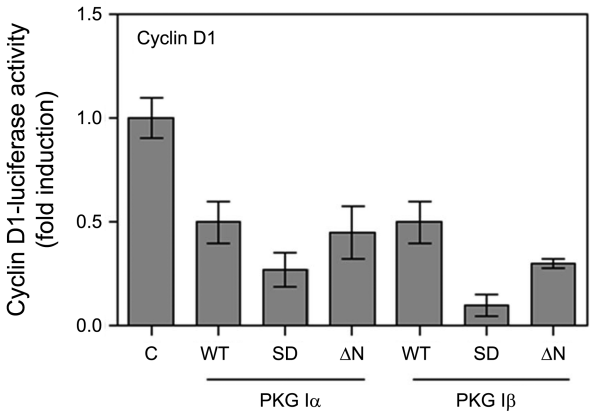

Figure 2. Downregulation of cyclin D1 promoter by PKG. NIH3T3 cells were transfected with the pCD1-luciferase reporter plasmid, which has a full-length cyclin D1 promoter linked to the luciferase gene, together with either empty control vector (*C*) or various expression vectors of PKG Iα and PKG Iβ, as indicated in the figure. Cell extracts were prepared after growing the cells for 24 h, and luciferase activities were measured and normalized with respect to parallel β-galactosidase activities. Luciferase activities are expressed as fold induction, after correction for β-galactosidase activities. The *error bars* indicate the standard deviations of triplicate assays. Similar results were obtained in three independent experiments.

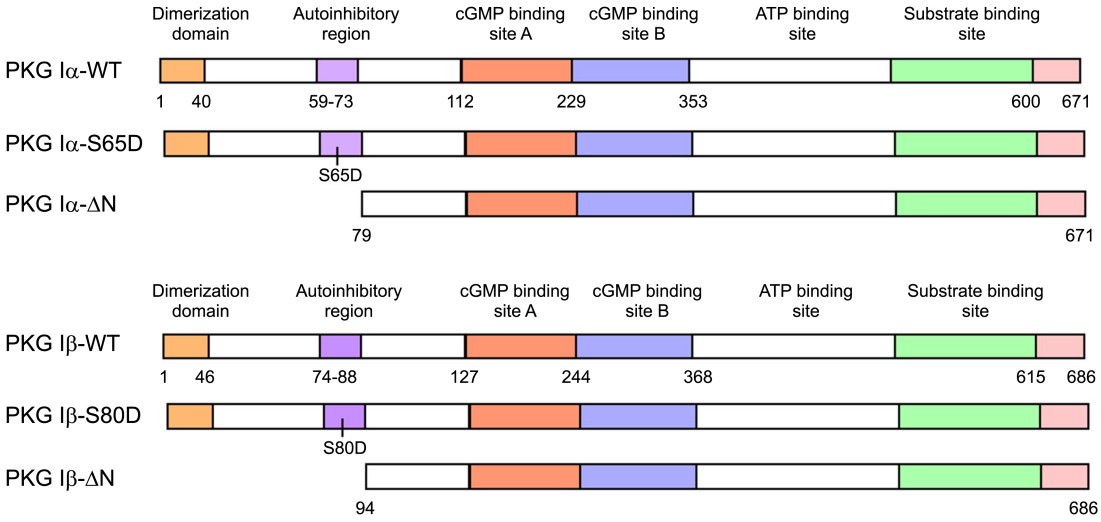

Figure 1. Schematic diagrams of structures of PKG mutants. PKG Iα-WT (wild type) constructs encode a full-length PKG open reading frame of 671 amino acid, which contain dimerization domain, autoinhibitory region, cGMP binding site A and B, ATP and substrate binding site. PKG Iα-S65D and PKG Iα-ΔN (constitutively active) constructs encode a full-length PKG with a point mutation in the autoinhibitory region and a truncated PKG from which dimerization and autoinhibitory region have been deleted, respectively. PKG Iβ-WT (wild type) constructs, which contain different regions like PKG Iα-WT, encode a full-length PKG open reading frame of 686 amino acid. PKG Iβ-S80D and PKG Iβ-ΔN (constitutively active) mutants encode point mutated and truncated PKG, respectively. All constructs were subcloned into the HA tagged mammalian expression vector pHACE.

promoter. After growing the cells for 24 h, extracts were prepared and assayed for luciferase activity.

We found that the S80D and ΔN mutants of PKG Iβ downregulated the transcription of cyclin D1 by 90% and 70%, respectively. In contrast, cyclin D1 was downregulated by 73% and 55%, respectively by the S65D and ΔN mutants of PKG Iα (Fig. 2). Taken together, our data indicates that although the constitutively activated mutants of both PKG Iα and PKG Iβ downregulate the transcription of cyclin D1, PKG Iβ is more significant in the downregulation of cyclin D1 transcription.

PKG Downregulates the Serum Response Element in the c-fos Promoter. Because the above findings suggest that the constitutively active mutants of PKG cause attenuation of cyclin D1 transcription, we were interested in determining whether overexpresion of PKG Iα or PKG Iβ inhibits c-fos transcription in NIH3T3 derivatives. Therefore, NIH3T3 derivatives that express wild type or mutant forms of PKG were transfected with the c-fos promoter-luciferase reporter plasmid. The cells were grown for twenty-four hours and cell extracts were prepared and assayed for luciferase activity. We found that the derivatives that express the constitutively activated PKG Iα or PKG Iβ displayed decreased transcriptional activity of the c-fos promoter (Fig. 3(a)).

The serum response element (SRE), transcriptional control element in the *c-fos* promoter, plays an important role in the transcription of c-fos. So, to study the upstream events of c-fos inhibition the NIH3T3 derivatives were transiently transfected with pSRE-luciferase reporter plasmid.[28] We found that in the NIH3T3 derivatives that express constitutively activated PKG Iα or PKG Iβ, there was a decrease in the activity of the SRE transcriptional control element, as shown in Figure 3(b). However, the constitutively activated mutants of PKG Iβ were more potent in inhibiting the SRE transcription. These findings suggest that PKG downregulates gene expression in NIH3T3 cells possibly by integrating multiple signal transduction pathways that may inhibit cell proliferation.

Role of NF-κB in the Downregulation of Cyclin D1. Previous studies have mentioned NF-κB as an important positive regulator of cyclin D1 transcription in different cell types.[20,21] Therefore, to examine whether PKG inhibited the transactivation at the NF-κB site, we transiently transfected the NIH3T3 derivatives that express wild type or mutant forms of PKG with NF-κB-luciferase plasmid. We found that the constitutively activated mutants of PKG Iβ decreased NF-κB transactivation. However, the constitutively activated mutants of PKG Iα showed weak inhibition of NF-κB (Fig. 4). Taken together these data suggests that PKG, at least PKG Iβ, can inhibit the activation and transcriptional activity of NF-κB and thereby downregulates cyclin D1 transcription.

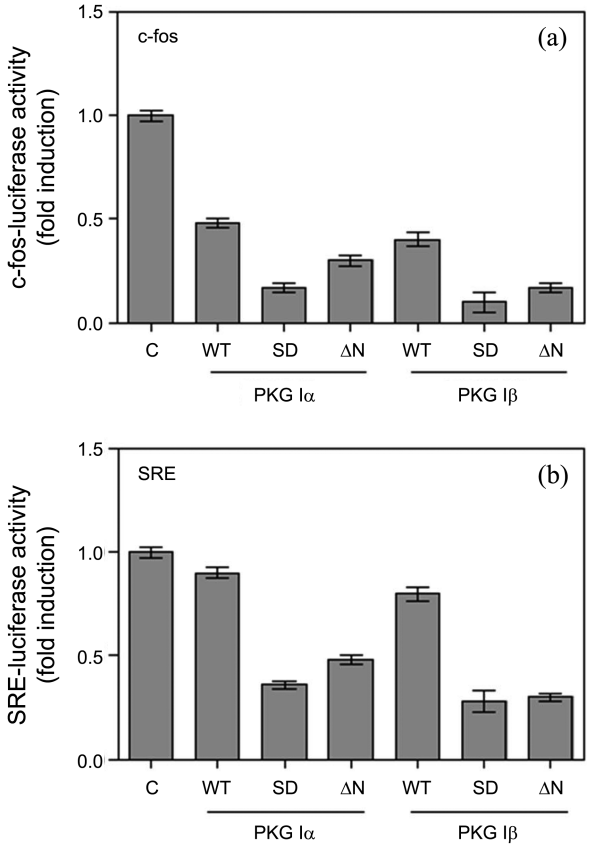

Figure 3. Downregulation of c-fos and SRE by PKG. (a) NIH3T3 cells were transfected with the pFos-luciferase reporter plasmid, containing a full length *c-fos* promoter linked to the luciferase gene, together with either empty control vector, *C* (pcDNA3) or various expression vectors of PKG Iα and PKG Iβ, as indicated. Cell extracts were prepared after growing the cells for 24 h, and luciferase activities were measured and normalized with respect to parallel β-galactosidase activities. (b) Assays were performed as in Figure 2(a) but with the pSRE-luciferase reporter plasmid, which contains a full-length SRE promoter linked to the luciferase gene.

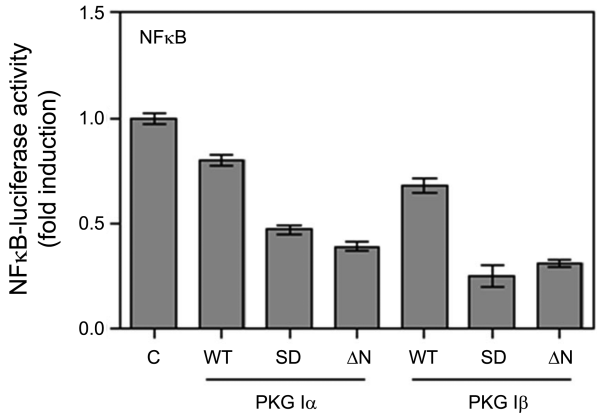

Figure 4. Inhibition of NF-κB transactivation by PKG. NIH3T3 cells were transiently cotransfected with the pNFκB-luciferase reporter plasmid, which has a full-length cyclin D1 promoter linked to the luciferase gene, and either empty vector or various expression vectors of PKG Iα and PKG Iβ that encode wild type (WT) or activated mutants (SD, ΔN). Cell extracts were prepared after growing the cells for 24 h, and luciferase activities were measured and normalized with respect to parallel β-galactosidase activities. Luciferase activities are expressed as fold induction after correction for β-galactosidase activities. The *error bars* indicate the standard deviations of triplicate assays. Similar results were obtained in three independent experiments.

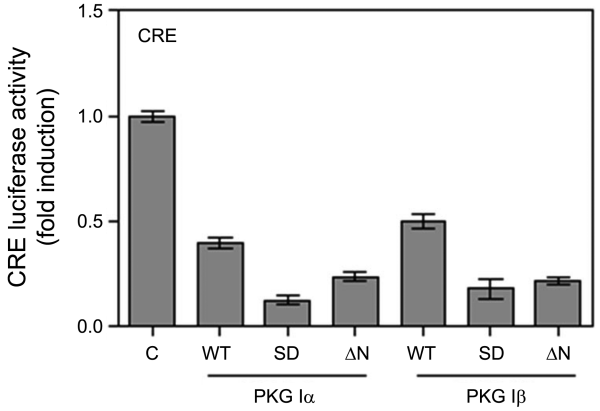

Figure 5. Inhibition of CRE transactivation by PKG. NIH3T3 cells were transfected with the pCRE-luciferase reporter plasmid, which has a full length CRE promoter linked to the luciferase gene, together with either empty control vector (*C*) or various expression vectors of PKG Iα and PKG Iβ, as indicated in the figure. Cell extracts were prepared after growing the cells for 24 h, and luciferase activities were measured and normalized with respect to parallel β-galactosidase activities. Luciferase activities are expressed as fold induction, after correction for β-galactosidase activities. The *error bars* indicate the standard deviations of triplicate assays. Similar results were obtained in three independent experiments.

PKG Inhibits Transactivation at the CRE Site. To examine the role of PKG in CRE transactivation, we transiently transfected the NIH3T3 cells with a CRE reporter plasmid along with either control vector or the constitutively active mutants of PKG. We found that the constitutively active mutants of both PKG Iα and PKG Iβ inhibited the CRE site, an effect which can be expected to result in the downregulation of cyclin D1 expression (Fig. 5).

Discussion

In this study, we explored the possible roles of specific isoforms of PKG in the transcriptional control of cyclin D1 in NIH3T3 mouse fibroblast cells. To examine the downstream effectors of PKG that mediate cyclin D1 regulation, we used a series of expression vectors that encode wild type and constitutively active mutants of PKG Iα and PKG Iβ, as described in Figure 1. PKG Iα-S65D and PKG Iβ-S80D constructs encode full length PKG with a point mutation that substitutes aspartic acid to serine in the pseudosubstrate site of the autoinhibitory region, whereas DN constructs of PKG Iα and PKG Iβ encode dimerization and autoinhibitory region deleted PKG. We found that the overexpression of PKG causes downregulation of cyclin D1 in NIH3T3 cells.

Cyclin D1, the regulatory subunit of several cyclin-dependent kinases, is required for the progression of the G1 phase and is associated with cell cycle progression and cell proliferation.[29] Transcriptional downregulation of cyclin D1 by the PKG led us to study the immediate early regulatory elements of cyclin D1. It has been found that the CREB/ATF2, NF-κB, AP1, TCF/LEF1, SP1, Ets, and E2F sites of cyclin D1 promoter play pivotal role in the regulation of cyclin D1 expression.[19,23] Our study suggests that PKG regulates the transcription of cyclin D1 mainly through the c-fos, NF-κB, and CRE sites of cyclin D1 promoter. Furthermore, we found that the PKG Iβ isoform is more potent in downregulation of cyclin D1 (Fig. 2). It is also of interest that the SD mutants of both PKG Iα and Iβ displayed higher constitutive kinase activities than the corresponding ΔN mutants. These data suggest that the regulatory region of PKG contain additional regulatory sequences other than pseudosubstrate sequences.

NF-κB, a transcriptional activator of genes involved in cell proliferation and survival, is inactive when bound to cytoplasmic IκB protein but becomes active when IκB is phosphorylated and degraded. This activated NF-κB translocates to the nucleus and modulates transcription of several target genes, including cyclin D1. Previous studies have demonstrated that in NIH3T3 cells Rac1-mediated cyclin D1 promoter activity requires the presence of the -39 to -33 NF-κB binding site.[21] It has also been found that the activated NF-κB can stimulate transcription from the cyclin D1 promoter in COS-7 cells and mouse embryo fibroblasts, whereas mutation of NF-κB-responsive elements attenuates serum induced transcription.[20] Our results suggest that the overexpression of PKG inhibits the activation and transcription of NF-κB, which is consistent with the previous findings that NF-κB is a positive regulator of cyclin D1 promoter (Fig. 3). However, the precise mechanism of NF-κB inhibition by PKG is still not clear.

We also found that PKG inhibits the transactivation of c-fos by the downregulation of SRE (Fig. 4). However, transcriptional activity of SRE is mediated by two transcription factors, serum response factor (SRF) and ternary complex factor (TCF). SRF binds as a dimer to the c-fos SRE, whereas TCF interacts with the SRE only when it is occupied by SRF.[28] PKG can inhibit different MAP kinases which in turn can attenuate TCF. For example, recent evidence suggests that PKG directly phosphorylates Ser-43 of Raf-1 *in vivo* and *in vitro* and this phosphorylation uncouples the Ras-Raf interaction and thereby prevents mitogen-induced Raf-1 activation.[30] Thus our results are consistent with the previous studies which mention that PKG inhibits SRF-dependent transcription by interfering with RhoA signaling in cardiomyocytes and VSMCs.[31] Previous studies have also established the role of CRE in the transcriptional regulation of cyclin D1. It has been found that in NIH3T3 cells SV40 small t antigen-induced cyclin D1 transcription is mediated by the CRE site of cyclin D1 promoter.[24] In colon carcinoma cells, the CRE site is positively regulated by p21Ras activation and in MCF-7 human mammary carcinoma cells estrogen activates the cyclin D1 promoter *via* the CRE site.[22,23] Consistent with the previous findings, we found that in NIH3T3 cells the CRE site plays important role in the downregulation of cyclin D1 by PKG.

Even though our study has confirmed the involvement of c-fos, CRE and NF-κB, immediate early genes of cyclin D1 promoter, in the downregulation of cyclin D1, the precise pathways and the underlying molecular mechanism by which

PKG regulates these sites remain unclear. In summary, our study provides evidence that the increased expression of PKG, predominantly PKG Iβ, in NIH3T3 mouse fibroblasts play a pivotal role to inhibit cell cycle progression by the downregulation of cyclin D1 *via* the regulation of three distinct *cis*-acting regulatory elements.

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