Regulation of Gene Expression by cGMP-dependent Protein Kinase

TRANSCRIPTIVE ACTIVATION OF THE c-fos PROMOTER*

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The cAMP/cAMP-dependent protein kinase (A-kinase) and Ca2+/calmodulin-dependent protein kinase (Cam- kinase) signal transduction pathways are well known to regulate gene transcription, but this has not been demonstrated directly for the cGMP/cGMP-dependent protein kinase (G-kinase) signal transduction pathway. Here we report that transfection of G-kinase into G-kinase-deficient cells causes activation of the human c-fos promoter in a strictly cGMP-dependent manner. The effect of G-kinase appeared to be mediated by several sequence elements, most notably the serum response element (SRE), the AP-1 binding site (FAP), and the cAMP response element (CRE). The magnitude of G-kinase transactivation of the fos promoter was similar to that of A-kinase, but there were significant differences between G-kinase and A-kinase activation of single enhancer elements and of a chimeric Gal4-CREB transcription factor. Our results indicate that G-kinase transduces signals to the nucleus independently of A-kinase or Ca2++, although it may target some of the same transcription factors as A-kinase and Cam-kinase.

The c-fos gene product is part of the AP-1 transcription factor complex which plays a central role in regulating cell growth and differentiation (1). c-fos transcription is rapidly induced by a variety of stimuli including activation of A-kinase by cAMP, protein kinase C by phorbol esters, or Cam-kinase IV by Ca2++ (2, 3). We (4) and others (5, 6) have shown that treating cells with cGMP analogs or increasing the intracellular cGMP concentration induces c-fos mRNA expression; in some instances co-induction with Ca2++ ionophores or antioxidants was required (7, 8). Unlike cAMP which acts predominantly through A-kinase activation, cGMP has several intracellular target proteins: G-kinase, cGMP-gated ion channels, and cGMP-stimulated or cGMP-inhibited phosphodiesterases (9). To test whether c-fos induction by cGMP analogs was mediated by G-kinase, we used G-kinase-deficient baby hamster kidney (BHK) cells and a chloramphenicol acetyltransferase (CAT) reporter construct under control of the human fos promoter (pFOS-CAT, 10). We found that reporter gene expression from this construct was stimulated by cGMP analogs only in BHK cells co-transfected with a G-kinase expression vector, but not in cells co-transfected with empty vector. Transactivation of the fos promoter in the presence of G-kinase was characterized further and found to be independent of A-kinase or Ca2++.

EXPERIMENTAL PROCEDURES

Plasmids—The G-kinase expression vector encoding human G-kinase type I β has been described (11); the parent vector is pRC/CMV (Invitrogen). pFC400, pFC225, pFC122, and pFC87 were derived from pFOS-CAT by deletion of the appropriate restriction fragment (10); the remaining fos promoter constructs, pTRE-CAT, pCRE-CAT, pSRE-CAT, Gal4-CREB, pRSV-PKIwt, and pRSV-PKImut were described previously (12-16).

Western Blot Analysis—Western blots were prepared as described previously using a G-kinase-specific antibody and G-kinase purified from bovine lung as a standard (11).

Transfections and Reporter Gene Assays—BHK cells were transfected with 1 μg of plasmid DNA plus 6 μl of LipofectAMINE™ (Life Technologies, Inc.) per ml of serum-free medium as described (4). Six hours after the addition of DNA, cells were placed in low serum-containing media (Dulbecco’s modified Eagle’s medium supplemented with 0.1% serum and 0.1% bovine serum albumin); at 24 h, drugs were added and cells were harvested 8 h later. The transfection efficiency was 67-80% as determined by histochemical staining for β-galactosidase of cells transfected with pRSV-βGal (4). CAT activity was measured as described (4). Luciferase activity was measured in the presence of 0.1 mM luciferin and 5 mM ATP using a photon-counting luminometer (17).

β-Galactosidase activity was measured using the chemiluminescent substrate GalactoSTAR™ as recommended by the supplier (Tropix, Inc.).

Protein Kinase Assays—Total G-kinase activity in cell extracts was measured at 30 °C as the difference between phosphorylation of Kemptide (LRRASLG) in the presence and absence of 10 μM 8-Bromo-cGMP. The assay was performed in the presence of the specific A-kinase inhibitor peptide PKI (16) as described previously (18) except that 750 μM Kemptide was used as substrate. To determine the amount of G-kinase or A-kinase that was activated in vivo in BHK cells treated with 8-Bromo-cGMP or 8-Bromo-cAMP, respectively, cells were washed rapidly in situ in large volumes of ice-cold phosphate-buffered saline and snap-frozen on dry ice. Cells were disrupted by several 10-s pulses of sonication, centrifuged at 11,000 × g for 30 s, and assays were performed at 4 °C immediately thereafter to minimize 8-Bromo-cGMP dissociation from G-kinase (19). To correct for the activity of A-kinase and other protein kinases, G-kinase activity in G-kinase-transfected cells was measured in the presence of PKI, and Kemptide phosphorylation in mock-transfected cells was subtracted from that in the G-kinase-transfected cells. A-kinase activity was determined as the difference between Kemptide phosphorylation in the absence and presence of 10 μM PKI (16).

RESULTS AND DISCUSSION

We have previously shown that NO-releasing agents and membrane-permeable cGMP analogs induce c-fos mRNA expression in rat embryonal fibroblasts; these cells contain NO-
stimulatable guanylate cyclase activity and G-kinase activity (4). To test whether c-fos mRNA induction by cGMP analogs was mediated by G-kinase, we co-transfected the reporter plasmid pFOS-CAT (10) with a G-kinase expression vector (11) into BHK cells which contain extremely low endogenous G-kinase activity. In G-kinase-transfected cells, the enzyme was easily detected by Western blotting (Fig. 1), and total cellular G-kinase activity was 0.83 ± 0.09 nmol/min/mg of protein compared to 0.02 ± 0.01 nmol/min/mg of protein in cells transfected with control expression vector (“empty” pRC/CMV). From Western blots using bovine lung G-kinase as a standard and by comparing enzyme activity in transfected BHK cells to that of the purified bovine lung enzyme (18), we estimate the intracellular concentration of G-kinase in the entire transfected BHK cell population to be 3–6 pmol/mg of protein; taking into consideration that the transfection efficiency was 67–80%, the concentration of G-kinase in successfully transfected cells is in the range of physiological G-kinase concentrations found in smooth muscle cells, neuronal cells, and platelets (1–9 pmol/mg of protein) (9).

CAT expression from the pFOS-CAT reporter was induced by 8-Br-cGMP only in G-kinase-transfected cells and was strictly dependent on the amount of G-kinase vector transfected (Fig. 2A) and the 8-Br-cGMP concentration in the culture medium (Fig. 2B). Thus, activation of the fos promoter by 8-Br-cGMP required transfected G-kinase and was not due to cross-activation of A-kinase. Maximal induction of pFOS-CAT was ~5-fold at 1 mM 8-Br-cGMP, a concentration consistent with that reported to be required for various physiological responses in whole cells (4, 20, 21). When 8-chlorophenylthio-cGMP was used to replace 8-Br-cGMP, maximal pFOS-CAT expression in G-kinase-transfected cells occurred at 0.25 mM (data not shown). In comparison, activation of endogenous A-kinase by 1 mM 8-Br-cAMP maximally induced pFOS-CAT expression 7-fold (Fig. 2B). For both G-kinase and A-kinase there was an excellent correlation between pFOS-CAT activity (Fig. 2B) and the amount of total cellular G-kinase activity (inset, Fig. 2B). These data are further evidence that 8-Br-cGMP induced pFOS-CAT expression by activating G-kinase and show that the amount of G-kinase activity in the G-kinase-transfected cells was comparable with the amount of endogenous A-kinase activity (taking into consideration that the transfection efficiency was 67–80%). CAT expression from the promoterless parent plasmid of pFOS-CAT (10), and from several other promoter constructs containing the Rous sarcoma virus long terminal repeat, the chicken β-actin promoter, and the SV40 early promoter (4) was not influenced by G-kinase in the absence or presence of 8-Br-cGMP, indicating that the effect of G-kinase required sequence elements present in the fos promoter.

The pFOS-CAT reporter construct contains approximately 700 base pairs of the human fos gene 5'-flanking region including several known enhancer elements: a sIS-inducible element, a serum-inducible element (SRE), an AP-1 binding site (FAP), which resembles the phorbol ester response element, (TRE), and a cAMP-response element (CRE) (Fig. 3) (2, 10, 12). fos promoter induction by A-kinase is mediated by the SRE, FAP, and CRE, with each element functioning independently without synergism (12, 22). A series of promoter deletions and mutations generally gave a similar pattern for both G-kinase- and A-kinase-mediated transactivation (Fig. 3); deletions of the fos promoter up to ~72 nucleotides from the transcription start were inducible, but deletion of the CRE (pFC53) abolished inducibility by both kinases. However, the constructs pFC87 and pTF5/53 showed much greater induction by A-kinase than G-kinase suggesting that the two kinases do not regulate the fos promoter via identical mechanisms. The high inducibility of
G-kinase Regulation of Gene Expression

**Methods**

tionstartsite(arrow directs repeats (D.R.) regions corresponding to the shown containing 700 base pairs of the human c-ate the effect of G-kinase.

**CRE** is indicated by 5 sites are indicated. A linker scanning mutation in pFC60LS (in the pTRE-CAT, 50 ng of the indicated CAT reporter plasmid, and either 400 ng of control vector or G-kinase expression vector; cells were left untreated (−) or treated with 1 mM 8-Br-cGMP (cGMP) or 8-Br-cAMP (cAMP) for 8 h as indicated. The parent plasmid pBL-CAT-5 (open bars) contains an enhancerless thymidine kinase promoter; CAT expression from this plasmid was very low and not influenced by G-kinase or A-kinase. pTE-CAT (narrow diagonal bars) contains 10 copies of the SV40 enhancer AP-1 binding site (13), pCRE-CAT (filled bars) contains four copies of the fos CRE (14), and pSRE-CAT (wide diagonal bars) contains four copies of the fos SRE (14). Reporter gene expression from all three constructs was stimulated by G-kinase and A-kinase by 8-Br-cGMP (which has been noted before (12)) may be mediated by the C/EBP-related transcription factor NFIL-6 (23).

To study G-kinase transactivation of specific enhancer elements, we used synthetic promoter constructs containing multimerized TRE, CRE, or SRE consensus sequences in the context of an enhancerless thymidine kinase promoter (13, 14). Reporter gene expression from all three constructs was stimulated 4-5-fold by 8-Br-cAMP (8-Br-cGMP; cAMP) or of transfected G-kinase by 8-Br-cGMP (8-Br-cGMP/GK) was calculated as the difference in CREB expression in the presence and absence of 1 mM cyclic nucleotide. Results are the mean of at least three independent experiments which differed by <15% of the mean.

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**CREB** binds constitutively to the fos promoter CRE (22), and its transactivation potential is modulated by phosphorylation, especially phosphorylation of Ser133 by A-kinase or Cam-kinase IV (2, 3, 15). To determine if G-kinase modulates the transactivation potential of CREB, we used a Gal4-CREB fusion construct containing the DNA binding domain of the yeast transcription factor Gal4 and lacking the CREB COOH-terminal leucine zipper dimerization motif (Gal4-CREBΔzip (15, 24)). This construct was co-transfected with either G-kinase or control vector and the reporter plasmid pGAL4-Luc, which contains Gal4 DNA binding sites (3). Treatment of G-kinase transfected cells with 8-Br-cGMP increased transactivation of pGAL4-Luc by Gal4-CREBΔzip 3.5-fold (Fig. 5A). 8-Br-cGMP had no effect on CREB transactivation in cells transfected with control vector indicating no significant cross-activation of A-kinase by 8-Br-cGMP. However, A-kinase activation by 8-Br-cAMP caused a 16-fold increase in CREB-mediated transactivation. A Ser133 → Ala mutation in CREB (Gal4-CREBΔzip-M1) (15) rendered CREB unresponsive to either G-kinase or A-kinase activation (Fig. 5A). These results suggest that G-kinase modulation of CREB activity requires Ser133 and are consistent with the finding that G-kinase phosphorylates a synthetic peptide derived from the sequence surrounding Ser133 of CREB with a 4-fold lower $V_{\text{max}}$ than that of A-kinase (25). At present, we do not know whether G-kinase directly phosphorylates CREB in vivo; the enzyme could also modulate the activity of proteins which cooperate with CREB, e.g. CBP (26). We did not observe any effect of G-kinase on the transactivation potential of Gal4-Fos, Gal4-c-Jun, or Gal4-JunB fusion constructs (27) (data not shown).

To exclude that G-kinase activation of CRE-containing promoters required endogenous A-kinase activity, we co-transfected an expression vector encoding either the specific A-kinase inhibitor peptide PKI (PKIwt), or a noninhibitory mutant of PKI (PKImut (16), together with pFOS-CAT and either G-kinase or control vector. Co-transfection of PKImut inhibited cAMP-induced reporter gene expression by >70%, but had no significant effect on G-kinase-mediated transactivation (Fig. 5B).

Since 8-Br-cGMP has been reported to amplify Ca$^{2+}$ induction of the fos promoter in PC12 cells (7), we examined the effect of the Ca$^{2+}$ ionophores A23187 and ionomycin (7) on pFOS-CAT activity in BHK cells transfected with G-kinase or control vector. There was no significant effect of the ionophores (0.1–3 μM) on pFOS-CAT expression in the presence or absence of 8-Br-cGMP (data not shown), suggesting that BHK cells may
PKImut (encoding for a PKImutant which does not inhibit A-kinase (16), open bars) were added. Cells were treated with cyclic nucleotides as lacking the Cam-kinase isozyme required for Ca\textsuperscript{2+} and A-kinase. and an effect of PKIon pFOS-CAT expression induced by G-kinase these proteins can form a large number of different complexes, including heterodimers between proteins from both families which bind to TRE- and CRE-like consensus sequences (1, 30, 31). Nuclear localization of G-kinase has not been established, but nuclear staining by a G-kinase-specific antibody has been observed in immunofluorescence studies of human macrophages (32), and G-kinase contains an amino acid motif (residues 404 - 410 of G-kinase (\beta) which closely resembles the nuclear localization signal of human interleukin \textalpha (33)). c-fos is the first gene whose expression is regulated by G-kinase; however, other candidate genes include junB, tumor necrosis factor \alpha, and elastin since their mRNA expression is induced by cGMP analogs (4, 20, 34).

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Fig. 5. Effect of G-kinase on CREB-mediated transactivation and effect of PKI on pFOS-CAT expression induced by G-kinase and A-kinase. A, BHK cells were co-transfected with 100 ng of the reporter plasmid pGAL4-Luc (3), 100 ng of pRSV-\betaGal (\beta-galactosidase expression vector serving as internal control), 3 ng of either pGal4-CREB\Deltazip (a chimeric transcription factor containing the CREB transactivation domain fused to the Gal4 DNA binding domain, filled bars) or pGal4-CREB\Deltazip-M1 (pGal4-CREB\Deltazip with a Ser\textsuperscript{133} \rightarrow Ala mutation in CREB, diagonal bars), and 400 ng of either G-kinase or control vector. Cells were treated with 1 mM 8-BrcGMP (GMP) or 8-BrcAMP (cAMP) for 8 h as indicated. CAT activity was normalized to \beta-galactosidase activity expressed from pRSV-\betaGal; the ratio of CAT/\beta-galactosidase activity in the absence of G4-CREB constructs was assigned a value of 1. B, cells were co-transfected with pOS-CAT and G-kinase or control vector as described in Fig. 4 except that 200 ng of either pRSV-PKI\textsuperscript{mut} (encoding for wild type PKI (16), cross-hatched bars) or pRSV-PKI\textsuperscript{mut} (encoding for a PKI mutant which does not inhibit A-kinase (16), open bars) were added. Cells were treated with cyclic nucleotides as described above. A and B, results are the mean \pm S.D. of three independent experiments.

being lack the Cam-kinase isozyme required for Ca\textsuperscript{2+} induction of the fos promoter (3). G-kinase is important in the regulation of smooth muscle tone, platelet function, neuronal long-term potentiation, and hematopoietic cell differentiation (9, 28, 29). Some of these functions involve lowering of cytosolic Ca\textsuperscript{2+}, inhibition of phosphatidylinositol breakdown, and phosphorylation of cytoskeletal proteins (9, 21). This report demonstrates that G-kinase can regulate gene expression and that the transcription factor CREB and the c-fos gene are important common nuclear targets for the cGMP-, cAMP- and Ca\textsuperscript{2+}-activated signal transduction pathways. Additional transcription factors which may mediate transactivation of the fos promoter by G-kinase include other members of the CREB/ATF or Fos/Jun families; these proteins can form a large number of different complexes, including heterodimers between proteins from both families which bind to TRE- and CRE-like consensus sequences (1, 30, 31). Nuclear localization of G-kinase has not been established, but nuclear staining by a G-kinase-specific antibody has been observed in immunofluorescence studies of human macrophages (32), and G-kinase contains an amino acid motif (residues 404 - 410 of G-kinase (\beta) which closely resembles the nuclear localization signal of human interleukin \textalpha (33)). c-fos is the first gene whose expression is regulated by G-kinase; however, other candidate genes include junB, tumor necrosis factor \alpha, and elastin since their mRNA expression is induced by cGMP analogs (4, 20, 34).

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