MOLECULAR AND CELLULAR BIOLOGY, Sept. 1997, p. 5244–5254 Vol. 17, No. 9
0270-7306/97/$04.00+0

Regulation of Gene Expression by Cyclic GMP-Dependent Protein Kinase Requires Nuclear Translocation of the Kinase: Identification of a Nuclear Localization Signal

TANIMA GUDI,[1] SUZANNE M. LOHMANN,[2] AND RENATE B. PILZ[1]*

University of California, San Diego, La Jolla, California 92093-0652,[1] and Institut für Klinische Biochemie und Pathobiochemie, Medizinische Universitätsklinik, 97080 Würzburg, Germany[2]

Received 26 March 1997/Returned for modification 28 April 1997/Accepted 4 June 1997

We recently demonstrated that cyclic GMP (cGMP)-dependent protein kinase (G-kinase) activates the human *fos* promoter in a strictly cGMP-dependent manner (T. Gudi et al., J. Biol. Chem. 271:4597–4600, 1996). Here, we demonstrate that G-kinase translocates to the nucleus by an active transport mechanism which requires a nuclear localization signal (NLS) and is regulated by cGMP. Immunofluorescent staining of G-kinase was predominantly cytoplasmic in untreated cells, but intense nuclear staining appeared in 8-bromo (Br)-cGMP-treated cells. We identified a putative NLS in the G-kinase ATP binding domain which resembles the NLS of the interleukin-1α precursor. Fusion of the G-kinase NLS to the N terminus of β-galactosidase produced a chimeric protein which localized to the nucleus. Mutation of a single amino acid residue ($K^{407}\rightarrow E$) within the G-kinase NLS produced an enzyme with normal cGMP-dependent activity in vitro which did not translocate to the nucleus and did not transactivate the *fos* promoter in the presence of 8-Br-cGMP in vivo. In contrast, N-terminally truncated versions of G-kinase with constitutive, cGMP-independent activity in vitro localized to the nucleus and transactivated the *fos* promoter in the absence of 8-Br-cGMP. These results indicate that nuclear localization of G-kinase is required for transcriptional activation of the *fos* promoter and suggest that a conformational change of the kinase, induced by cGMP binding or by removal of the N-terminal autoinhibitory domain, functionally activates an otherwise cryptic NLS.

The NO/cyclic GMP (cGMP) signal transduction pathway is present in many mammalian cells and involved in the regulation of important physiological functions such as neurotransmission, cell differentiation and proliferation, changes in vascular smooth muscle tone, endothelial cell permeability, and platelet aggregation (13, 36, 37, 39, 40, 44, 45). Whereas the cyclic AMP (cAMP) signal transduction pathway is well known to regulate gene transcription, regulation of gene expression by the NO/cGMP signal transduction pathway has been demonstrated only recently in different cell types (2, 5, 16, 18, 19, 24, 38, 41, 47). In rat embryo fibroblasts and thyroid epithelial cells, we showed that NO-releasing agents and membrane-permeable cGMP analogs increase c-*fos* and *junB* mRNA expression, AP-1 DNA binding, and the transcriptional activity of promoters containing phorbol ester response elements (41). We subsequently demonstrated that transfection of G-kinase Iβ into G-kinase-deficient baby hamster kidney (BHK) cells causes induction of endogenous c-*fos* mRNA and activation of a cotransfected human *fos* promoter construct in a strictly cGMP-dependent manner (18, 18a). The effect of G-kinase is mediated by several sequence elements in the *fos* promoter, most notably the cAMP response element, the AP-1 binding site, and the serum response element with adjacent C/EBP-β binding site (18). The magnitude of G-kinase transactivation of the *fos* promoter was similar to that of cAMP-dependent protein kinase (A-kinase), but there were significant differences between G-kinase and A-kinase transactivation of single enhancer elements (18). G-kinase transduced signals to the nucleus independently of A-kinase and Ca^{2+}/calmodulin-dependent protein kinase, although G-kinase appeared to utilize some of the same transcription factors as those kinases (18).

Regulation of gene expression by A-kinase requires dissociation of the A-kinase tetramer, translocation of the free catalytic (C) subunits of A-kinase into the nucleus, and direct phosphorylation of transcription factors like CREB (26). Nuclear entry of the 38-kDa C subunit of A-kinase is thought to occur by passive diffusion but appears to be the rate-limiting step in coupling hormonal stimulation of cAMP synthesis to changes in transcription of cAMP-responsive genes (20). In contrast to A-kinase, G-kinase I is a homodimer with a total molecular mass of about 150 kDa which does not dissociate upon binding of cGMP; the regulatory and catalytic domains of G-kinase are located in tandem on each polypeptide. When cGMP binds to the regulatory domain, the kinase undergoes a major conformational change which results in activation of its catalytic function (13, 22, 28). Since nuclear translocation of proteins of >45 to 60 kDa requires a nuclear localization signal (NLS) for docking at the nuclear pore complex (17), we examined G-kinase I for the presence of such a signal sequence. Although there is no single established NLS consensus sequence, most NLS sequences consist of short stretches of positively charged amino acids (10), and we identified a cluster of positively charged amino acids in the ATP binding domain of G-kinase which is similar to the previously described NLS of the interleukin-1α precursor (32, 51). In the present work, we show that G-kinase translocates to the nucleus and that nuclear localization of G-kinase is regulated by cGMP, requires the putative NLS, and is necessary for transactivation of a target gene.

MATERIALS AND METHODS

Cell culture and transient transfections. BHK cells were maintained in Dulbecco's minimal essential medium (DMEM) as described previously (18). Early-passage A10 rat embryonal smooth muscle cells were provided by W. Dillmann (University of California, San Diego) and were cultured in DMEM supplemented with 20% fetal bovine serum (25). For subcellular localization studies,

* Corresponding author. Phone: (619) 534-8805. Fax: (619) 534-1421. E-mail: rpilz@ucsd.edu.

BHK cells were transfected with 6 μg of Lipofectamine (Life Technologies) and 500 ng of the indicated G-kinase expression vector or the indicated β-galactosidase construct; for transactivation studies, cells were cotransfected with 50 ng of pFos-CAT, 50 ng of pRSV-luciferase (internal control), and 500 ng of the indicated G-kinase expression vector for 36 h as described previously (18). For maximal activation of G-kinase, some cultures were treated with 1 mM 8-bromo (Br)-cGMP for the indicated times (8 h for transactivation assays). Chloramphenicol acetyltransferase (CAT) and luciferase activities were measured as described previously (18). Histochemical staining for β-galactosidase activity was performed with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (41).

Immunofluorescent staining of G-kinase. Cells were fixed for 10 min in 1% paraformaldehyde–75 mM cacodylate buffer–0.72% sucrose (pH 7.4) followed by 10 min in 3.7% formaldehyde–phosphate-buffered saline at room temperature, 4 min in methanol (−20°C), and 1 min in acetone (−20°C) as described by Pryzwansky et al. (42). After blocking with 5% bovine serum albumin, cells were incubated with a previously characterized G-kinase I-specific antibody (diluted 1:500 for BHK and 1:200 for A10 cells) (33) followed by fluorescein-5-isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Cappel). In some cases, nuclei were counterstained with propidium iodide (1 μg/ml). Neither incubation of mock-transfected BHK cells with G-kinase-specific antibody nor incubation of G-kinase-expressing BHK and A10 cells with preimmune rabbit serum produced any fluorescent signal. Cells were visualized with a Zeiss fluorescence microscope or a Bio-Rad MRC-1024 laser scanning confocal system equipped with a krypton-argon laser.

To determine the temperature and energy dependence of nuclear translocation, cells were transferred to glucose-deficient DMEM supplemented with either 6 mM glucose or 6 mM deoxyglucose plus 10 mM NaN_3; cells were cultured in this medium at either 30 or 4°C (glucose-supplemented only) for 2 h prior to addition 1 mM 8-Br-cGMP. After an additional 2-h incubation period, cells were fixed for immunofluorescence studies; in replicate cultures, cells were extracted to determine the intracellular ATP concentration, using a luciferase-based assay (30).

Plasmid constructs. Wild-type G-kinase Iβ cDNA (43) and all other G-kinase constructs were expressed from the cytomegalovirus early promoter (34). Site-directed mutagenesis was performed with wild-type G-kinase cDNA template in pBluescript KS (Stratagene), using the principle of unique-site elimination (9), or with the template in pAlter, using a selection primer which restores antibiotic resistance (Promega Altered Sites kit). To introduce the desired mutation, the following primers were used: 5′-AAG ATT CTC GAG AAA CGT CAC ATT GTG GAC-3′ (K^{407}→E, with generation of an *Xho*I site); 5′-AAG ATT CTC **A**AG AAA CGT CAC ATT GTG GAC-3′ (E^{407}→K, back mutation); 5′-AGT GAA GAA TCC **G**AA ACG TT**C** GC**G** ATG AAG ATT C-3′ (K^{399}→E, with generation of an *Nru*I site); 5′-GTA GA**G** CTC GTC CAG TTG **G**AA AGT GAA G-3′ (K^{394}→E, with generation of an *Xho*I site); 5′-ATG AAG ATT CTC **GC**G AAA CGT CAC ATT GTG GAC-3′ (K^{407}→A, with generation of an *Nru*I site); 5′-CGA ATG AAG ATT CTC **GC**G AAA **G**CT CAC ATT GTG GAC AC-3′ (K^{407}→A/R^{409}→A, with generation of an *Nru*I site); and 5′-CTG GTT G**C**T TTT GGC TT**C** GC**G** AAG AAA ATA GG-3′ (D^{516}→A, with generation of an *Nru*I site). In each case, the mutation was confirmed by DNA sequence analysis.

For the NLS–β-galactosidase fusion construct, the following primer was used to introduce an *Xba*I site followed by a translation initiation consensus sequence and the sequence encoding the putative NLS of G-kinase fused in frame with amino acid 8 of β-galactosidase: 5′-CTCTAGACCACC ATG GCT AAG ATT CTC AAG AAA CGT CAC ATT CCC GTC GTT TTA CAA CGT CGT-3′ (the G-kinase sequence is underlined). This primer was used together with the antisense primer 5′-CGCATCGTAACCGTGCATCTG-3′ in a PCR with the template pMC1871 (Pharmacia) containing the coding sequence of β-galactosidase. The resulting PCR fragment was sequenced and spliced into the vector pCMV-βgal, using *Xba*I and *Bsu*36I to replace the 5′ end of β-galactosidase.

To generate an expression vector for the catalytic domain of G-kinase I, the PCR primer 5′-AAGCGGCCGCC ATG GCA TAT GAA GAT GCA GAA GCT AAA GC-3′ was used to introduce a *Not*I site and a translation initiation consensus sequence upstream of the codon for Ala^{349} of G-kinase Iβ. For N-terminal truncation of the kinase at Val^{93}, the PCR primer 5′-CACTCTAGA CCACC ATG GTG ACC CTG CCC TTC TAC CCC-3′ was used to introduce an *Xba*I site and a translation initiation consensus sequence upstream of the codon for Val^{93} of G-kinase Iβ. In both cases, the antisense primer was 5′-CT CGGTATGCTTGATGAGTCCTG-3′. The PCR products were sequenced and spliced into the wild-type G-kinase Iβ expression vector, using *Not*I or *Xba*I and *Nco*I, respectively, to replace the 5′-end of the coding sequence with the new sequence.

G-kinase activity assay. G-kinase activity was measured in transfected BHK cells by using the synthetic peptide Kemptide and the specific A-kinase inhibitor PKI as previously described (18) except that the ATP concentration in the assay was 300 μM. The apparent K_a for 8-Br-cGMP and the apparent K_m for ATP were measured as described previously (4, 34).

Western blots. Whole-cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes as described previously (18). Western blots were incubated with G-kinase-specific antibody (1:2,000), and bound antibody was detected by enhanced chemiluminescence using a horseradish peroxidase-coupled secondary antibody (18).

RESULTS

G-kinase translocates to the nucleus in response to activation by 8-Br-cGMP. The subcellular localization of G-kinase I in neutrophils, macrophages, and primary aortic smooth muscle cells appears to be predominantly cytoplasmic; however, association of some G-kinase with cytoskeletal and nuclear structures has been observed (8, 42, 52). These observations suggest that G-kinase I may translocate to the nucleus, but the mechanism and regulation of this process have not been investigated. In this study, we examined the subcellular localization of G-kinase Iβ in BHK cells which were transfected with a G-kinase Iβ expression vector; the transfected cells express G-kinase activity at a level that is within the physiological range of G-kinase activities found in smooth muscle, neuronal cells, and platelets (18). We used a G-kinase I-specific antibody which showed no cross-reactivity with other cellular proteins, since mock-transfected BHK cells demonstrated no fluorescent signal. We found diffuse cytoplasmic localization of the kinase in logarithmically growing or serum-starved, G_0-arrested BHK cells; however, within 30 min of exposure to 1 mM 8-Br-cGMP, prominent nuclear staining of G-kinase appeared and a marked change in cell morphology from flat to a more rounded shape was observed (Fig. 1A; results are shown for transiently transfected BHK cells, but similar results were obtained with stably transfected cells). The 8-Br-cGMP-induced change in cell shape did not occur in G-kinase-deficient BHK cells treated with 8-Br-cGMP and was therefore interpreted to be a direct result of G-kinase activation; it was reminiscent of the changes in cell morphology induced by membrane-permeable cAMP analogs in different cell types (21, 27). Immunostaining of G-kinase I at various times after exposure to 8-Br-cGMP showed prominent perinuclear staining at 15 min; nuclear staining was maximal at 30 min to 1 h and persisted for more than 4 h in the presence of 8-Br-cGMP (Fig. 1A). Nuclear staining of G-kinase in 8-Br-cGMP-treated BHK cells was confirmed by confocal laser scanning microscopy (Fig. 2).

In cells treated with 8-Br-cGMP for 2 h, washed, and returned to stimulant-free culture media, intense nuclear G-kinase staining was still observed up to 8 h later (data not shown). The presence of nuclear G-kinase many hours after removal of exogenous 8-Br-cGMP may be due to lack of nuclear export and a long half-life of the nuclear kinase, or it may be because 8-Br-cGMP is poorly metabolized by phosphodiesterases and active G-kinase is continuously transported into the nucleus (15, 50).

To examine whether nuclear translocation of G-kinase was a temperature- and energy-dependent process, cells were incubated at 4°C or ATP-depleted prior to addition of 8-Br-cGMP. In cells at 4°C and cells in which the intracellular ATP concentration was decreased to 20% of control levels, 8-Br-cGMP failed to induce nuclear translocation of the kinase (Fig. 1B).

To study the effect of 8-Br-cGMP on the subcellular localization of G-kinase in cells expressing endogenous G-kinase activity, we examined A10 rat embryonal smooth muscle cells (25). In 8-Br-cGMP-treated A10 cells, the kinetics of nuclear translocation of endogenous G-kinase were similar to those observed for transfected G-kinase in BHK cells (Fig. 3).

Characterization of an NLS in G-kinase I. Nuclear translocation of proteins of >45 to 60 kDa through the nuclear pore complex is a highly selective, energy- and temperature-dependent process which involves recognition of NLSs in the translocated protein by the cytosolic transport factors importin-α

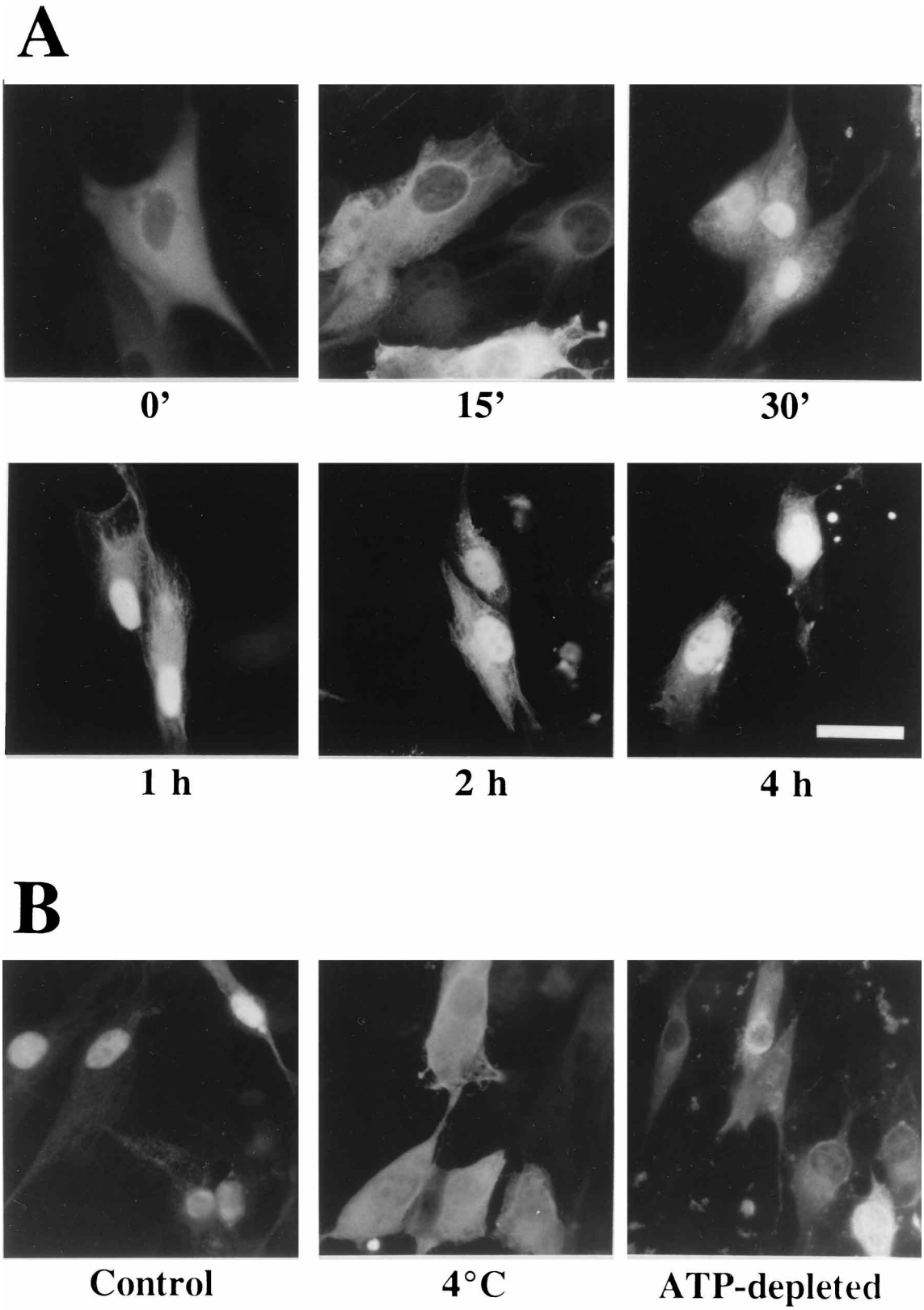

FIG. 1. Transfected G-kinase activated by 8-Br-cGMP translocates to the nucleus of BHK cells: time course and temperature and energy dependence. (A) BHK cells were transiently transfected with wild-type G-kinase vector as described in Materials and Methods. At 36 h after transfection, cells were treated with 1 mM 8-Br-cGMP for the indicated time prior to fixation and staining with a G-kinase-specific antibody and a fluorescently labeled secondary antibody. There was no fluorescent signal in mock-transfected BHK cells (not shown). (B) Cells were transfected as described above, but 36 h after transfection they were either incubated as described above (control), transferred to 4°C, or subjected to ATP depletion as described in Materials and Methods prior to the addition of 1 mM 8-Br-cGMP for 2 h. Photographs were taken with a 40× objective lens (1.4 numerical aperture); the bar represents 20 μm.

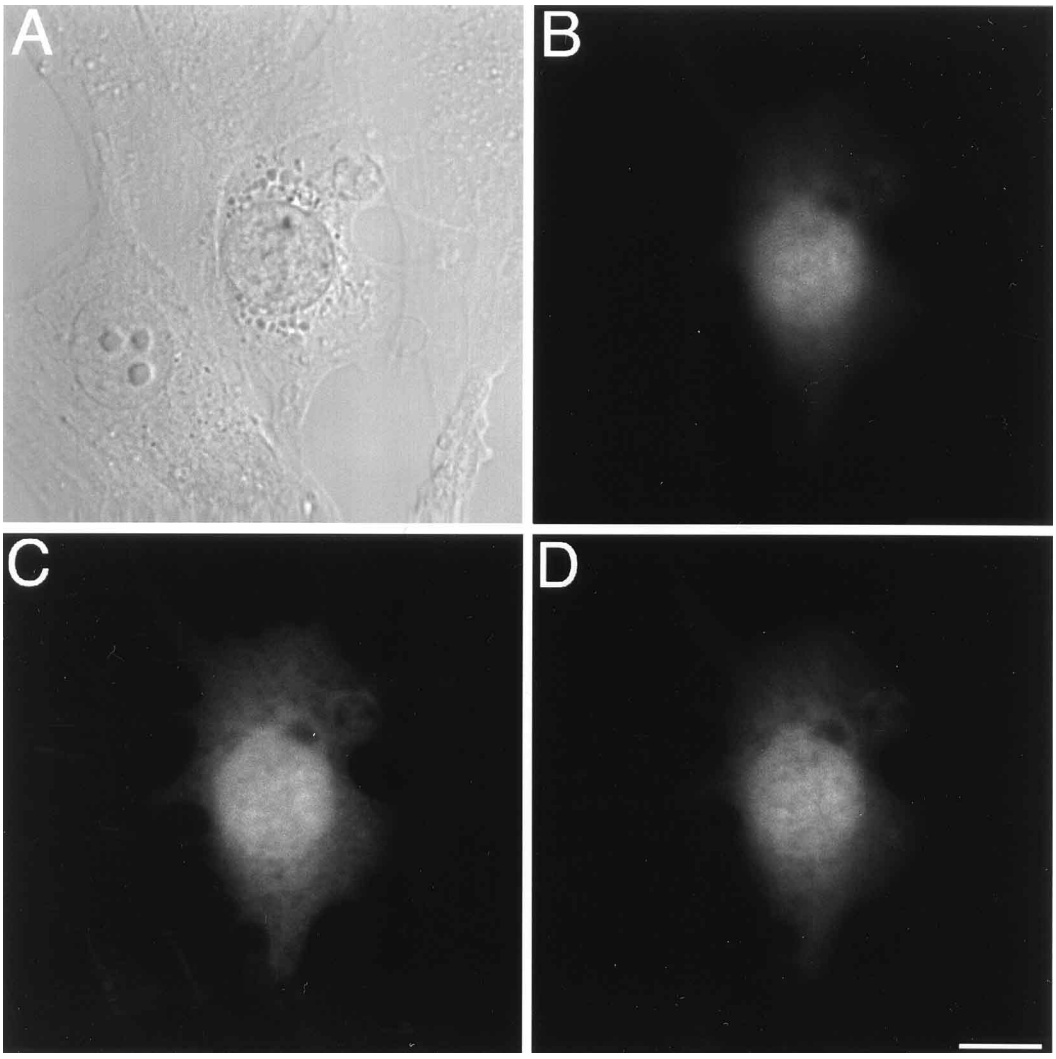

FIG. 2. Laser scanning confocal microscopy. BHK cells were transfected with wild-type G-kinase expression vector, treated with 8-Br-cGMP for 1 h, and stained as described for Fig. 1; cells were optically sectioned at 0.5-μm intervals, using a Bio-Rad MRC-1024 laser scanning confocal microscope. (A) Phase-contrast photograph; (B to D) immunofluorescence photographs of three consecutive sections through the nucleus. Photographs were taken with a 40× objective lens (1.3 numerical aperture); the bar represents 8 μm.

and -β (17). Examination of G-kinase I for the presence of a potential NLS revealed a stretch of eight amino acids in the ATP binding domain which closely resembles the NLS of the interleukin-1α precursor with five of eight amino acids identical and the other three conservatively substituted (references 32 and 51 and Fig. 4).

To determine whether the putative NLS of G-kinase is required for nuclear localization of the kinase, we performed site-directed mutagenesis to alter several of the basic amino acids (the variant G-kinase constructs are summarized in Fig. 5). We first substituted Glu for Lys^{407} of G-kinase Iβ ($K^{407}\rightarrow$ E), a mutation analogous to the one which renders the interleukin-1α precursor exclusively cytoplasmic (51). We expected that this mutation would not alter the catalytic activity of G-kinase because the counterpart of Lys^{407} in G-kinase Iβ is an acidic amino acid in the C-subunit of A-kinase (Asp^{75}) (22, 46). When the mutant $K^{407}\rightarrow$E G-kinase was expressed in BHK cells, we found that the kinase localized to the cytoplasm in the absence or presence of 8-Br-cGMP (Fig. 5 and 6). To rule out that the lack of nuclear translocation could be due to other, unwanted mutations created during site-directed mutagenesis, we showed that the phenotype of the $K^{407}\rightarrow$E G-kinase mutant could be reversed by mutating Glu^{407} back to Lys. The back-mutated $E^{407}\rightarrow$K G-kinase underwent nuclear translocation in response to 8-Br-cGMP exactly like the wild-type enzyme (data not shown). To test the specificity of the putative NLS $K^{407}\rightarrow$E mutation, we performed Lys→Glu substitutions just upstream of the putative NLS ($K^{399}\rightarrow$E and $K^{394}\rightarrow$E). These control mutants performed like wild-type G-kinase with respect to cGMP-dependent nuclear translocation (Fig. 6 shows the $K^{399}\rightarrow$E G-kinase only). Interestingly, when we mutated Lys^{407} to Ala, the $K^{407}\rightarrow$A mutant enzyme was able to translocate to the nucleus in response to 8-Br-cGMP, suggesting that the change of Lys^{407} to a neutral amino acid was not sufficient to disrupt the NLS (Fig. 6). However, when we replaced a second basic amino acids in the NLS with Ala, the $K^{407}\rightarrow A/R^{409}\rightarrow$A double-mutant enzyme remained predominantly cytoplasmic when cells were exposed to 8-Br-cGMP (Fig. 6).

Amino acids 404 to 411 of G-kinase Iβ can direct β-galactosidase to the nucleus. To determine whether the putative

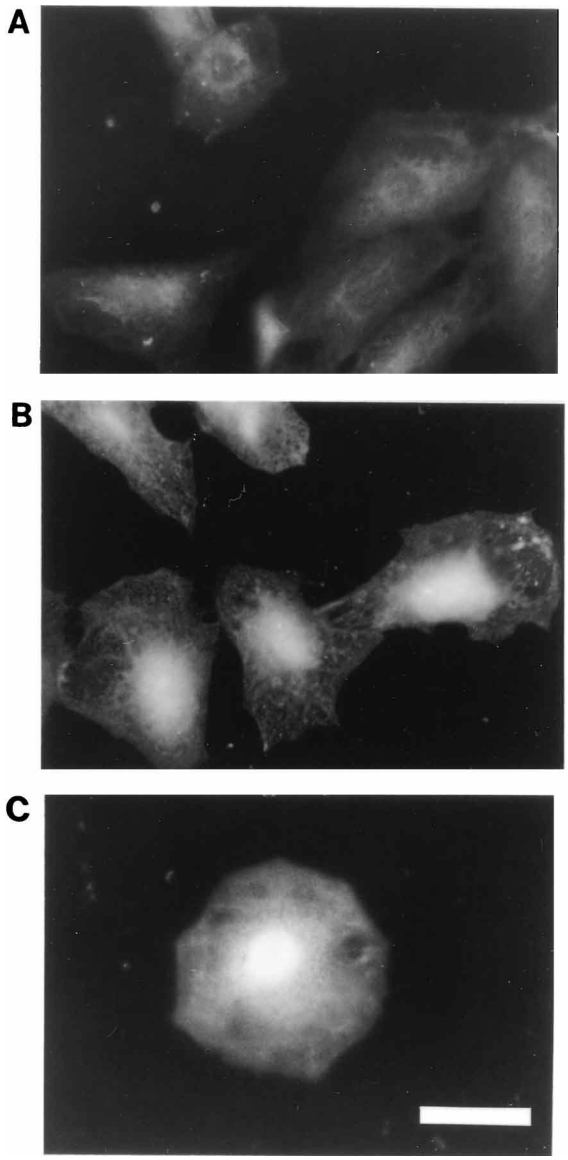

FIG. 3. Nuclear translocation of endogenous G-kinase in A10 cells. A10 cells were left untreated (A) or treated with 1 mM 8-Br-cGMP for 30 (B) or 60 (C) min and subjected to immunostaining with a G-kinase I-specific antibody as described in Materials and Methods. Photographs were taken under the same magnification as those in Fig. 1; the bar represents 20 μm.

NLS of G-kinase can function as an NLS on a heterologous protein, we fused amino acids 404 to 411 of G-kinase Iβ to the N terminus of β-galactosidase. When the fusion protein was expressed in BHK cells, histochemical staining showed a significant amount of nuclear β-galactosidase activity (compare cells expressing the NLS fusion protein in Fig. 7A to cells expressing wild type β-galactosidase in Fig. 7B). These results indicate that the putative NLS of G-kinase is sufficient to direct β-galactosidase to the nucleus.

Subcellular localization of constitutively active or inactive G-kinase constructs. Since nuclear translocation of G-kinase requires activation of the enzyme by 8-Br-cGMP, we next examined truncated versions of the kinase which are cGMP independent and constitutively active because they lack the N-terminal autoinhibitory domain (13). First, we truncated the G-kinase Iβ cDNA at the codon corresponding to Ala^{349} to produce an expression vector for the carboxyl-terminal catalytic domain of G-kinase (Fig. 4 and Fig. 5). When the truncated enzyme (ΔA^{349}) was expressed in BHK cells, it was found to localize to the nucleus in the absence as well as in the presence of 8-Br-cGMP (Fig. 5 and 8). Substitution of Lys^{407} with Glu in the catalytic domain of G-kinase did not change the constitutive nuclear localization of the truncated enzyme (Fig. 5). Since this isolated catalytic domain of G-kinase I is only 38 kDa in size (Fig. 9), it may enter the nucleus by passive diffusion in the absence of a functioning NLS (17).

Next, we removed the dimerization and autoinhibitory domains of G-kinase by N-terminal truncation of G-kinase Iβ at Val^{93} (ΔV^{93}) to generate a monomeric enzyme which is cGMP independent and constitutively active (reference 13 and Table 1). Although this approximately 65-kDa protein (Fig. 9) should be too large to enter the nucleus by passive diffusion, the subcellular localization of the Val^{93}-truncated kinase was predominantly nuclear in the absence as well as in the presence of 8-Br-cGMP (Fig. 5 and 8). Substitution of Lys^{407} with Glu in the Val^{93}-truncated G-kinase ($\Delta Val^{93}/K^{407}\rightarrow E$) rendered the enzyme exclusively cytoplasmic in the presence or absence of 8-Br-cGMP, suggesting that nuclear localization of the Val^{93}-truncated kinase was dependent on the presence of an intact NLS (Fig. 5).

Substitution of Asp^{516} with Ala in the catalytic domain of G-kinase Iβ resulted in a catalytically inactive enzyme (Table 1); Asp^{516} of G-kinase Iβ corresponds to Asp^{184} of the C subunit of A-kinase, which coordinates with the Mg^{2+} that is complexed with the α- and β-phosphates of ATP in the active center of the enzyme (14, 46). The dead (inactive) G-kinase $D^{516}\rightarrow A$ was found to localize to the nucleus in the presence of 8-Br-cGMP (Fig. 5), suggesting that catalytic activity of the kinase is not required for nuclear translocation.

Expression levels and enzymatic activities of the variant G-kinases. Since amino acid substitutions and truncations of G-kinase could alter the activity and/or stability of the enzyme, we measured G-kinase activity by using a synthetic peptide substrate and examined G-kinase protein levels by Western blotting in BHK cells transfected with the various G-kinase constructs. The in vitro activities and protein expression levels of the G-kinase mutant $K^{407}\rightarrow E$, the back mutant $E^{407}\rightarrow K$, the mutants $K^{399}\rightarrow E$ and $K^{407}\rightarrow A$, and the double mutant $K^{407}\rightarrow A/R^{409}\rightarrow A$ were very similar to those of wild-type G-kinase (Table 1 and Fig. 9; compare lanes 3 to 7 with lane 2 [wild-type G-kinase]). The protein expression level of the dead kinase $D^{516}\rightarrow A$ was also similar to that of wild-type G-kinase (Fig. 9, lane 8); however, G-kinase activity in cells transfected with the dead kinase was the same as that found in mock-transfected cells (Table 1).

Since the putative NLS of G-kinase is located in the ATP binding domain of the enzyme, mutagenesis of Lys^{407} and Arg^{409} could potentially influence ATP binding. The apparent K_m values for ATP of wild-type and mutant G-kinases were determined to be 10 μM (wild type), 110 μM ($K^{407}\rightarrow E$), 9 μM ($K^{407}\rightarrow A$), and 12 μM ($K^{407}\rightarrow A/R^{409}\rightarrow A$). Thus, substitution of Lys^{407} and Arg^{409} with Ala did not alter ATP binding significantly, whereas substitution of Lys^{407} with Glu decreased the mutant enzyme's affinity for ATP. However, this increase in K_m should not significantly influence the activity of the mutant $K^{407}\rightarrow E$ G-kinase inside the cell, since the intracellular ATP concentration is ≥1 mM (reference 3 and unpublished observation). The apparent K_a for 8-Br-cGMP was 1 μM for the wild type and for all mutant G-kinases tested, in agreement with previous reports for G-kinase Iβ (15, 34).

Expression levels of the truncated kinases appeared consis-

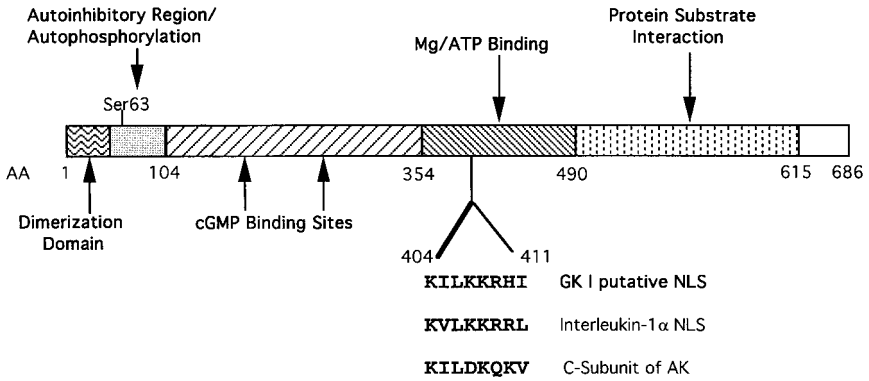

FIG. 4. Structural features of G-kinase Iβ. G-kinase Iα and Iβ appear to be splice products of the same gene which differ only in the first ~100 amino acids (AA) (the dimerization and autoinhibitory domains) (13). Shown enlarged is the sequence of a putative NLS of G-kinase (GK). The NLS of the interleukin-1α precursor (amino acids 79 to 86) and amino acids 72 to 79 of the C subunit of A-kinase (AK) which correspond to amino acids 404 to 411 of G-kinase Iβ are shown for comparison (32, 46, 51).

tently lower than that of wild-type G-kinase (Fig. 9; compare lanes 10 and 11 to lane 9; five times more protein was loaded in lanes 10 and 11 than in lane 9). This could be secondary to altered antibody recognition of the kinases because of the truncation; however, the cGMP-independent activity of the truncated kinases was less than half of the cGMP-dependent activity of the full-length kinases (Table 1), suggesting that indeed less protein was present intracellularly, possibly because of altered stability of the truncated enzymes, corresponding to the short half-life of the free C subunit of A-kinase (29). The K^{407}→E mutant versions of the truncated kinases showed protein expression levels and cGMP-independent protein kinase activities very similar to those of the corresponding truncated kinases without mutation.

Transactivation of the *fos* promoter by the variant G-kinases. We have previously shown that transfection of G-kinase into G-kinase-deficient BHK cells causes transactivation of the *fos* promoter in a strictly cGMP-dependent manner (18). To determine whether transcriptional activation of the *fos* promoter by G-kinase requires nuclear translocation of the kinase,

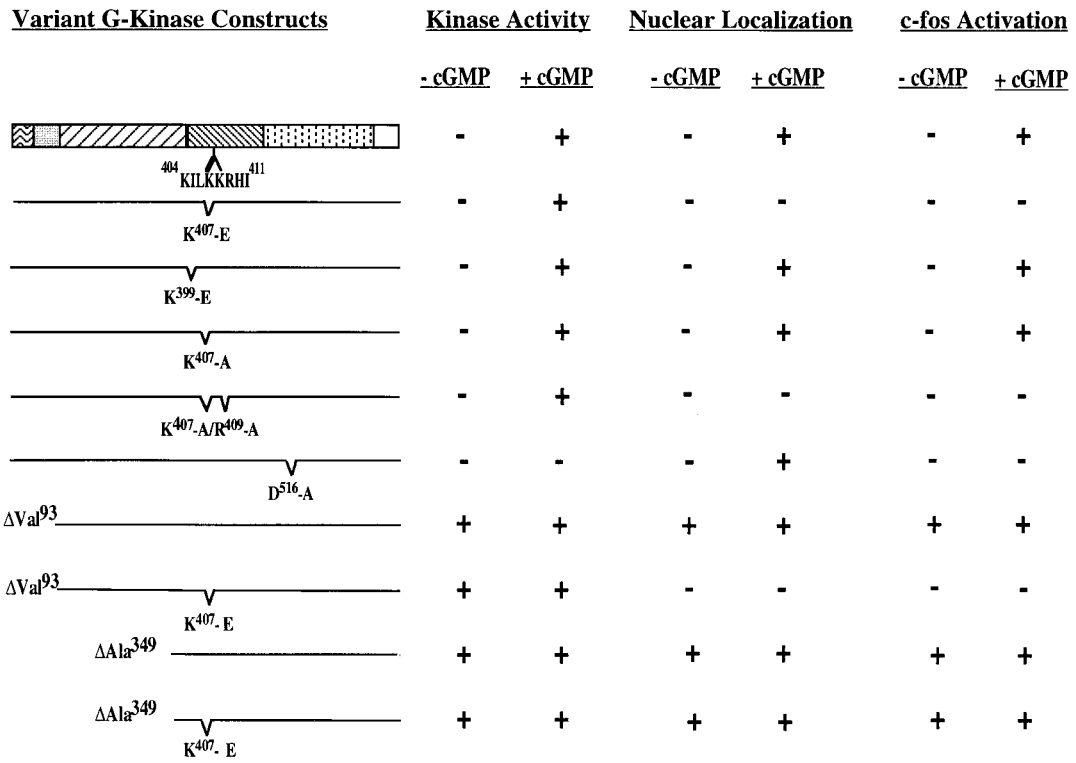

FIG. 5. Summary of the variant G-kinases. Wild-type G-kinase is shown with its functional domains in various shading patterns as in Fig. 4; the NLS sequence is shown enlarged (amino acids 404 to 411). The mutations and N-terminal truncations of the variant G-kinases are indicated. Kinase activity, nuclear localization, and c-*fos* promoter transactivation in the presence or absence of cGMP are indicated for all variant G-kinase constructs. For kinase activity, − denotes the activity of mock-transfected cells as described in Table 1; for nuclear localization, − indicates not observed; and for c-*fos* transactivation, − indicates <2-fold c-*fos* promoter transactivation as described for Fig. 10.

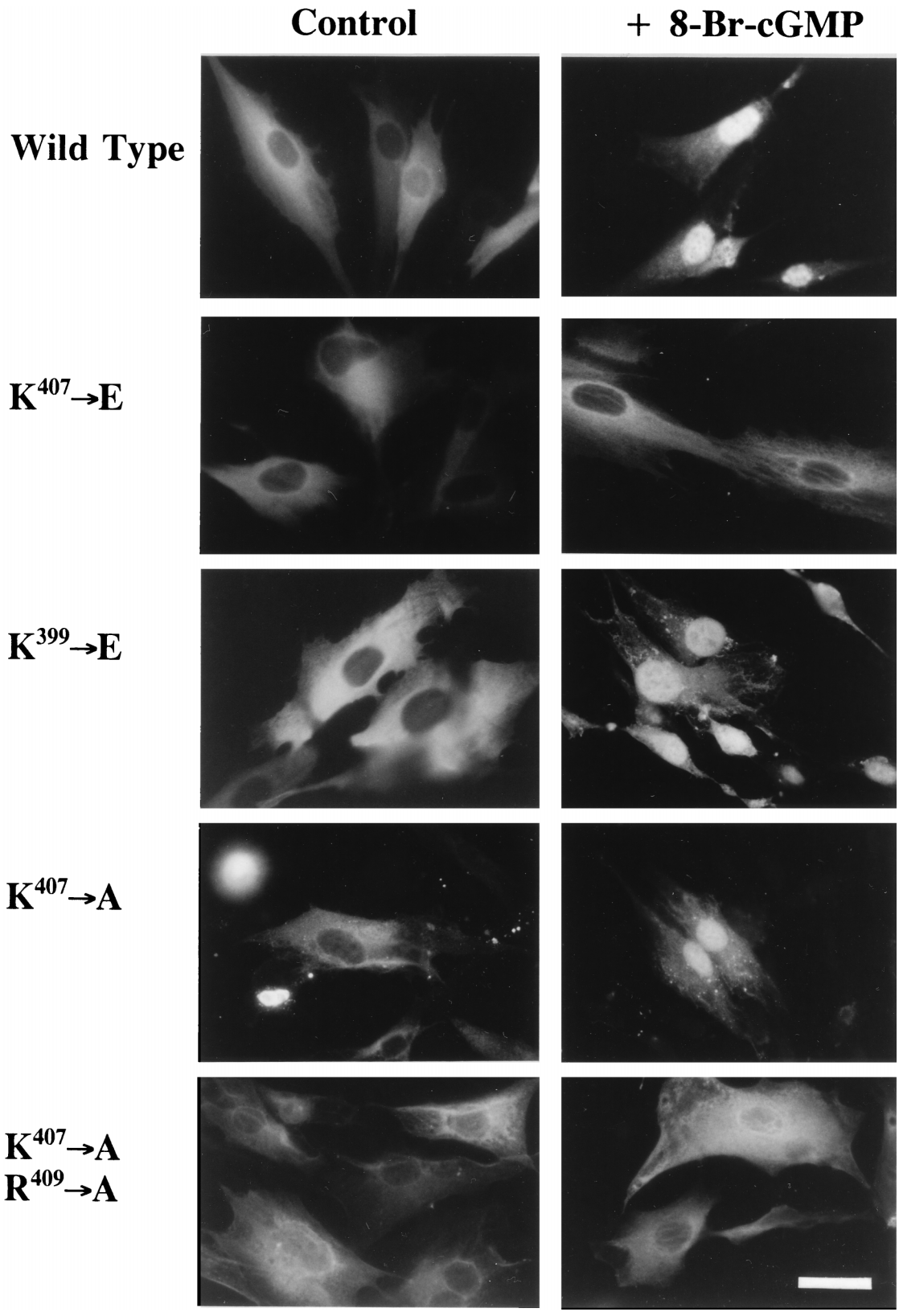

FIG. 6. Specific amino acid substitutions in the putative NLS of G-kinase prevent nuclear localization of the enzyme. BHK cells were transfected with expression vectors encoding wild-type G-kinase Iβ or the indicated mutant G-kinase. Cells shown on the left were left untreated, whereas cells shown on the right were treated with 1 mM 8-Br-cGMP for 1 h prior to fixation. Immunostaining for G-kinase was performed as described in Materials and Methods. Photographs were taken under the same magnification as those in Fig. 1; the bar represents 20 μm.

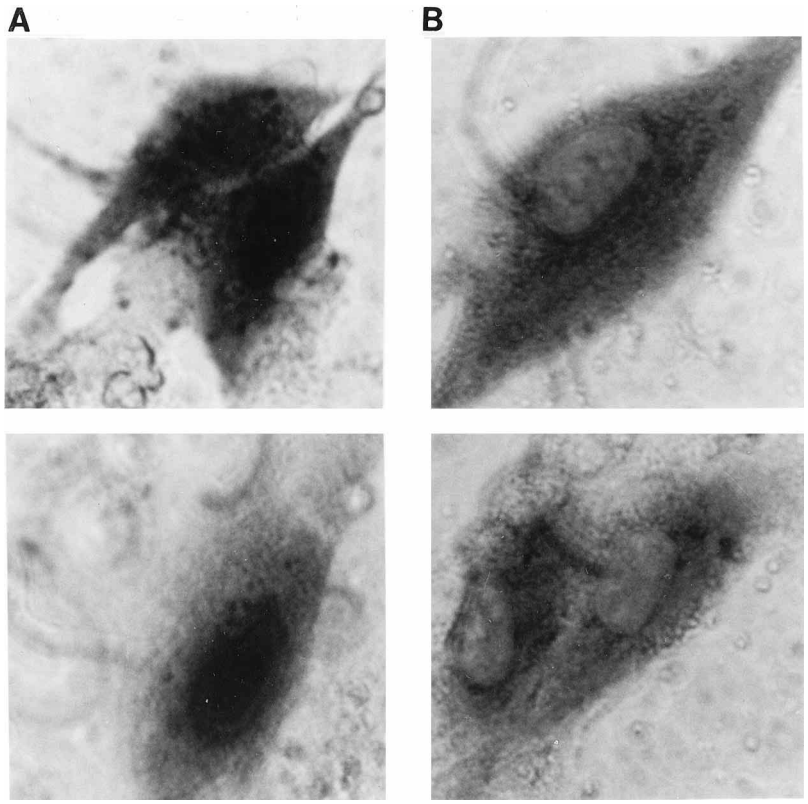

FIG. 7. The putative NLS of G-kinase directs β-galactosidase to the nucleus. BHK cells were transfected with a construct in which the putative NLS of G-kinase (amino acids 404 to 411, KILKKRHI) was fused to the N terminus of β-galactosidase (A; the two panels show cells from two independent experiments). BHK cells transfected with the wild-type β-galactosidase vector are shown for comparison (B). Histochemical staining for β-galactosidase activity was performed as described in Materials and Methods. Photographs were taken at a magnification of ×80.

we cotransfected BHK cells with the reporter pFos-CAT and the various G-kinase constructs described above. While wild-type G-kinase activated the *fos* promoter approximately fivefold in the presence of 1 mM 8-Br-cGMP, the mutant, $K^{407}\rightarrow E$ G-kinase was ineffective, consistent with its predominantly cytoplasmic localization (Fig. 5 and 10). The back mutant $E^{407}\rightarrow K$ and the mutant $K^{399}\rightarrow E$ performed similarly to wild-type G-kinase. Mutant $K^{407}\rightarrow A$ G-kinase transactivated the *fos* promoter approximately fourfold in the presence of 8-Br-cGMP, whereas the double mutant $K^{407}\rightarrow A/R^{409}\rightarrow A$ transactivated less than twofold, again consistent with the ability of the mutant kinases to translocate to the nucleus. The constitutively active, N-terminally truncated kinases (ΔV^{93} and ΔA^{349}) both efficiently transactivated the *fos* promoter and localized to the nucleus in the absence of 8-Br-cGMP (Fig. 8 and 10). In cells transfected with the ΔA^{349} truncation, there was no effect of

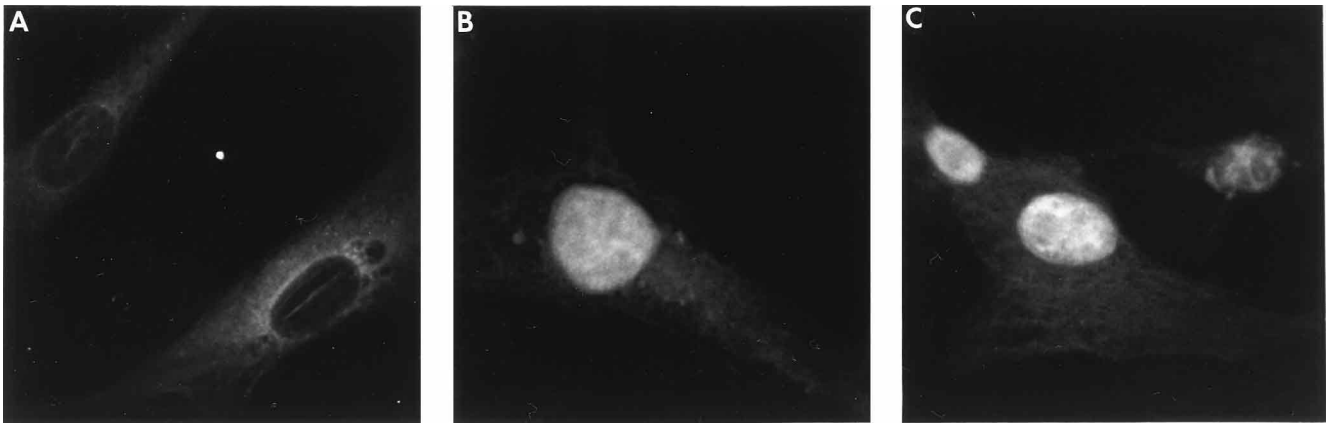

FIG. 8. Subcellular localization of constitutively active G-kinases. BHK cells were transfected with expression vectors encoding wild-type G-kinase Iβ (A), ΔV^{93} (B), or ΔA^{349} (C). Immunostaining for G-kinase and laser scanning confocal microscopy were performed as described for Fig. 2; shown are optical sections through the middle of nuclei with photographs taken under the same magnification as shown in Fig. 2. Unstimulated cells are shown, but similar results were obtained when cells transfected with the truncated kinases were treated with 8-Br-cGMP (not shown).

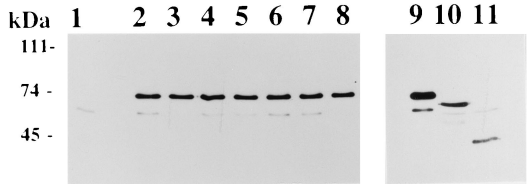

FIG. 9. Protein expression levels of the variant G-kinases. BHK cells were transfected with 0.5 μg of each G-kinase construct; Western blots were prepared with whole-cell lysates and probed with a G-kinase-specific antibody as described in Materials and Methods. Lane 1, mock-transfected cells; lanes 2 and 9, wild-type G-kinase; lane 3, NLS mutant $K^{407}\rightarrow E$; lane 4, back-mutant $E^{407}\rightarrow K$; lane 5, mutant $K^{399}\rightarrow E$; lane 6, NLS mutant $K^{407}\rightarrow A$; lane 7, NLS double mutant $K^{407}\rightarrow A/R^{409}\rightarrow A$; lane 8, dead kinase $D^{516}\rightarrow A$; lane 10, ΔV^{93}; lane 11, ΔA^{349}. Lanes 1 to 9 were loaded with whole-cell extracts corresponding to 10^4 cells, whereas lanes 10 and 11 were loaded with extracts corresponding to 5×10^4 cells; note an empty lane between lanes 1 and 2. The exposure time for lanes 9 to 11 was twice the exposure time for lanes 1 to 8.

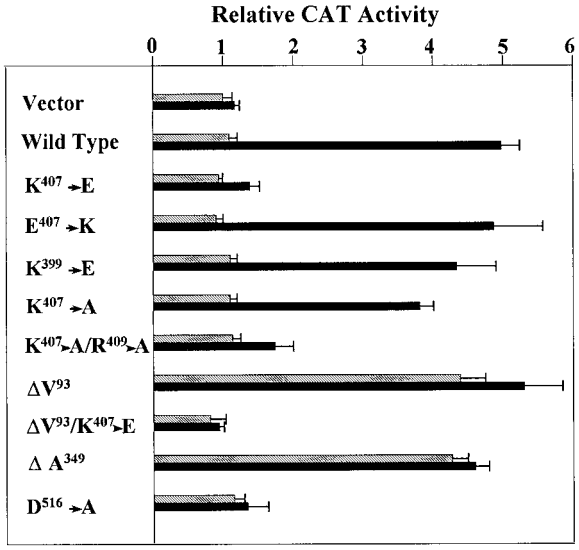

FIG. 10. Transactivation of the *fos* promoter by variant G-kinases. BHK cells were cotransfected with 50 ng of pFos-CAT, 50 ng of pRSV-luciferase (internal control), and 500 ng of the indicated G-kinase expression vector as described in Materials and Methods. Cells were either left untreated (striped bars) or treated with 1 mM 8-Br-cGMP (black bars). CAT activity was normalized to the luciferase activity in each sample, and the CAT/luciferase ratio of untreated cells transfected with empty vector (Vector) was assigned a value of 1.

8-Br-cGMP, whereas in cells transfected with the ΔV^{93} truncation, there was a small but reproducible increase in pFos-CAT expression when cells were treated with 8-Br-cGMP. The $K^{407}\rightarrow E$ mutant version of the ΔV^{93}-truncated G-kinase ($\Delta Val^{93}/K^{407}\rightarrow E$) did not transactivate the *fos* promoter in the presence or absence of 8-Br-cGMP, consistent with its exclusively cytoplasmic localization (Fig. 5 and 10). In contrast, the $K^{407}\rightarrow E$ mutant version of the ΔA^{349}-truncated kinase transactivated the *fos* promoter independently of cGMP and to the same degree as the ΔA^{349}-truncated enzyme without mutation; this observation is consistent with the constitutive nuclear localization of the mutant ΔA^{349}-truncated kinase (Fig. 5). As expected, the dead kinase $D^{516}\rightarrow A$ did not show any transactivation of the *fos* promoter in spite of its nuclear localization in the presence of 8-Br-cGMP.

DISCUSSION

The widespread importance of the NO/cGMP signal transduction pathway is recognized, but our knowledge of the molecular mechanisms mediating the downstream effects of this pathway is still limited. In particular, there is very little information concerning the mechanisms how NO and cGMP regulate gene expression, although NO-releasing agents and cGMP analogs have been shown to increase the mRNA levels of c-*fos*, *junB*, microtubule-associated protein 2, cyclooxygenase 2, and tumor necrosis factor alpha α and to decrease mRNA levels of gonadotropin-releasing hormone and the atrial natriuretic peptide receptor (2, 5, 16, 19, 24, 38, 41, 47). In some cases, the effect of cGMP requires the presence of calcium ionophores (2, 38). We have recently shown that G-kinase, one of the major intracellular targets of the NO/cGMP signal transduction pathway, mediates cGMP stimulation of the *fos* promoter independently of changes in calcium or cAMP (18). We now show that G-kinase translocates to the nucleus in response to activation by 8-Br-cGMP and that this translocation is required for transactivation of the *fos* promoter.

We have identified an NLS sequence located in the ATP binding domain of G-kinase which is necessary for nuclear transport of the kinase and sufficient to mediate nuclear transport of a heterologous protein. Interestingly, replacing a single basic amino acid with a negatively charged residue or replacing two basic amino acids with neutral residues disrupted the NLS, whereas replacing a single basic amino acid with a neutral residue did not. These results suggest that the function of the NLS relies on its total positive charge rather than on a single crucial amino acid residue. The fact that nuclear translocation of G-kinase requires activation of the kinase by cGMP suggests that the NLS is cryptic in the inactive enzyme. It is possible that the NLS is exposed by the conformational change that occurs when cGMP binds to the native enzyme or when the autoinhibitory domain is removed by truncation (28); the latter would explain the predominantly nuclear localization of the ΔV^{93}-truncated, constitutively active G-kinase (Fig. 8). The predominant cytoplasmic localization of the $K^{407}\rightarrow E$ mutant version of the Val^{93}-truncated G-kinase suggests that a single NLS which includes Lys^{407} is functional and constitutively active in the monomeric enzyme.

Our results with G-kinase Iβ should be directly applicable to

TABLE 1. G-kinase activities in BHK cells transfected with different G-kinase constructs

Cells[a]	G-kinase activity (nmol/min/mg of protein)[b]
Mock transfected	0.02 ± 0.01[c]
Transfected with:	
Wild-type G-kinase	0.98 ± 0.10[c]
$K^{407}\rightarrow E$	0.97 ± 0.09[c]
$E^{407}\rightarrow K$ (back mutant)	1.16 ± 0.18[c]
$K^{399}\rightarrow E$	1.09 ± 0.17[c]
$K^{407}\rightarrow A$	0.98 ± 0.13[c]
$K^{407}\rightarrow A/R^{409}\rightarrow A$	0.90 ± 0.05[c]
$D^{516}\rightarrow A$ (dead kinase)	0.02 ± 0.01[c]
ΔA^{349} (truncated kinase)	0.55 ± 0.10[d]
ΔV^{93} (truncated kinase)	0.57 ± 0.04[d]
$\Delta V^{93}/K^{407}\rightarrow E$	0.50 ± 0.03[d]

[a] BHK cells were transfected with 0.5 μg of the indicated G-kinase expression vector as described in Materials and Methods.

[b] Data represent the mean ± standard deviation of three independent transfections performed in duplicate.

[c] G-kinase activity in mock-transfected cells and in cells transfected with the full-length kinases was calculated as the difference between Kemptide phosphorylation in the presence and absence of 8-Br-cGMP (see Materials and Methods).

[d] Activity of the truncated, cGMP-independent kinases was calculated as the difference between Kemptide phosphorylation in transfected and mock-transfected cells (see Materials and Methods).

G-kinase Iα, since these two isoforms differ only in the N-terminal ~100 amino acids and have the same mechanism of activation (13). Our results may also be relevant for G-kinase II, because the cluster of basic amino acids which comprise the G-kinase I NLS is conserved in G-kinase II, except that a Cys residue replaces Ile in the second position of the putative NLS (KCIRKKHI) (23). It will be interesting to determine whether G-kinase II can translocate to the nucleus and serve to regulate gene expression; however, this enzyme is tightly associated with the plasma membrane and differs from G-kinase I in its tissue distribution and function (23, 48).

The subcellular localization of A-kinase and protein kinase C, the two serine/threonine kinases most closely related to G-kinase, is determined by specific anchoring proteins designated A-kinase anchoring proteins and receptors for activated protein kinase C, respectively (11, 35). Since certain A-kinase anchoring proteins appear to be important for cAMP signalling to the nucleus (7, 12), it is plausible that G-kinase localization in different subcellular compartments is relevant for the effects of cGMP on gene expression. Preliminary experiments from our laboratory suggest that about 30% of G-kinase Iβ is associated with particulate fractions of unstimulated A10 cells or transfected BHK cells, and Western blots probed with purified, radioactively labeled G-kinase Iβ indicate the presence of several G-kinase binding proteins in these two cell types (reference 6 and unpublished observation). One of these G-kinase binding proteins may be the intermediate filament protein vimentin (31). Nuclear transport of inactive G-kinase could be prevented by extranuclear anchoring proteins, and cGMP activation of the kinase could release the enzyme from anchoring proteins. Theoretically, truncation or site-directed mutagenesis of G-kinase could alter the interaction of the kinase with important anchoring proteins.

The kinetics of nuclear entry of G-kinase I are similar to those of the C subunit of A-kinase: upon cAMP binding to the regulatory subunit of A-kinase, the C subunit of A-kinase is released and accumulates in the nucleus over a 30-min period (20). However, the C subunit of A-kinase is rapidly and actively exported from the nucleus by its physiological inhibitor PKI (50), whereas G-kinase appears to remain in the nucleus for a prolonged period of time. G-kinase does not bind to PKI with high affinity, and the sequence of G-kinase I does not reveal any similarity to known nuclear export sequences (22, 43, 50). Therefore, removal of G-kinase from the nucleus may be determined by the rate of proteolytic breakdown in the nucleus. Additionally, G-kinase action in the nucleus could be terminated by protein phosphatases, as described for A kinase (1, 49). Clearly, more work needs to be done to identify the nuclear targets of G-kinase, which may include CREB-related transcription factors (18, 38). The work presented here establishes a link between the activation of receptor guanylate cyclases by peptide hormones or of soluble guanylate cyclase by NO leading to an increase in the intracellular cGMP concentration and cGMP-dependent nuclear translocation of G-kinase leading to changes in gene expression.

ACKNOWLEDGMENTS

T.G. was supported in part by a grant from the Charles H. and Anna S. Stern Foundation; S.M.L. was supported by Deutsche Forschungsgemeinschaft grant SFB 355; R.B.P. was supported by National Science Foundation grant MCB-9506327. The Bio-Rad MRC-1024 laser scanning confocal system is part of the San Diego Microscopy and Imaging Resource at UCSD supported by NIH grant RR04050 (principal investigator, M. H. Ellisman).

We thank C. Buckmaster and J. Feramisco for assistance with fluorescence microscopy and T. Deerinck for assistance with the laser scanning confocal system. We are also indebted to G. R. Boss for helpful discussions and to I. Huvar for technical assistance.

REFERENCES

1. **Alberts, A. S., M. Montminy, S. Shenolikar, and J. R. Feramisco.** 1994. Expression of a peptide inhibitor of protein phosphatase 1 increases phosphorylation and activity of CREB in NIH 3T3 fibroblasts. Mol. Cell. Biol. **14:**4398–4407.
2. **Belsham, D. D., W. C. Wetsel, and P. L. Mellon.** 1996. NMDA and nitric oxide act through the cGMP signal transduction pathway to repress hypothalamic gonadotropin-releasing hormone gene expression. EMBO J. **15:** 538–547.
3. **Boss, G. R., and R. W. Erbe.** 1982. Decreased purine synthesis during amino acid starvation of human lymphoblasts. J. Biol. Chem. **257:**4242–4247.
4. **Butt, E., K. Abel, M. Krieger, D. Palm, V. Hoppe, J. Hoppe, and U. Walter.** 1994. cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) *in vitro* and in intact human platelets. J. Biol. Chem. **269:**14509–14517.
5. **Cao, L., J. Wu, and D. G. Gardner.** 1995. Atrial natriuretic peptide suppresses the transcription of its guanylyl cyclase-linked receptor. J. Biol. Chem. **270:**24891–24897.
6. **Carr, D. W., and J. D. Scott.** 1992. Blotting and band-shifting: techniques for studying protein-protein interactions. Trends Biochem. Sci. **17:**246–249.
7. **Cassano, S., A. Gallo, V. Buccigrossi, A. Porcellini, R. Cerillo, M. E. Gottesman, and E. V. Avvedimento.** 1996. Membrane localization of cAMP-dependent protein kinase amplifies cAMP signaling to the nucleus in PC12 cells. J. Biol. Chem. **47:**29870–29875.
8. **Cornwell, T. L., K. B. Pryzwansky, T. A. Wyatt, and T. M. Lincoln.** 1991. Regulation of sarcoplasmic reticulum protein phosphorylation by localized cyclic GMP-dependent protein kinase in vascular smooth muscle cells. Mol. Pharmacol. **40:**923–931.
9. **Deng, W. P., and J. A. Nickloff.** 1992. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. Anal. Biochem. **200:**81–88.
10. **Dingwall, C., and R. A. Laskey.** 1992. Nuclear targeting sequences—a consensus? Trends Biochem. **16:**478–481.
11. **Faux, M. C., and J. D. Scott.** 1996. Molecular glue: kinase anchoring and scaffold proteins. Cell **85:**9–12.
12. **Feliciello, A., P. Giuliano, A. Porcellini, C. Garbi, S. Obici, E. Mele, E. Angotti, D. Grieco, G. Amabile, S. Cassano, Y. Li, A. M. Musti, C. S. Rubin, M. E. Gottesman, and E. V. Avvedimento.** 1996. The v-Ki-Ras oncogene alters cAMP nuclear signaling by regulating the location and the expression of cAMP-dependent protein kinase IIβ. J. Biol. Chem. **41:**25350–25359.
13. **Francis, S. H., and J. D. Corbin.** 1994. Progress in understanding the mechanism and function of cyclic GMP-dependent protein kinase. Adv. Pharmacol. **26:**115–170.
14. **Francis, S. H., and J. D. Corbin.** 1994. Structure and function of cyclic nucleotide-dependent protein kinases. Annu. Rev. Physiol. **56:**237–272.
15. **Francis, S. H., B. D. Noblett, B. W. Todd, J. N. Wells, and J. D. Corbin.** 1988. Relaxation of vascular and tracheal smooth muscle by cyclic nucleotide analogs that preferentially activate purified cGMP-dependent protein kinase. Mol. Pharmacol. **34:**506–517.
16. **Gong, J.-H., H. Renz, H. Sprenger, M. Nain, and D. Gemsa.** 1990. Enhancement of tumor necrosis factor-α gene expression by low doses of prostaglandin E_2 and cyclic GMP. Immunobiology **182:**44–55.
17. **Gorlich, D., and I. W. Mattaj.** 1996. Nucleocytoplasmic transport. Science **271:**1513–1518.
18. **Gudi, T., I. Huvar, M. Meinecke, S. M. Lohmann, G. R. Boss, and R. B. Pilz.** 1996. Regulation of gene expression by cGMP-dependent protein kinase. J. Biol. Chem. **271:**4597–4600.

18a. **Gudi, T., and R. B. Pilz.** Unpublished data.

19. **Haby, C., F. Lisovoski, D. Aunis, and J. Zwiller.** 1994. Stimulation of the cyclic GMP pathway by NO induces expression of the immediate early genes *c-fos* and *junB* in PC12 cells. J. Neurochem. **62:**496–501.
20. **Hagiwara, M., P. Brindle, A. Harootunian, R. Armstrong, J. Rivier, W. Vale, R. Tsien, and M. R. Montminy.** 1993. Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A. Mol. Cell. Biol. **13:**4852–4859.
21. **Han, J.-D., and C. S. Rubin.** 1996. Regulation of cytoskeleton organization and paxillin dephosphorylation by cAMP. J. Biol. Chem. **271:**29211–29215.
22. **Hofmann, F., W. Dostmann, A. Keilbach, W. Landgraf, and P. Ruth.** 1992. Structure and physiological role of cGMP-dependent protein kinase. Biochim. Biophys. Acta **1135:**51–60.
23. **Jarchau, T., C. Haeusler, T. Markert, D. Poehler, J. Vandekerckhove, H. R. De Jonge, S. M. Lohmann, and U. Walter.** 1994. Cloning, expression, and *in situ* localization of rat intestinal cGMP-dependent protein kinase II. Proc. Natl. Acad. Sci. USA **91:**9426–9430.
24. **Johnston, H. M., and B. J. Morris.** 1994. NMDA and nitric oxide increase microtubule-associated protein 2 gene expression in hippocampal granule cells. J. Neurochem. **63:**379–381.
25. **Kimes, B. W., and B. L. Brandt.** 1976. Characterization of two putative smooth muscle cell lines from rat thoracic aorta. Exp. Cell Res. **98:**349–366.
26. **Lalli, E., and P. Sassone-Corsi.** 1994. Signal transduction and gene regula-

tion: the nuclear response to cAMP. J. Biol. Chem. **269:**17359–17362.
27. **Lamb, N. J., A. Fernandez, M. A. Conti, R. Adelstein, D. B. Glass, W. J. Welch, and J. Feramisco.** 1988. Regulation of actin microfilament integrity in living nonmuscle cells by the cAMP-dependent protein kinase and the myosin light chain kinase. J. Cell Biol. **106:**1955–1971.
28. **Landgraf, W., F. Hofmann, J. T. Pelton, and J. P. Huggins.** 1990. Effects of cyclic GMP on the secondary structure of cyclic GMP-dependent protein kinase and analysis of the enzyme's amino-terminal domain by far-ultraviolet circular dichroism. Biochemistry **29:**9921–9928.
29. **Lee, S.-L., and R. A. Steinberg.** 1996. Pathways for degradation of the catalytic subunit of cAMP-dependent protein kinase differ in wild-type and kinase-negative S49 mouse lymphoma cells. J. Biol. Chem. **271:**16553–16558.
30. **Lemasters, J. J., and C. R. Hackenbrock.** 1978. Firefly luciferase assay for ATP production by mitochondria. Methods Enzymol. **57:**36–50.
31. **MacMillan-Crow, L. A., and T. M. Lincoln.** 1994. High-affinity binding and localization of the cyclic GMP-dependent protein kinase with the intermediate filament protein vimentin. Biochemistry **33:**8035–8043.
32. **Maier, J. A. M., M. Statuto, and G. Ragnotti.** 1994. Endogenous interleukin 1 alpha must be transported to the nucleus to exert its activity in human endothelial cells. Mol. Cell. Biol. **14:**1845–1851.
33. **Markert, T., A. B. Vaandrager, S. Gambaryan, D. Poehler, C. Haeusler, U. Walter, H. R. De Jonge, T. Jarchau, and S. M. Lohmann.** 1995. Endogenous expression of type II cGMP-dependent protein kinase mRNA and protein in rat intestine. J. Clin. Invest. **96:**822–830.
34. **Meinecke, M., J. Geiger, E. Butt, M. Sandberg, T. Jahnsen, T. Chakraborty, U. Walter, T. Jarchau, and S. Lohmann.** 1994. Human cyclic GMP-dependent protein kinase Iβ overexpression increases phosphorylation of an endogenous focal contact-associated vasodilator-stimulated phosphoprotein without altering the thrombin-evoked calcium response. Mol. Pharmacol. **46:**283–290.
35. **Mochly-Rosen, D.** 1995. Localization of protein kinases by anchoring proteins: a theme in signal transduction. Science **268:**247–251.
36. **Moncada, S., and E. A. Higgs.** 1995. Molecular mechanisms and therapeutic strategies related to nitric oxide. FASEB J. **9:**1319–1330.
37. **Ouaaz, F., B. Sola, F. Issaly, J. P. Kolb, F. Davi, F. Mentz, M. Arock, N. Paul-Eugène, M. Körner, B. Dugas, P. Debr', and M. D. Mossalayi.** 1994. Growth arrest and terminal differentiation of leukemic myelomonocytic cells induced through ligation of surface CD23 antigen. Blood **84:**3095–3104.
38. **Peunova, N., and G. Enikolopov.** 1993. Amplification of calcium-induced gene transcription by nitric oxide in neuronal cells. Nature **364:**450–453.
39. **Peunova, N., and G. Enikolopov.** 1995. Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells. Nature **375:**68–73.
40. **Pilz, R. B., M. Berjis, S. D. Idriss, J. S. Scheele, M. Suhasini, L. Gao, I. E. Scheffler, and G. R. Boss.** 1994. Isolation and characterization of HL-60 cells resistant to nitroprusside-induced differentiation. J. Biol. Chem. **269:**32155–32161.
41. **Pilz, R. B., M. Suhasini, S. Idriss, J. L. Meinkoth, and G. R. Boss.** 1995. Nitric oxide and cGMP analogs activate transcription from AP-1-responsive promoters in mammalian cells. FASEB J. **9:**552–558.
42. **Pryzwansky, K. B., S. Kidao, T. A. Wyatt, W. Reed, and T. M. Lincoln.** 1995. Localization of cyclic GMP-dependent protein kinase in human mononuclear phagocytes. J. Leukocyte Biol. **57:**670–678.
43. **Sandberg, M., V. Natarajan, I. Ronander, D. Kalderon, U. Walter, S. M. Lohmann, and T. Jahnsen.** 1989. Molecular cloning and predicted full-length amino acid sequence of the type Iβ isozyme of cGMP-dependent protein kinase from human placenta. FEBS Lett. **255:**321–329.
44. **Schmidt, H. H. H. W., S. M. Lohmann, and U. Walter.** 1993. The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. Biochim. Biophys. Acta **1178:**153–175.
45. **Schmidt, H. H. H. W., and U. Walter.** 1994. NO at work. Cell **78:**919–925.
46. **Taylor, S. S., J. A. Buechler, and W. Yonemoto.** 1990. cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annu. Rev. Biochem. **59:**971–1005.
47. **Tetsuka, T., D. Daphna-Iken, B. W. Miller, Z. Guan, L. D. Baier, and A. R. Morrison.** 1996. Nitric oxide amplifies interleukin 1-induced cyclooxygenase-2 expression in rat mesangial cells. J. Clin. Invest. **97:**2051–2056.
48. **Vaandrager, A. B., and H. R. De Jonge.** 1996. Signalling by cGMP-dependent protein kinases. Mol. Cell. Biochem. **157:**23–30.
49. **Wadzinski, B. E., W. H. Wheat, S. Jaspers, Jr., Peruski, L. F., R. L. Lickteig, G. L. Johnson, and D. J. Klemm.** 1993. Nuclear protein phosphatase 2A dephosphorylates protein kinase A-phosphorylated CREB and regulates CREB transcriptional stimulation. Mol. Cell. Biol. **13:**2822–2834.
50. **Wen, W., J. L. Meinkoth, R. Y. Tsien, and S. S. Taylor.** 1995. Identification of a signal for rapid export of proteins from the nucleus. Cell **82:**463–473.
51. **Wessendorf, J. H. M., S. Garfinkel, X. Zhan, S. Brown, and T. Maciag.** 1993. Identification of a nuclear localization sequence within the structure of the human interleukin-1α precursor. J. Biol. Chem. **268:**22100–22104.
52. **Wyatt, T. A., T. M. Lincoln, and K. B. Pryzwansky.** 1991. Vimentin is transiently co-localized with and phosphorylated by cyclic GMP-dependent protein kinase in formyl-peptide-stimulated neutrophils. J. Biol. Chem. **266:** 21274–21280.