Cyclic AMP- and Cyclic GMP-dependent Protein Kinases Differ in Their Regulation of Cyclic AMP Response Element-dependent Gene Transcription*

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The ability of cGMP-dependent protein kinases (cGKs) to activate cAMP response element (CRE)-dependent gene transcription was compared with that of cAMP-dependent protein kinases (cAKs). Although both the type I cGKI (cGKII) and the type II cAKII translocate to the nucleus following activation by 8-bromo-cyclic AMP, however, cGKIb did not translocate to the nucleus upon activation by 8-bromo-cyclic GMP. Replacement of an autophosphorylated serine (Ser79) of cGKIβ with an aspartic acid resulted in a mutant kinase with constitutive kinase activity in vitro and in vivo. The cGKIbS79D mutant localized to the cytoplasm and only a weak activator of CRE-dependent gene transcription. However, an amino-terminal deletion mutant of cGKIβ was found in the nucleus as well as the cytoplasm and was a strong activator of CRE-dependent gene transcription. These data suggest that the inability of cGKs to translocate to the nucleus is responsible for the differential ability of cAKs and cGKs to activate CRE-dependent gene transcription and that nuclear redistribution of cGKs is not required for NO/cGMP regulation of gene transcription.

The cyclic nucleotides, cAMP and cGMP, are intracellular second messengers mediating the actions of a large number of hormones and neurotransmitters. These cyclic nucleotides act to allosterically regulate the action of a small number of important proteins. Unlike cAMP, which acts mainly through cAMP-dependent protein kinases (cAKs),1 cGMP is able to activate three classes of proteins: ion channels, phosphodiesterases, and cGMP-dependent protein kinases (cGKs). The cAKs and the cGKs are highly homologous protein kinase families with similar substrate specificities. Phosphorylation of cellular proteins by both families of kinases leads to alterations in calcium mobilization, protein phosphatase activity, ion channel function, gene transcription, smooth muscle contractility, and platelet aggregation (1–4).

The cGKs are classified into two types based on their historical order of characterization. The type I enzymes (cGKIs) are highly expressed in lung (5), cerebellum (6), platelets (7), and smooth muscle (8). Two type I isoforms, Iα and Iβ, arise from the alternative splicing of a single gene (9–12). These two forms differ in their amino-terminal autoinhibitory domains but share the same cGMP-binding sites and catalytic domains (4, 13). In Purkinje cells (6), smooth muscle cells (14, 15), monocytes (16), and neutrophils (17, 18), the majority of the cGKI immunoreactivity is soluble and localized to the cytoplasm. A second type of cGK, termed the type II cGK (cGKII), is highly expressed in intestinal microvilli (19) and is encoded by a gene distinct from that encoding cGKI proteins (20, 21). While cGKI isoforms are soluble proteins, cGKII is particulate and associated with cellular membranes (19). Both types possess amino-terminal leucine zipper motifs and exist as homodimers in native tissues (13). In contrast to the cAKs, which have separate catalytic and regulatory subunits, each monomer of the cGKs consists of both a regulatory domain and a catalytic domain contained in the same polypeptide (13).

Many mammalian tissues coexpress isoforms of cAK and cGK, where the cAK and cGK proteins are thought to play distinct roles in cellular regulation. The in vitro substrate specificities of the cAKs and the cGKs are very similar, although a number of proteins have been identified as specific substrates for either cAKs or cGKs. For example, the type I regulatory (R) subunit of cAK (22), G-substrate (23), histone H2B (24), and the bovine lung cAMP-binding cGMP-specific phosphodiesterase (25, 26) are specific substrates of cGKI in vitro, while the cAMP response element-binding protein (CREB) has been shown to be a specific in vitro substrate of cAK (76). Although in vitro substrate specificity may be an important indicator of in vivo substrate specificity, recent evidence suggests that colocalization of kinase and substrate in the cell is at least an equally important factor (27).

The cAMP signaling pathway is used to regulate the tran...
Cytoplasmic Localization of Activated cGKI

scription of many genes and involves the phosphorylation of specific transcription factors by the C subunit of cAK (28). In the absence of cAMP, cAK exists predominantly as an inactive tetramer. The inactive holoenzyme composed of two R subunits and two C subunits. The inactive holoenzyme complex may be localized diffusely in the cytoplasm or localized to specific subcellular compartments by interaction of the R subunits with protein kinase A anchoring proteins (27). Upon cytoplasmic elevation of cAMP, cAMP binds to each R subunit, causing the holoenzyme complex to dissociate into a homodimer of R subunits and two catalytically active C subunits. Once released, the C subunit can phosphorylate cytoplasmic substrate proteins, that remain to be characterized.

Activity Determination—10-cm plates of CV-1 or HEK293 cells were transiently transfected using a calcium phosphate coprecipitation method (37). 48 h after application of DNA precipitates, plates were washed twice with ice-cold phosphate-buffered saline (PBS). Following the addition of 200 μl of a homogenization buffer (10 mM sodium phosphate, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol), the homogenate was centrifuged and the supernatant was sonicated twice for 10 s. For kinase activity determinations, cyclic nucleotide (50 μM) was added to or omitted from separate tubes containing a phosphotransferase assay mixture consisting of 20 mM Tris HCl pH 8.0, 5 mM MgCl2, 100 μM ATP and 11 μM [γ-32P]ATP (ICN) (specific activity = 200–300 cpm/pmol), 10 mM NaF, 10 mM dithiothreitol, and the synthetic acceptor peptide Kemptide (LLRASLG; 50 μM) or H2Tide (RKRRIAE; 110 μM). When assaying cGKI activity, the phosphotransfer reaction was allowed to proceed for 10 min at 30 °C. The assay was initiated by the addition of cell extracts (0.2 mg/ml), and the phosphorylation reaction was terminated by spotting aliquots onto P81 phosphocellulose (Whatman). The P81 phosphocellulose was washed with 10 mM phosphoric acid and counted.

Site-directed Mutagenesis of cGKI and Construction of cGKI Deletion Mutant—The mutations in pCMV.mCGKI J75A, pCMV.mCGKI β579D, pCMV.mCGKI J404R, and pCMV.mCGKI K407R/R409Q were created by a two-step PCR method using mutagenic primers as described previously (38). The resulting PCR fragments were subcloned into pCMV.mCGKI using convenient restriction sites, and each construct was verified by DNA sequencing of the PCR-amplified region.

MATERIALS AND METHODS

Screening of Mouse Brain cDNA Library for Full-length Mammalian cGKI—cDNA—cDNA library screening was performed essentially as described previously (20). A 1.0-kilobase pair EcoRI-SalI restriction fragment from C.9/2.6 was isolated and labeled by random primer labeling, primer extension in the presence of [α-32P]ATP. The resulting radiolabeled DNA fragments were used to screen a mouse brain cDNA library. Clones mcGKIβ3D and mcGKI2.2 were isolated, and their inserts were restriction-mapped. mcGKIβ3D, which contains the entire open reading frame (ORF) of murine cGKI, was fully sequenced in both directions using Sequenase DNA polymerase (U.S. Biochemical Corp.). mcGKI2.2, which lacks the first 1130 bp of the cGKI ORF, was partially sequenced to confirm a 2-bp deletion in the ORF of mcGKIβ3D. The murine cGKI cDNA sequence derived from the sequencing of both clones has been submitted to the GenBank™ database. Sequence analyses were performed using DNASTAR software.

Construction of Murine cGKI Mammalian Expression Vector—The pCMV.mCGKI mammalian expression vector was constructed by the polymerase chain reaction (PCR) method using the oligonucleotides 5′-GGA GAT CCT CAC CAT GGC CAG CCT GGC GGA TTT TCG AC-3′ and 5′-GGA GAT CCA C-3′ as a template. The resulting radiolabeled DNA fragments were used to screen a mouse brain cDNA library. Clones mcGKIβ3D and mcGKI2.2 were isolated, and their inserts were restriction-mapped. mcGKIβ3D, which contains the entire open reading frame (ORF) of murine cGKI, was fully sequenced in both directions using Sequenase DNA polymerase (U.S. Biochemical Corp.). mcGKI2.2, which lacks the first 1130 bp of the cGKI ORF, was partially sequenced to confirm a 2-bp deletion in the ORF of mcGKIβ3D. The murine cGKI cDNA sequence derived from the sequencing of both clones has been submitted to the GenBank™ database. Sequence analyses were performed using DNASTAR software.

Construction of Murine cGKI and cGKI G2A Mammalian Expression Vectors and Generation of Polyclonal Antibodies to Murine cGKI—pCMV.mCGKI and pCMV.mCGKI G2A were constructed by PCR. PCR fragments were generated using the oligonucleotides as forward primers: 5′-GAG GAT CCT CAC CAT GGC CAG CCT GGC GGA TTT TCG AC-3′ and the reverse primer, and the plasmid pGEM-T-Flag-Ca1 as a template. The resulting PCR fragment was ligated into pGEM-T (Promega) to create pGEM-T.Flag-Ca1. To generate the cAK/cGKI chimera, PCR fragments coding for the Flag-tagged amino terminus of murine Ca (39) and the carboxyl terminus of murine cGKI were amplified in separate PCRs. A PCR fragment coding for the amino terminus of murine Ca was used as the template in the PCRs to amplify 5′-AGG GAT CCT CAC ATT ACC CC-3′ and the reverse primer, and the plasmid pGEM-T-Flag-Ca1 as a template. The resulting PCR fragment was ligated into pGEM-T (Promega) to create pGEM-T.Flag-Ca1 as a template. The resulting PCR fragment was cut with BamHI and ligated into the BamHI site of pCMV Neo (30) to create pCMV.mCGKI G2A. The resulting fragment was cut with BamHI and ligated into the BamHI site of pCMV Neo (30) to create pCMV.mCGKI G2A. The resulting fragment was cut with BamHI and ligated into the BamHI site of pCMV Neo (30) to create pCMV.mCGKI G2A.
His<sub>e</sub>cGKI was expressed in Spodoptera frugiperda (Sf9) cells and purified as described previously (41). Purified His<sub>e</sub>cGKI was used to immunize rabbits for polyclonal antibody production (Research Genetics Inc.).

Lysotracer Assays—CV-1 and HEK293 cells were grown separately on 10-cm plates to 30% and 50% confluency, respectively, and transfected using a standard calcium phosphate method (37) with 0.5 μg of the cAMP-responsive reporter construct human chorionic gonadotropin-luciferase (HCG-luciferase) (42) as well as the indicated amounts of pRSV, pGAL and expression vectors. The total amount of plasmid DNA was brought to 30 μg with the parent vector pCMV. Neo. 24 h after transfection, cells were washed three times with PBS, cells were incubated with a rabbit polyclonal antibody generated against the carboxy-terminal 15 amino acids of cGKI (anti-cGMP-PK CT) (Upstate Biotechnology, Inc.) at a 1:1000 dilution or an anti-Flag epitope antibody (M2) (Eastman Kodak) at a 1:2000 dilution in blocking buffer (PBS supplemented with 0.1% bovine serum albumin, 3% goat serum, and 0.1% saponin (Sigma)) for 1 h at room temperature. After four washes with wash buffer (PBS supplemented with 0.1% saponin) followed by a 1:1000 dilution of Alexa-488-conjugated streptavidin (Molecular Probes, Inc., Eugene, OR) was incubated with the cells for 1 h in the dark in blocking buffer. Prior to examination by fluorescence microscopy, cells were washed three times for 2 min in wash buffer and twice for 2 min in PBS.

To confirm the specificity of the anti-cGMP-PK CT antibody for endogenous cGKI in A7r5 cells, the anti-cGMP-PK CT antibody (40 nM) was preincubated for 1 h at room temperature with a 1:1000 dilution of Alexa-488-conjugated streptavidin. Cells were washed, and Alexa-488 conjugate was incubated with the cells for 1 h in the dark in blocking buffer. Finally, cells were washed three times for 2 min in wash buffer and twice for 2 min in PBS.

In control experiments, to verify the specificity of the anti-cGMP-PK CT antibody for endogenous cGKI in A7r5 cells, the anti-cGMP-PK CT antibody was preincubated with 10 μM cGMP for 1 h at room temperature with a 1:1000 dilution of Alexa-488-conjugated streptavidin. Cells were washed, and Alexa-488 conjugate was incubated with the cells for 1 h in the dark in blocking buffer. Finally, cells were washed three times for 2 min in wash buffer and twice for 2 min in PBS.

RESULTS

Cloning and Sequencing of Marine cGKI—A single full-length cGKiβ cDNA clone was isolated from a mouse brain cDNA library. The cDNA was fully sequenced and shown to contain 2841 bp (Fig. 1). The murine cGKiβ cDNA contains a short 89-bp 5'-untranslated region, an ORF of 2061 nucleotides, and a 691-bp 3'-untranslated region. The predicted murine cGKI protein contains 886 amino acids with a calculated molecular mass of 77.8 kDa and shows greater than 99% amino acid identity to human cGKI (9). Despite 139 nucleotide differences in the ORF sequence, there are only three amino acid differences between murine cGKI and human cGkiβ: Glu<sup>242</sup> to Asp, Thr<sup>590</sup> to Gln, and Asn<sup>571</sup> to Ser (Fig. 1).

Effect of cGKiβ Overexpression on CRE-dependent Gene Transcription—To determine whether cGkiβ was capable of regulating CRE-dependent gene transcription, we examined the ability of cGkiβ to transactivate the cAMP-responsive HCG promoter. CV-1 cells (Fig. 2A) or HEK293 cells (Fig. 2B) were transfected with a constant amount of the cGKiβ expression vector along with the HCG-luciferase reporter plasmid (42). Control cells were transfected with the HCG-luciferase reporter plasmid alone. Transfected cells were treated with or without 8-Br-cGMP (1 mM) for 24 h. CV-1 and HEK293 cells were chosen for this experiment because regulation of CRE-dependent gene transcription by cAK has been characterized by experiments previously in these cell lines (36, 43, 47). In both CV-1 and HEK293 cells, transfection of wild-type cGKIβ only minimally stimulated luciferase gene transcription in the absence of cyclic nucleotide treatment (Fig. 2, A and B). CV-1 and HEK293 cells transfected with the HCG-luciferase reporter plasmid alone showed minimal responses to 8-Br-cGMP treatment (Fig. 2, A and B). When CV-1 cells overexpressing cGKIβ were stimulated with 8-Br-cAMP (1 mM) or 8-Br-cGMP (1 mM) and 3-isobutyl-1-methylxanthine (50 μM) in DMEM for various times at 37 °C, following stimulation, cells were washed twice with ice-cold PBS and fixed with 4% formaldehyde in PBS for 10 min at room temperature followed by a 1:1 mixture of methanol and acetone for 5 min. After air drying, the cells were incubated with 0.5% normal goat serum for 1 h and then with the goat anti-mouse IgG (Jackson) or a 1:3000 dilution of Cy5-labeled streptavidin (Jackson) was incubated with the cells for 1 h in the dark in blocking buffer. Prior to examination by fluorescence microscopy, cells were washed four times for 2 min in wash buffer and twice for 2 min in PBS.
pressing cGKIβ were treated with 8-Br-cGMP (1 mM), a small but reproducible increase in luciferase activity was observed (Fig. 2A). In HEK293 cells, overexpression of cGKIβ increased luciferase activity following 8-Br-cGMP treatment 13-fold (Fig. 2B). Thus, the HCG-luciferase reporter plasmid is more sensitive to overexpression of cGKIβ in HEK293 cells than in CV-1 cells. The greater sensitivity of the luciferase reporter assay in HEK293 cells may be due to a number of factors including the levels of accessory transcription factors (48, 49) or cellular phosphatases (50, 51). The data from both CV-1 and HEK293 cells indicate that cGKIβ is capable of inducing CRE-dependent gene transcription in mammalian cell lines.

**cGKIβ Is a Relatively Weak Activator of CRE-dependent Gene Transcription**—For comparison of cGKIβ and cAK activation of CRE-dependent gene transcription, the same HCG-luciferase reporter assay was employed. To generate comparable levels of cGKIβ and cAKII in transfected cells, the quantity of each expression vector transfected was carefully titrated to produce similar amounts of cGKIβ and cAKII protein and kinase activity (data not shown). For cGK expression, CV-1 cells were transfected with 10 μg of pCMV.mcGKIβ. For cAK expression, cells were transfected with both 1 μg of pCMV.Flag-Cα3 and 4 μg of pCMV.RIIα (52). Control cells were transfected with the parental pCMV.Neo vector alone. Extracts from cGKIβ- and cAKII-transfected cells possess similar levels of cGKIβ and cAKII protein by quantitative Western blotting using purified proteins as standards (data not shown). The extracts from cGKIβ-transfected cells and cAKII-transfected cells showed similar specific activities with in vitro kinase assays in which the nonspecific peptide Kemptide (150 μM) was used as the phosphoacceptor (extracts from cGKIβ transfected cells 50.73 nmol/min/mg and extracts from cAKII transfected cells 50.84 nmol/min/mg). The maximal cGMP- or cAMP-stimulated protein kinase activities of extracts from cGKIβ-transfected cells and extracts from...
Cytoplasmic Localization of Activated cGKI

Once similar levels of kinase activity were obtained, transcrip-
tional regulation was examined (Fig. 2C). In cells expressing cGKIβ or cAKII, stimulated luciferase activity was half-
maximal at 100 µM 8-Br-cGMP and 30 µM 8-Br-cAMP, respectively. Cotransfection with cGKIβ gave a maximal 2-fold stimulation of reporter gene expression over basal levels, whereas cotransfection with cAK gave a maximal 14-fold stim-
ulation (Fig. 2C). cAKII-transfected cells showed significantly higher luciferase activity at all cyclic nucleotide concentrations examined (Fig. 2C). Although CV-1 cells express both endoge-

nous cGKI and cAK, cells transfected with the reporter gene alone showed no response to 8-Br-cGMP treatment but a sig-
nificant response to 8-Br-cAMP treatment (4-fold) (data not shown). A reduced potency of cGKIβ relative to cAKII was also observed in HEK293 cells, where cotransfection with cGKIβ gave a maximal 13-fold induction in comparison with the 250-
fold induction seen with the C subunit of cAK (data not shown).

cAK's maximal induction of luciferase activity was 52-fold higher than cGKIβ in CV-1 cells and 25-fold higher than cGKIβ's in HEK293 cells (Fig. 2D). Our results in both CV-1 and HEK293 cells indicate that when compared with cAK, cGKIβ is only a weak activator of CRE-dependent gene transcription.

VASP Is Phosphorylated Efficiently by Both cGKIβ and cAK in Vivo—Since cGKIβ and cAKII were expressed at equal amounts, but showed a 10-fold difference in their ability to regulate transcription, experiments were designed to compare the ability of cGKIβ and cAK to phosphorylate a mutually recognized substrate in vivo.

A cytoplasmic substrate for cGK and cAK has been identified in human platelets (53). This 46-kDa protein, termed VASP (vasodilator- and A kinase-stimulated phosphoprotein), has been shown to be widely expressed and localized to cytoplasmic focal adhesions and stress fibers (54). The human VASP protein was demonstrated to be phosphorylated at three sites to varying extents by both cGK and cAK. Phosphorylation of one of these sites, Ser157, caused a shift in mobility from 46 to 50 kDa in a SDS-PAGE gel. VASP has been shown to be widely expressed and localized to cytoplasmic focal adhesions and stress fibers (55). A full-length murine VASP cDNA was sequenced (see “Ma-
terials and Methods”), and the murine VASP protein predicted by the ORF was 87% identical to the human VASP protein (56). All three human VASP cyclic cAMP-dependent phospho-
rylation sites are conserved in murine VASP (46). To differen-
tiate transfected VASP from endogenous VASP, we con-
structed an amino-terminal Flag-tagged VASP mammalian expression vector, pCMV.Flag-VASP (see “Materials and Methods”).

To verify that Flag-tagged murine VASP was expressed and properly localized, cells were transiently transfected with pCMV.Flag-VASP (20 μg) or a com-

bination of pCMV.Flag-VASP (20 μg), pCMV.pCMVFlag-PKI (2 μg), and pCMV-

V.Flag-PKI (2 μg). 24 h after transfection, cells were serum-starved for an additional 24 h and then treated for the indicated number of minutes with 8-Br-cAMP (1 μM) and IBMX (500 μM) or 8-Br-cGMP (1 μM) and IBMX (500 μM). Extracts were generated (see “Materials and Methods”), resolved by 10% SDS-PAGE and immunoblotted with the anti-Flag antibody (M2) as the primary antibody and an 125I-labeled sheep anti-mouse antibody as the secondary antibody.

aK are expressed at similar levels in extracts from CV-1 cells transfectected with 2 μg of pCMV.Flag-VASP, as determined by in vitro kinase assays (data not shown). Western blot analysis of extracts from untreated cells using an anti-Flag antibody detected two bands, a major 46-kDa band and a minor 50-kDa band (Fig. 3B), indicating that cGKIβ has significant basal activity toward VASP in vivo. Within 5 min of 8-Br-cGMP treatment, nearly all of the remaining 46-kDa band was con-
verted to the 50-kDa form and remained in that form for at least 1 h (Fig. 3B). To exclude the possibility that 8-Br-cGMP was cross-activating endogenous cAK, we cotransfected sufficient PKI expression vector to completely block cAK activation (data not shown). Coexpression of PKI had no effect on 8-Br-cGMP-dependent increases in VASP phosphorylation (data not shown). These findings suggest that both cGKIβ and cAK are equally capable of phosphorylating protein substrates in cells treated with membrane soluble cyclic nucleotides. Thus, the lack of significant regulation of CRE-dependent gene transcrip-
tion by cGKIβ is not due to a deficit in cGKIβ activation.

Effect of Cyclic Nucleotides on the Subcellular Distribution of cGKIβ and the C Subunit of cAKII—Nuclear pore complexes mediate the transport of proteins between the cytoplasm and the nucleus of mammalian cells. These pore complexes allow passive diffusion of small proteins (<40–65 kDa) in and out of the nucleus, while larger proteins must be actively transported (58). cAKs consist of separate R subunits and C subunits, while cGKS possess a linker sequence, which connects the regulatory domain and catalytic domain into a single polypeptide chain. Therefore, unlike the cAKs, activation of the cGKS by cGMP does not result in the dissociation of the regulatory and the catalytic components of the enzyme and does not cause the release of a small catalytic subunit (13). The actual size of active cGKS is increased further by dimerization of cGK sub-
units (13). Hence, while the free C subunit of cAK (40 kDa) is able to passively diffuse into the nucleus to phosphorylate specific transcription factors (29), cGKIβ (150 kDa) would be expected either to be restricted to the cytoplasm or to require a nuclear localization sequence (NLS) for transport to the nucleus.

Because CREB is primarily a nuclear protein (28), a re-
stricted cytoplasmic localization could explain cGKIβ’s mini-
mal ability to activate CRE-dependent gene transcription. To exa-
nine the subcellular localization of cGKIβ, we transiently transfectCV-1 cells with pCMV.pCMVFlag-VASP. CV-1 cells express low levels of endogenous cGKI and possess a flat morphology. Anti-cGKIβ serum, which recognizes the carboxy-terminal 15 amino acids of murine cGKI (anti-cGMP-PK CT), detected a single major band with an apparent molecular mass of 46 kDa (Fig. 3A), the size expected for nonphosphorylated VASP. No band was seen in nontransfect-
ced CV-1 cell extracts (data not shown).

When Flag-tagged VASP-transfected cells were treated with 8-Br-cAMP, two immuno-reactive species were detected on Western blots. The most rapidly migrating species corre-
sponded to the previously observed 46-kDa VASP band, and an additional 50-kDa VASP band corresponded to a phosphoryl-
ated form of the VASP protein (Fig. 3A). Phospho-VASP was observed following 5 min of cyclic nucleotide treatment and remained at the maximal level for at least 1 h (Fig. 3A). For-
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tor for mouse PKI protein (data not shown), which specifically inhibits cAKs but not cGKS (57). Formation of the 50-kDa band was also prevented by site-directed mutagenesis of Ser153 to an alanine (data not shown).

To demonstrate activation of transiently expressed cGKIβ by 8-Br-cGMP, cells were cotransfected with expression vectors for Flag-tagged VASP, cGKIβ, and PKI. cGKIβ and endogenous

FIG. 3. Time course of Flag-tagged VASP mobility shift induced by membrane soluble cyclic nucleotides. CV-1 cells were transiently transfected with pCMV.Flag-VASP (20 μg) (A) or a combination of pCMV.Flag-VASP (20 μg), pCMV.pCMVFlag-PKI (2 μg), and pCMV-V.Flag-PKI (2 μg) (B). 24 h after transfection, cells were serum-starved for an additional 24 h and then treated for the indicated number of minutes with 8-Br-cAMP (1 μM) and IBMX (500 μM) (A) or 8-Br-cGMP (1 μM) and IBMX (500 μM) (B). Extracts were generated (see “Materials and Methods”), resolved by 10% SDS-PAGE and immunoblotted with the anti-Flag antibody (M2) as the primary antibody and an 125I-labeled sheep anti-mouse antibody as the secondary antibody.

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Fected HEK293 cells. No bands were detected in extracts from parent molecular mass of 86 kDa in extracts from cGKII-trans-
length His-tagged cGKII purified from SF9 cells as the antigen
cGKII isoform, rabbit antiserum was prepared using full-
tirely consistent with its being a weak activator of CRE-de-
m tion of cGKI

C

A

B

D

FIG. 4. Effect of cyclic nucleotides on the subcellular distribution of cGKIB and the C subunit of cAKII. Indirect immunofluorescence microscopy analysis of CV-1 cells transiently transfected with either pCMV.mcGKIB (cGKIB; a and b) or pCMV.Flag-Ca3 and pCM-
V.RlII (cGKII; c and d). 24 h post-transfection, 8-Br-cAMP (1 mM) and IBMX (500 μM) (b) or 8-Br-cAMP (1 mM) and IBMX (500 μM) (d) were added to (8-Br-cNMP; b and d) or omitted (Cont.; a and c) for 1 h. Cells were fixed with a 4% paraformaldehyde solution and a 1:1 solution of acetone and methanol and then labeled with the anti-cGMP-PK CT antibody (a and b) or the anti-Flag antibody (M2) (c and d) as the primary antibody and Cy3-Fab′ goat anti-rabbit (a and b) or Cy3-
F(ab′)2 goat anti-mouse (c and d) as the secondary antibody.

(d) not shown). A similar restriction of cGKIB to the cyto-
plasm was observed in transfected COS-1, HEK293, and baby hamster kidney mammalian cell lines (data not shown).

In contrast, as shown previously (30), the C subunit of cAK was found to quickly diffuse into the nucleus upon treatment of cells with 8-Br-cAMP (Fig. 4, C and D). Thus, unlike the C subunit of cAK, which can translocate into the nucleus upon activation, cGKIB is restricted to the cytoplasm independent of its activation state.

In CV-1 cells, immunofluorescence microscopy revealed that both endogenous CREB and a GFP-hCREB chimera protein were targeted to the nucleus. No significant immunofluorescence was discernible in the cytoplasm (data not shown).

Hence, the restricted cytoplasmic localization of cGKIB is entirely consistent with its being a weak activator of CRE-de-
pendent gene transcription.

cGKII Does Not Translocate to the Nucleus upon Activation by cGMP.—To determine the subcellular localization of the cGKII isoform, rabbit antiserum was prepared using full-
length His-tagged cGKII purified from SF9 cells as the antigen
(41). Anti-cGKII serum recognized a single band with an apparent molecular mass of 86 kDa in extracts from cGKII-transfected HEK293 cells. No bands were detected in extracts from control or cGKIB-transfected HEK293 cells. Preimmune serum from the rabbit did not detect bands in any extracts (data not shown).

Immunostaining for transfected wild type murine cGKII in
CV-1 cells using cGKII antiserum, revealed a crescent-shaped, perinuclear staining pattern (Fig. 5A). This staining was spec-
ific for cGKII, since no significant anti-cGKII immunofluores-
ence was observed in cells transfected with parental vector alone (data not shown). Treatment with 8-Br-cGMP for 1 h did not affect the staining pattern and did not cause an increase in nuclear staining (Fig. 5B).

To rule out the possibility that cGKII was not translocating to the nucleus because it was strongly membrane-bound, we

mutated the penultimate glycine of murine cGKII to an ala-
line. The cGKIIIG2A mutant is a nonmyristoylated and soluble protein that is enzymatically similar to wild type cGKII (59). In contrast to wild type cGKII, the cGKIIIG2A mutant was localized diffusely in the cytoplasm (Fig. 5C). As described for wild type cGKII, treatment with 8-Br-cGMP had no effect on the subcellular distribution of the cGKIIIG2A protein (Fig. 5D). These data indicate that restriction to the cytoplasm is not cGKIB-specific but a general property of both cGKIB and cGKII.

Localization of Endogenous cGKI in A7r5 Smooth Muscle Cells—Since overexpression of proteins can result in aberrant subcellular localization, several cell lines were screened for detectable expression of cGKI and cGKII. The anti-cGMP-PK CT antibody detected a single cGKI band with an apparent molecular mass of 75 kDa in cell extracts from N1E-115 neuroblastomas, N2A neuroblastomas, NG-108 neuroblastomas, CV-1 cells, HEK293 cells, A10 smooth muscle cells, and A7r5 smooth muscle cells. The highest levels were detected in extracts from NG-108 neuroblastomas and A7r5 smooth muscle cells. No band was detected in extracts from COS-1 cells or Y1 adrenal tumor cells or when identical blots were probed with anti-cGKI serum (data not shown).

Localization of endogenous cGKI in A7r5 smooth muscle cells by indirect immunofluorescence revealed diffuse cytoplasmic staining (Fig. 6A), and treatment with 1 mM 8-Br-cGMP had no effect on this staining pattern (Fig. 6B). The cytoplasmic staining was specific for cGKI, since it was abolished by preabsorp-
tion of the anti-cGMP-PK CT antibody with a peptide coding for the carboxyl-terminal 18 amino acids of cGKI (Fig. 6C).

Generation of Full-length Constitutively Active cGKIB Mu-
nants—The difference in the ability of cGKs and cAKs to activate CRE-dependent gene transcription could be due to differ-
ences in their substrate specificities, their subcellular localizations, or both. To define the mechanism(s) by which cGKs and cAKs differentially regulate gene transcription, a full-length and a small sized constitutively active cGKIB mu-
tant were generated.
In efforts to produce a full-length constitutively active kinase, three different classes of cGKI point mutants were generated. For the first class of mutant, mutations were made in cGKI analogous to those that have been identified in the C subunit of cAK and result in constitutive activity of the C subunit even in the presence of excess R subunit (60–64). These residues, His119 and Trp350 of cGKIβ were mutated to glutamine and arginine, respectively, in accordance with the mutations found for the R subunit-insensitive C subunit mutants (60–64). A second class of constitutively active mutant was designed based on the observation that regulatory domain arginine residues in both cAK and cGKI may mimic substrate site arginines. These pseudosubstrate arginine residues have been shown to be important in the high affinity of several kinases’ regulatory domains for their catalytic domains (65). Specifically, proteolytic cleavage of cGKβ after its pseudosubstrate arginine (Arg75) generates a kinase with high constitutive activity (66). Thus, to generate the second class of constitutively active mutant, we mutated Arg75 to a neutral residue, alanine. Finally, for the third class of mutation, it was reported that cGKIβ undergoes autophosphorylation at two sites in its regulatory domain in the presence of cyclic nucleotides. Phosphorylation of the second site, Ser79, leads to a large increase in basal kinase activity (67). For this third class of constitutively active mutant, we mutated Ser79 to an acidic residue, aspartic acid, to mimic the effect of autophosphorylation. One advantage of the second and third approaches is that mutations in the regulatory domain are less likely to affect the substrate specificity or specific activity of the enzyme.

The effects of these mutations were tested by measuring the basal and cGMP-activated kinase activities in extracts made from HEK293 cells transiently transfected with the mutant expression vectors (Fig. 7). Extracts from HEK293 cells transfected with pCMVNeo or a mammalian expression vector encoding a catalytically inactive mutant (cGKIβK404R) showed no significant cGMP-dependent kinase activity (Fig. 7). Except for the cGKβH419Q mutant, which shows a 5-fold reduction in specific activity, the cGMP-dependent kinase activities (Fig. 7) and protein expression levels (data not shown) of all of the constitutively active mutants were similar to wild type cGKIβ. Both the cGKIβR75A and cGKIβS79D mutants exhibited high basal activity when compared with wild type cGKIβ. The increases in basal kinase activity of the cGKIβR75A and cGKIβS79D mutants were 12- and 14-fold, respectively. Specifically, mutation of Ser79 to an aspartic acid increased the basal kinase activity from 5 to 72% of the total cGMP-dependent kinase activity (Fig. 7). These results support the hypotheses that Arg75 is important in the high affinity of cGKIβ’s regulatory domain for its catalytic domain (65) and that autophosphorylation of Ser79 is capable of activating cGKIβ in the absence of cGMP (67). Of interest, the cGKIβH419Q and cGKIβW530R mutants showed only small increases in basal kinase activity (Fig. 7), suggesting that the amino acid residues in the cAK catalytic domain responsible for the tight interaction between cAK’s R subunit and C subunit are less important to inhibition of cGKI’s catalytic domain by its regulatory domain. The cGKIβS79D mutant showed the highest basal activity and was therefore employed in further studies of cGKI regulation of transcription.

**Generation of a Constitutively Active cGKI Deletion Mutant**—An additional mutant was sought to determine the effect of cGKI’s protein size on its ability to regulate transcription. Previous studies have shown that truncation of cGKI’s autoinhibitory regulatory domain results in a fully active, cGMP-independent catalytic domain that possesses a similar substrate specificity as the cGKI holoenzyme (68). Because of its small size (40 kDa), it was predicted that the catalytic domain of cGKI would be able to passively diffuse into the nucleus and directly phosphorylate Ser130 of CREB in a manner similar to the C subunit of cAK (29).

The entire amino-terminal regulatory domain of cGKIβ was deleted, and only the catalytic domain was expressed in HEK293 cells. To increase the stability of the cGKI catalytic domain, we appended the amino terminus of the C subunit of cAK to the cGKI catalytic domain. Previously, the first 23 amino acids of cAK’s C subunit were reported to stabilize PKC’s catalytic domain and allow for a high level of expression in mammalian cells (69). To assess its stability and the extent of its activation in transfected cells, the basal and cGMP-dependent kinase activities of this chimera were examined in extracts made from transiently transfected HEK293 cells (Fig. 8A). Wild type cGKIβ, the cGKIβS79D mutant, and the catalytic domain of cGKI were found to be expressed at equivalent levels by Western blot analysis (data not shown) and in vitro kinase assays (Fig. 8A). As expected, the truncated catalytic domain of
were transiently transfected with pCMV.Flag-VASP (20 ng).

Western blot analysis of Flag-tagged VASP phosphorylation. CV-1 cells were transiently transfected with either pCMV mcGKI S79D (S79D; a and b) or pCMV.FC/CD (CD; c). 24 h post-transfection, 8-Br-cGMP (1 mM) and IBMX (500 μM) were added (a; b) or omitted (a and c) for 1 h. Cells were then labeled with the anti-cGMP-PK CT antibody as the primary antibody and Cy3-Fab’2 goat anti-rabbit as the secondary antibody.

FIG. 8. In vitro and in vivo activity of constitutively active cGKIα mutants. A, in vitro kinase activity. HEK293 cells were transiently transfected with 30 ng of pCMV.neo (Neo), pCMV.mcGKIα (WT), pCMV.mcGKIαS79D (S79D), or pCMV.FC/CD (CD). 48 h after transfection, protein extracts were generated (see "Materials and Methods") and assayed for kinase activity in the presence (gray bar) or absence (black bar) of cGMP (50 μM) using the heptapeptide substrate H2Ride. Protein kinase inhibitor peptide (1 μM) was included in all assay tubes to inhibit endogenous cAK activity. Specific kinase activity is expressed as nmol/min/mg. B and C, in vivo kinase activity. B, Western blot analysis of Flag-tagged VASP phosphorylation. CV-1 cells were transiently transfected with pCMV.Flag-VASP (20 μg) and either no cGKIα expression vector (Neo; lane 1), 1 μg of pCMV.mcGKIα (WT; lane 2), 1 μg of pCMV.mcGKIαS79D (S79D; lane 3), or 1 μg of pCMV.FC/CD (CD; lane 4). 24 h after transfection, cells were serum starved for an additional 24 h. Extracts were generated and blotted as in Fig. 3. C, PhosphorImager quantitation of level of Flag-tagged VASP phosphorylation. Statistical analysis of experiment was as described for B. The percentage of VASP phosphorylation was calculated by dividing the quantity of the 50-kDa band by the sum of the 46-kDa band and the 50-kDa band.

cGKIα was found to be fully active in the absence of cyclic nucleotides (Fig. 8A).

In Vivo Activity of Constitutively Active cGKIα Mutants—To determine if the cGKIαS79D mutant and the catalytic domain of cGKI have increased basal kinase activity in vivo, Flag-tagged VASP was transfected alone or cotransfected with cGKIα, cGKIαS79D, or the catalytic domain. The level of VASP phosphorylation on Ser153 was low in serum-starved CV-1 cells expressing Flag-tagged VASP alone with 3% of the total VASP in the 50-kDa form (Fig. 8, B and C). Expression of the cGKIαS79D mutant converted 30% of VASP to the 50-kDa form, while the catalytic domain converted 70% of VASP to the 50-kDa form (Fig. 8, B and C). Wild type cGKIα stimulated VASP phosphorylation significantly less, with only 6% conversion of VASP to the 50-kDa form (Fig. 8, B and C). Thus, both the cGKIαS79D mutant and the catalytic domain of cGKI showed constitutive activity in vivo, with the catalytic domain being slightly more active toward VASP than the cGKIαS79D mutant.

Subcellular Localization of Constitutively Active cGKIα Mutants—In agreement with the earlier localization studies, which used 8-Br-cGMP to activate wild type cGKIα, the subcellular localization of the cGKIαS79D mutant was cytoplasmic in the absence or presence of 8-Br-cGMP (Fig. 9, A and B). Unlike full-length cGKIα, the Flag-tagged catalytic domain of cGKI was found both in the nucleus and in the cytoplasm (Fig. 9C). The catalytic domain was evenly distributed between the nucleus and the cytoplasm, consistent with the passive diffusion previously reported for the C subunit of cAK (29). The catalytic domain did not concentrate preferentially in the nucleus (Fig. 9C). As expected, the addition of 8-Br-cGMP to the medium had no discernible effect on its localization (data not shown). The catalytic domain of cGKI was detected both in the cytoplasm and in the nucleus with the M2 anti-Flag antibody (data not shown) and the Anti-cGMP-PK CT antibody (Fig. 9C), demonstrating that the anti-cGMP-PK CT antibody was capable of detecting cGKI in the nucleus. These data corroborate the cytoplasmic restriction of active full-length cGKIα and strongly suggest that cGKIα is restricted to the cytoplasm because of its large size.

Activation of CRE-dependent Gene Transcription in Cells Overexpressing Constitutively Active cGKIα Mutants—To correlate the localization of the various cGKIα mutants with their ability to increase CRE-dependent gene transcription, the abilities of wild type cGKIα, the cGKIαs79D mutant, and the catalytic domain of cGKI to regulate CRE-dependent gene expression were measured using the HCG-luciferase reporter. Expression of cGKIα or the cGKIαS79D mutant did not stimulate CRE-dependent transcription in CV-1 cells (Fig. 10A) under conditions where a substantial increase in the phosphorylation level of the cytoplasmic substrate VASP was observed (Fig. 8, B and C). In contrast, the catalytic domain, which could enter the nucleus, activated transcription from the HCG-luciferase reporter construct 4-fold (Fig. 10A). Unlike full-length cGKIα and the cGKIαS79D mutant, the catalytic domain was effective at both elevating the phosphorylation state of VASP (Fig. 8, B and C) and inducing CRE-dependent gene transcrip-
tion in CV-1 cells (Fig. 10A), suggesting that the ability of the catalytic domain to passively enter the nucleus allows it to be a more efficient activator of CRE-dependent gene transcription.

Regulation of HCG-luciferase activity was also examined in HEK293 cells, because the reporter assay was shown to be more sensitive in this cell line. When assayed for induction of luciferase gene expression upon transient transfection into HEK293 cells, wild type cGKI showed essentially no stimulation of luciferase activity in the absence of nucleotide treatment (Fig. 10B). In contrast, transfection of the cGKI S79D mutant elevated the basal luciferase activity 66-fold (Fig. 10B). This induction is consistent with the induction observed in HEK293 cells expressing wild type cGKI and treated with 8-Br-cGMP (13-fold). In HEK293 cells, cGKI S79D was significantly less potent as a regulator of gene transcription than the catalytic domain of cGKI (Fig. 10B). Whereas the cGKI S79D mutant stimulated CRE-dependent gene transcription 66-fold, the catalytic domain of cGKI induced luciferase activity 1071-fold (Fig. 10B). The C subunit of cAK and the catalytic domain of cGKI activated CRE-dependent gene transcription to a similar extent (Fig. 10C), with the 3-fold difference in activation being primarily due to the low stability of the cGKI catalytic domain compared with the C subunit of cAK (data not shown). As observed in the CV-1 cells, full-length cGKIβ was cytoplasmic in the presence or absence of 8-Br-cGMP in HEK293 cells (data not shown). Therefore, both the CV-1 and HEK293 cell data strongly support the conclusion that active full-length cGKIβ is a relatively weak activator of CRE-dependent gene transcription primarily because it is restricted to the cytoplasm.

Characterization of cGKIβ ATP-binding Domain Mutant—Unlike CV-1 cells, transfection of full-length cGKIβ into HEK293 cells confers readily measurable CRE-dependent transcriptional responses to 8-Br-cGMP. When cotransfected with HCG-luciferase into HEK293 cells, wild type cGKIβ can mediate a 13-fold increase in luciferase activity in response to 8-Br-cGMP (Fig. 2B). The magnitude of induction is dependent on the amount of cGKIβ expression vector transfected (data not shown) and the 8-Br-cGMP concentration in the media (Fig. 11). Although immunofluorescence microscopy localized a majority of the cGKI immunoreactivity to the cytoplasm in cGKIβ-transfected HEK293 cells, it was possible a small fraction of the total cGKIβ was present in the nucleus and responsible for the small increase in transcription (data not shown). Typically, proteins larger than 40–65 kDa require a basic NLS to be actively transported to the nucleus. Inspection of the cGKI protein sequence by other investigators for a potential NLS revealed a single cluster of basic amino acids in the ATP-binding domain (KILKKKRHI; residues 404–411) (33, 34).

To determine whether the induction of CRE-dependent gene transcription by full-length cGKIβ in HEK293 cells was dependent on the putative NLS sequence, this sequence was altered to the corresponding amino acids from the C subunit of cAK. The amino acids encoding this basic sequence are not highly conserved in the C subunit of cAK (KILDKQKV; resi-
Cytoplasmic Localization of Activated cGKI

8401

FIG. 11. 8-Br-cGMP activates CRE-dependent gene transcription in a concentration-dependent manner in HEK293 cells overexpressing cGKβ. A transcriptional assay measuring CRE-dependent gene transcription is shown. HEK293 cells were transiently transfected with (○) or without (●) 4 μg of pCMV.mcGKIβ. All plates received HCG-luciferase (0.5 μg), pRSV.gal (4 μg), and pCMV.PKI (2 μg). 24 h post-transfection, cells were treated for 20 h in the absence of serum with varying concentrations of 8-Br-cGMP and IBMX (500 μM), harvested, and assayed for luciferase and β-galactosidase activities. Luciferase activity was corrected for transfection efficiency by dividing by β-galactosidase activity (RLU/gal). Luciferase activity is expressed as the percentage of maximal activity attained. The error bars depict the S.D.

Figures 72–79 (39). Using site-directed mutagenesis, we changed two of the basic amino acids in cGKIβ’s putative NLS to the equivalent residues in the C subunit of cAK (Lys407 to Asp and Arg409 to Gln). These specific amino acid substitutions were chosen because the C subunit of cAK is not actively transported to the nucleus (29), and these mutations were unlikely to perturb the structure of the kinase since they are found in the C subunit of cAK.

To determine the effect of these mutations on the ability of cGKIβ to activate CRE-dependent gene transcription, we co-transfected wild type cGKIβ or the cGKIβK407D/R409Q mutant along with a CRE-dependent reporter construct into HEK293 cells. While cGKIβ increased luciferase activity 10-fold following 8-Br-cGMP treatment, the activated cGKIβK407D/R409Q mutant only induced a 4-fold increase in gene transcription (Fig. 12A). Therefore, substitution of two basic amino acids in the putative NLS sequence reduced but did not eliminate cGKIβ’s ability to activate CRE-dependent gene transcription.

Because this basic sequence is located within the ATP-binding region of the catalytic domain, it was possible that the mutations negatively affected cGKIβ’s catalytic activity. To determine the relative effect of the two amino acid substitutions on the catalytic activity of the cGKIβK407D/R409Q mutant, the basal and cGMP-dependent kinase activities of this mutant were compared with those of wild type cGKIβ in extracts made from the transiently transfected HEK293 cells. Although expression levels of wild type cGKIβ and the cGKIβK407D/R409Q mutant were similar as determined by Western blot analysis (data not shown), cGMP-dependent kinase activity was 60% less in the extract from cells overexpressing the cGKIβK407D/R409Q mutant as compared with the extract from the cells overexpressing wild type cGKIβ (Fig. 12B).

The cGKIβK407D/R409Q mutant showed a reduction in the phosphorylation of substrates in vivo, as determined by VASP transfection experiments (Fig. 12, C and D). Western blot analysis of extracts from cells transfected with VASP and PKI alone detected a single 46-kDa band (Fig. 12C). When identically transfected cells were treated with 8-Br-cGMP (1 mM) for 1 h, both a 46-kDa band and a 50-kDa band were detected (Fig. 12C). Formation of the 50-kDa phosphorylated VASP band with 8-Br-cGMP treatment was due to phosphorylation of Ser153 by endogenous cGKI, since its formation was not blocked by coexpression of PKI, although PKI did completely block 8-Br-cAMP induction of the 50-kDa band (data not shown). While 94% of Flag-tagged VASP was converted to the 50-kDa form in cells expressing wild type cGKIβ treated with 8-Br-cGMP, only 56% was converted in similarly treated cells expressing cGKIβK407D/R409Q (Fig. 12, C and D). Hence, the cGKIβK407D/R409Q mutant has significantly reduced basal and cGMP-stimulated kinase activity in vivo. These data suggest that mutation of basic residues in the putative NLS decreases the ability of cGKIβ to activate gene transcription, not because it prevents localization of cGKIβ in the nucleus but because it decreases the catalytic activity of the kinase. Thus, mutagenesis of the putative NLS of cGKIβ did not specifically alter either cGKIβ’s localization or its ability to regulate transcription.

Discussion

cAK and cGK regulation of CRE-dependent gene transcription was investigated in this study to determine if these two kinases differentially regulate this process. Because of the large size of active cGKs, we sought to determine whether, like the C subunits of cAKs, cGKs would translocate to the nucleus following activation by cyclic nucleotides. Analyses of the subcellular localization of endogenous cGKI in A7r5 cells as well as transfected cGKIβ and cGKI indicate that the cGKs localize to the cytoplasm regardless of their activation state. The inability of cGKIβ to translocate to the nucleus and directly phosphorylate CREB renders it a relatively weak activator of CRE-dependent gene transcription and suggests one mechanism by which cAK and cGK differentially regulate gene transcription. These findings strongly imply that the restricted cytoplasmic localization of the cGKs is an important mechanism for selective regulation of nuclear functions by the two families of cyclic nucleotide-dependent protein kinases.

In this study, constitutively active cGKI mutants were generated by two approaches to determine if cAK’s greater ability to activate CRE-dependent gene transcription was due to differences in cGK’s and cAK’s substrate specificities or differences in their subcellular localizations. First, mutation of an autophosphorylation site serine to an aspartic acid to mimic phosphorylation resulted in a constitutively active cGKI without significantly changing the size of the protein. Autophosphorylation of cGKIβ at Ser79 has been shown to activate the enzyme and to produce an elongation of the enzyme, suggesting that both cGMP binding and autophosphorylation activate the enzyme by a similar mechanisms (70). When this activated form of full-length cGKIβ, cGKIβS79D, was expressed and its ability to transactivate a CRE-responsive promoter was measured, only a minimal increase in luciferase expression was found when compared with catalytic domain of cGKI. Like activated wild type cGKIβ, the cGKIβS79D mutant was also restricted to the cytoplasm, suggesting a mechanism for its poor transactivating ability.

Each subunit of a cGK dimer consists of an amino-terminal regulatory domain and a carboxyl-terminal catalytic domain. Deletion of cGKI’s regulatory domain generates a monomeric, constitutively active kinase. In contrast to the cGKIβS79D mutant, the smaller, constitutively active catalytic domain of cGKI was found in both the cytoplasm and the nucleus. The catalytic domain strongly activated CRE-dependent gene transcription, demonstrating that the phosphotransferase activity
FIG. 12. The cGKIβ ATP-binding domain mutant is a weak inducer of both CRE-dependent gene transcription and Flag-tagged VASP phosphorylation. A, transcriptional assay measuring CRE-dependent gene transcription. HEK293 cells were transiently transfected with 20 μg of pCMV.Neo (Neo), pCMV.mcGKIβ (WT), or pCMV.mcGKIβ-K407R/R409Q (MUT). All plates received HCG-luciferase (0.5 μg), pRSV.βgal (5 μg), and pCMV.PKI (2 μg). 24 h post-transfection, cells were treated for 20 h in the absence of serum with (gray bar) or without (black bar) 8-Br-cGMP (1 mM) and IBMX (500 μM). B, in vitro kinase assay. Protein extracts from untreated cells (Fig. 12A) were assayed for kinase activity in the presence (gray bar) or absence (black bar) of cGMP (50 μM) using the heptapeptide substrate H2Btide (110 μM). Protein kinase inhibitor peptide (1 μM) was included in all assay tubes to inhibit endogenous cAK activity. C, Western blot analysis of Flag-tagged VASP phosphorylation. CV1 cells were transiently transfected with pCMV.Flag-VASP (20 μg), pCMV.Flag-PKI (2 μg) and no cGKIβ expression vector (Neo, lanes 1 and 2), 1 μg of pCMV.mcGKIβ (WT, lanes 3 and 4), or 1 μg of pCMV.cGKIβK407D/R409Q (Mut, lanes 5 and 6). 24 h after transfection, cells were serum-starved for an additional 24 h and then treated for 1 h with (+) or without (−) 8-Br-cGMP (1 mM). Extracts were generated and blotted as in Fig. 3. D, PhosphorImager quantitation of level of Flag-tagged VASP phosphorylation. Statistical analysis of experiment described for C. Black bar, without 8-Br-cGMP; gray bar, plus 8-Br-cGMP.
of cGKI is capable of recognizing members of the nuclear CREB-like transcription factor family. Likewise, the catalytic domain of cGKI activated gene transcription to a similar extent as the free C subunit of cAK, implying that cGK’s restricted cytoplasmic localization and not its unique substrate specificity is the major reason for cGKI’s weak transactivating activity. These data are entirely consistent with the conclusion that cGKI is a weak activator of CRE-dependent gene transcription because the large size of the active kinase prevents nuclear translocation.

During the course of these experiments, a report appeared describing the identification of an NLS in the ATP-binding domain of human cGKI that is only functional upon activation of the kinase by cGMP (34). The identified NLS (KILKKKH) does not closely resemble the well characterized monopartite nuclear targeting sequence of SV40 large T antigen (PKKKRKV) or the bipartite motif of nucleoplasmin (71). In an attempt to compare these findings with our own, the murine cGKI expression vector was transiently transfected into baby hamster kidney cells. Localization of murine cGKI in baby hamster kidney cells by indirect immunofluorescence revealed diffuse cytoplasmic staining either in the presence or absence of 8-Br-cGMP. Similar results were obtained in HEK293 cells, COS-1 cells, and CV-1 cells, suggesting that the restricted cytoplasmic localization of cGKI was common in mammalian cell lines. The discrepancy between our results and those published previously is not due to the overexpression of cGKI, since similar results were also obtained with endogenous cGKI in A7r5 cells. Finally, mutagenesis of the putative NLS reported by Gudi et al. (34) in our murine cGKI shows no specific effect on its ability to transactivate a CRE-dependent reporter construct (Fig. 12). Instead, the decreased ability of this ATP-binding site mutant to transactivate a CRE-dependent reporter construct correlated with its decreased catalytic activity in vitro and in vivo.

In this study, we demonstrate that cGKIβ is a weak inducer of CRE-dependent gene transcription. When HEK293 cells were transiently transfected with cGKIβ, 8-Br-cGMP treatment elevated CRE-dependent gene transcription 13-fold (Fig. 2B). 8-Br-cGMP treatment did not significantly activate CRE-dependent transcription in the absence of transfected cGKIβ (Fig. 2B). Transcriptional activation was also mediated by the cGKIβS79D constitutively active mutant alone, implying that cGKIβ kinase activity by itself was capable of elevating CRE-dependent transcription and that the 8-Br-cGMP was not affecting other signaling systems. Transient coexpression of PKI did not block cGKIβ-dependent increases in gene transcription, suggesting that this transactivation is independent of cAK activity. Experiments using a GAL4-CREB fusion construct suggest that transcriptional regulation by cGKI is at least partially mediated through CREB, since the cGKIβS79D mutant is capable of transactivating pGAL4-luc when coexpressed with the GAL4-CREB fusion protein (data not shown). These findings suggest that the pathway(s) by which cGKIβ activates CRE-dependent gene transcription may involve specific cytoplasmic cGK substrates that can signal through CREB.

The exact mechanism(s) by which cGKs weakly regulate CRE-containing promoters is unknown. It is possible that cGK activation results in the activation of other protein kinases that are able to translocate to the nucleus and phosphorylate CREB. A number of growth factor-stimulated kinases including the RSKs (72) and MAPKAP kinase 2 (73) as well CaM kinases (74) have recently been shown to translocate to the nucleus and phosphorylate CREB. Alternatively, although it is difficult to determine, it is possible that a small fraction of CREB is cytoplasmic and in equilibrium with the majority of nuclear CREB bound to DNA. Finally, novel transcription factors that can be phosphorylated in the cytoplasm and translocate to the nucleus may be the substrate of cGKs. In this regard, it is interesting to note that C/EBPβ can be phosphorylated in the cytoplasm of PC12 cells by cAK, and following phosphorylation it translocates to the nucleus and increases gene transcription (75).

Since the cGKs and the cAKs are highly homologous protein kinase families that possess similar substrate specificities, many questions remain regarding the unique roles these kinases may play in cells that coexpress cAK and cGK isoforms. The results of this study suggest that the physiological role(s) of these cyclic nucleotide-dependent protein kinases are more distinct than currently appreciated and that, in comparison with cAKs, cGKs play only a minor role in the regulation of CRE-dependent transcription. The findings as well as the agents generated within this study should be useful in characterization of the specific in vivo role(s) of the cyclic nucleotide-dependent protein kinase families.

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