Protein Kinase G Activates the JNK1 Pathway via Phosphorylation of MEKK1*

Received for publication, February 12, 2001, and in revised form, March 12, 2001
Published, JBC Papers in Press, March 14, 2001, DOI 10.1074/jbc.C100079200

Jae-Won Soh‡§, Yuehua Mao‡§, Li Liu¶, W. Joseph Thompson¶, Rifat Pamukcu¶, and I. Bernard Weinstein‡§

From the ‡Department of Medicine and ¶Herbert Irving Comprehensive Cancer Center, College of Physicians & Surgeons, Columbia University, New York, New York 10032 and *Cell Pathways, Inc., Horsham, Pennsylvania 19044

We recently obtained evidence that treatment of human colon cancer cells with exisulind (sulindac sulfone) and related compounds induces apoptosis by activation of protein kinase G (PKG) and c-Jun kinase (JNK1). The present study further explores this mechanism. We demonstrate that in NIH3T3 cells a constitutively active mutant of PKG causes a dose-dependent activation of JNK1 and thereby transactivates c-Jun and stimulates transcription from the AP-1 enhancer element. The activation of JNK1 and the transactivation of c-Jun by this mutant of PKG were inhibited by a dominant negative MEKK1. In vitro assays showed that a purified PKG directly phosphorylated the N-terminal domain of MEKK1. PKG also directly phosphorylated a full-length MEKK1, and this was associated with enhanced MEKK1 phosphorylation. Thus, it appears that PKG activates JNK1 through a novel PKG-MEKK1-SEK1-JNK1 pathway, by directly phosphorylating and activating MEKK1.

Cyclic GMP (cGMP) is an important second messenger that mediates several signal transduction pathways in mammalian cells (1). It is involved in the regulation of various physiological functions, including neurotransmission, cell differentiation, proliferation, and platelet aggregation (2). Cyclic GMP also modulates intracellular calcium levels in vascular smooth muscle cells and thereby modulates smooth muscle tone (3). Intracellular levels of cGMP are tightly regulated through synthesis by guanylate cyclases and hydrolysis by specific phosphodiesterases (PDEs) (4, 5). cGMP has several intracellular targets, including gated ion channels, cGMP-dependent protein kinases (PKG), cGMP-activated phosphodiesterases, and cGMP-inhibited phosphodiesterases (6, 7).

PDE2 and PDE5 are cGMP phosphodiesterases (5). In recent studies we obtained evidence that in human colon cancer cell lines, novel PDE2/5 inhibitors, including exisulind (sulindac sulfone) and the high affinity derivatives CP248 and CP461,

* This work was supported by grants from Cell Pathways, Inc., the T. J. Martell Foundation, and the National Foundation for Cancer Research (to I. B. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Herbert Irving Comprehensive Cancer Center, College of Physicians & Surgeons, Columbia University, HHSIC-1509, 701 West, 168th St., New York, NY 10032. Tel: 212-305-6921; Fax: 212-305-6889; E-mail: weinstein@cucfa.ccc.columbia.edu.

‡ The abbreviations used are: PDE, phosphodiesterase; PKG, protein kinase G; PRC, protein kinase C; DMEM, Dulbecco’s minimal essential medium; β-gal, β-galactosidase; DTT, dithiothreitol; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; WT, wild type; 8-Br-cGMP, 8-bromo-3′5′ cyclic GMP.

increase cellular levels of cGMP and that this leads to activation of PKG and c-Jun kinase (JNK1) and induction of apoptosis (8, 9). However, the biochemical details by which cGMP leads to activation of JNK1 were not definitely established. The present study presents evidence that the activation of PKG by cGMP leads to direct phosphorylation and activation of MEKK1. This then leads to activation of SEK1 and subsequently JNK1. This PKG-MEKK1-SEK1-JNK1 pathway represents a novel signal transduction pathway that may be important in understanding the roles of cGMP and PKG in cell proliferation and apoptosis.

EXPERIMENTAL PROCEDURES

Cell Cultures and Transfection and Reporter Assays—NIH3T3 mouse fibroblasts were routinely grown in Dulbecco’s minimal essential medium (DMEM) containing 10% calf serum. For reporter assays, triplicate samples of 1 × 10⁵ cells in 35-mm plates were transfected using Lipofectin (Life Technologies, Inc.) with 1 μg of the reporter plasmid, 0.05–5 μg of various expression vectors, and 1 μg of the control plasmid pCMV-β-gal. The pCMVβ-gal plasmid DNA was added to the transfections, as needed, to achieve the same total amount of plasmid DNA per transfection. Twenty-four hours after transfection, cell extracts were prepared, and luciferase assays were done using the Luciferase Assay System (Promega). Luciferase activities were normalized with respect to parallel β-gal activities, to correct for differences in transfection efficiency. β-gal assays were performed using the β-Galactosidase Enzyme Assay System (Promega). The plasmid pβRI/CMV-Δ93GK was kindly provided by R. Filz (University of California, San Diego) (10), the pPAP-1-luciferase plasmid by J. Pierce (NCI) (11), the pGAL4DB-c-Jun and pG5-luciferase plasmids by A. Minden (Columbia University), and the pCPE4-HA-MEKK1-WT and pCPE4-HA-MEKK1-D1369A plasmids by M. H. Cobb (University of Texas Southwestern Medical Center) (12). The pCMV-β-gal plasmid was purchased from Stratagene.

JNK1 Kinase Assays—The cells were lysed in a lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 20 μg/ml β-glycerophosphate, 25% glycerol) and then JNK1 was immunoprecipitated with an anti-FLAG antibody (Sigma). The lysates were boiled in 1 × Laemmli sample buffer and 20 μg of protein from each sample was separated by SDS-PAGE and transferred to nitrocellulose paper. The membrane was incubated with a 1:1000 dilution of a rabbit anti-phospho-JNK1 antibody (New England Biolabs) as the substrate, in a kinase reaction buffer (20 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 μM DTT, 20 μM ATP, 2 μM β-glycerophosphate, 1 μM of γ[32P]ATP), for 20 min, as described previously (8). The reaction mixture was then subjected to 10% PAGE.

For transient transfection experiments, subconfluent cultures of cells in 10-cm plates were transfected, using Lipofectin (Life Technologies, Inc.), with 2.5 μg of the pCMV3-M2-JNK1 plasmid and either 5 μg of pRe/CMV-Δ93GK or pCPE4-HA-MEKK1 plasmid DNA. The pcDNA3 plasmid DNA was used as a control. Twenty-four hours after transfection, the cells were lysed in the lysis buffer described above and then JNK1 was immunoprecipitated with an anti-FLAG antibody (Sigma) for 2 h and assayed for in vitro kinase activity, as described above.

The intensities of the bands on gels were determined with a Phosphoimager (Molecular Dynamics), and the ratio of the treated sample to the control untreated sample was expressed as “relative kinase activity” or “fold activation.” The experiments were repeated three times with similar results.

PKG Assays—Purified recombinant PKG I (100 units, Calbiochem)
was incubated with 2 μg of recombinant GST-MEKK1 (1–301) (Santa Cruz) in a reaction buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 10 μM dipyridamole, 10 mM DTT, 200 μM ATP, 10 mM NaF, 1 μCi of [γ-32P]ATP), for 20 min, in the absence or presence of 100 μM cGMP. The reaction mixture was subjected to 10% SDS-PAGE, and the intensities of the bands were determined as described above. The ratio of the treated sample to the control untreated sample was expressed as fold activation.

MEKK1 Assays—COS-7 cells were grown in DMEM containing 10% fetal bovine serum, and subconfluent cultures in 10-cm plates were transiently transfected with either a HA epitope-tagged MEKK1 WT (wild type) or the D1369A mutant plasmid, using Lipofectin (Life Technologies, Inc.), as described above. After 24 h, the cells were lysed in the above-mentioned lysis buffer. MEKK1 was then immunoprecipitated with an anti-HA antibody (Berkeley Antibody Company), and in vitro kinase assays were performed in the absence or presence of 100 units of purified PKG Iα (Calbiochem) in a kinase reaction buffer (20 mM HEPES, pH 7.5, 10 mM MgCl2, 1 mM DTT, 20 μM ATP, 20 μM β-glycerophosphate, 1 μCi of [γ-32P]ATP), for 20 min, as described previously (13). The pCEP4-HA-MEKK1-WT and pCEP4-HA-MEKK1-D1369A plasmids encode a wild type MEKK1 and a kinase-inactive mutant, respectively (12).

RESULTS AND DISCUSSION

PKG Activates JNK1—To directly address the role of PKG, we examined the effects of a constitutively active mutant of PKG on the JNK1 pathway in NIH3T3 mouse fibroblasts. For this purpose, we utilized the pRc/CMV-D93GK plasmid, which encodes a mutant PKG Iα sequence with an N-terminal truncation. Deletion of the N-terminal 93 amino acids renders this PKG independent of cGMP, and therefore, it is constitutively active (10).

We first established that in NIH3T3 cells, as in SW480 human colon cancer cells (8), increased levels of cGMP lead to activation of JNK1. Exponentially growing NIH3T3 cells were treated with various cGMP modulators for 1 h, and protein extracts were then collected for JNK1 assays (Fig. 1A). Anisomycin, a known activator of JNK1 (14), was used as a positive control. Treatment with 8-bromo-cyclic GMP (8-Br-cGMP, 500 μM), a cell permeable cGMP analog, led to rapid activation of JNK1. YC-1 (100 μM), a guanylate cyclase activator, and CP248 (1 μM), a PDE2/5 inhibitor (8, 9), also rapidly activated JNK1. Western blot analysis using a JNK1 antibody (Fig. 1A) ensured that equal amounts of the immunoprecipitated JNK1 protein were added to the kinase reactions. Therefore, increase in cellular levels of cGMP does lead to activation of JNK1 in NIH3T3 cells.

We then examined whether the constitutively active mutant of PKG causes JNK1 activation in NIH3T3 cells. The cells were transiently transfected with the pCMV5-M2-JNK1 plasmid and increasing amounts of the constitutively active PKG plasmid Δ93GK. The pCMV5-M2-JNK1 plasmid encodes a FLAG-tagged JNK1. The pCEP4-HA-MEKK1 plasmid, which encodes the wild type MEKK1, was used as a positive control, since when overexpressed it leads to JNK1 activation (12). Twenty-four hours after transfection, FLAG-tagged JNK1 was immunoprecipitated with an anti-FLAG antibody and assayed for JNK1 in vitro kinase activity, using GST-c-Jun as a substrate. As shown in Fig. 1B, transfection with Δ93GK activated JNK1, in a dose-dependent manner. Western blot analysis indicated that the kinase assays contained equal amounts of the FLAG-JNK1 protein (Fig. 1B). These data demonstrate that a constitutively active PKG mutant is sufficient to induce JNK1 activity in NIH3T3 cells.

MEKK1 Is Required for PKG-mediated JNK1 Activation—We previously reported that in SW480 cells submicromolar concentrations of the exuisulin analog CP248, a potent PDE2/5 inhibitor (8, 9), causes rapid (within 30 min) activation of MEKK1 (8). To determine whether MEKK1 is actually required for PKG-mediated JNK1 activation, we studied the effects of a dominant negative mutant of MEKK1, encoded by the plasmid pCEP4-HA-MEKK1-D1369A, on PKG-mediated activation of JNK1. The MEKK1-D1369A mutant encodes a kinase-inactive protein, due to an Asp1369 to Ala point mutation in its activation loop (12). NIH3T3 cells were transiently transfected with the pCMV5-M2-JNK1 plasmid and either Δ93GK or pCEP4-HA-MEKK1-D1369A, or both of the latter two plas-
In vitro intensities of the bands were quantitated with a PhosphorImager. WT and mutant proteins were present in the kinase reactions. The buffer and then the HA-MEKK1-WT and HA-MEKK1-D1369A mutant plasmids, using Lipofectin. After 24 h, the cells were lysed in a lysis medium. Twenty-four hours after transfection, the cells were lysed in a lysis buffer and then the HA-MEKK1-WT and HA-MEKK1-D1369A mutant proteins were immunoprecipitated with an anti-HA antibody. The immunoprecipitated MEKK1-WT or MEKK1-D1369A proteins were incubated in \([\gamma^{32P}]\text{ATP-phosphorylation assays}, with or without purified wild type PKG, and in the absence or presence of cGMP. Fig. 2B shows that in the presence of cGMP and PKG the MEKK1-WT protein underwent more extensive phosphorylation than the MEKK1-D1369A mutant protein, even though similar amounts of both proteins were immunoprecipitated. The panel on the right was exposed for a longer time than the panel on the left, because the bands in the panel on the right would otherwise not be well displayed. The more extensive phosphorylation of MEKK1-WT suggests that phosphorylation of this protein by cGMP-activated PKG leads to activation of the kinase activity of MEKK1, and subsequent MEKK1 autophosphorylation. This is consistent with evidence that when MEKK1 is activated in vitro it undergoes autophosphorylation (15), but this requires further studies.

These data suggest that phosphorylation of MEKK1-D1369A is due to phosphorylation by PKG and that phosphorylation of MEKK1-WT is due to both phosphorylation by PKG and MEKK1 autophosphorylation. The high level of phosphorylation of MEKK1-WT was dependent upon the addition of both PKG and cGMP (Fig. 2B), indicating that this was mediated by activated PKG. These results indicate that cGMP-activated PKG can directly phosphorylate MEKK1 and suggest that this activates MEKK1.

**PKG Activation Also Leads to Transactivation of c-Jun and Increased AP-1 Activity**—Since activated PKG resulted in the activation of JNK1 in NIH3T3 cells, it was of interest to look at immediate events downstream of JNK1. Activation of JNK1 leads to phosphorylation and thereby transactivation of the transcription factor c-Jun. Therefore, we examined whether transfection of NIH3T3 cells with the constitutively active mutant of PKG led to c-Jun transactivation, using transient transfection reporter assays (Fig. 3). The pG5-luciferase reporter plasmid has five copies of GAL4 binding sites, and the pGAL4-c-Jun plasmid encodes the GAL4 DNA binding domain protein fused to the transactivation domain of c-Jun (16). Activation of c-Jun by phosphorylation of its transactivation domain leads to activation of transcription of the pG5-luciferase reporter. NIH3T3 cells were transfected with the pG5-luciferase reporter plasmid together with the pGAL4-c-Jun plasmid, and with increasing amounts of the Δ93GK plasmid or the pCEP4-HA-MEKK1 plasmid. Luciferase assays (Fig. 3A) indicated that transfection with Δ93GK led to c-Jun transactivation, in a dose-dependent manner. Again, MEKK1 served as a positive control.

In additional studies, NIH3T3 cells were transfected with the pG5-luciferase reporter plasmid and the pGAL4-c-Jun plasmid, together with either Δ93GK, pCEP4-HA-MEKK1-D1369A, or both of the latter two plasmids (Fig. 3B). The constitutively active mutant of PKG again stimulated c-Jun activation, and this activation was markedly inhibited by the dominant negative MEKK1 construct (D1369A), in a dose-dependent manner (Fig. 3B). Therefore, the ability of PKG to cause transactivation of c-Jun is mediated through MEKK1, which then, presumably, activates SEK1 and then JNK1.

We then examined the ability of the Δ93GK mutant of PKG to mediate the activation of an AP-1 enhancer element. The AP-1 enhancer element is responsive to various stimuli that activate the c-Jun/c-Fos heterodimer (17, 18). NIH3T3 cells were transfected with a pAP-1-luciferase reporter plasmid, together with increasing amounts of the Δ93GK plasmid (Fig. 3C). Again the pCEP4-HA-MEKK1 plasmid was used as a positive control. Twenty-four hours after transfection, cell extracts were prepared and assayed for luciferase activity. We
Fig. 3. PKG transactivates c-Jun and also activates AP-1 transcription in NIH3T3 cells. A, NIH3T3 cells were transfected with the pG5-luciferase reporter plasmid (1 μg) and pGAL4-c-Jun plasmid (50 ng), together with increasing amounts (0, 0.1, 0.5, or 2 μg) of the pReCMV-Δ93GK plasmid or the pCEP4-HA-MEKK1 plasmid. The pG5-luciferase reporter plasmid has five copies of GAL4 DNA binding sites, and the pGAL4-c-Jun plasmid encodes the GAL4 DNA binding domain fused to the transactivation domain of c-Jun. The cells were also cotransfected with the pCMV-β-gal plasmid. The pcDNA3 plasmid DNA was used as a control (−). Twenty-four hours after transfection, cell extracts were prepared, and luciferase activities were measured and normalized with respect to β-galactosidase activities. For all experiments, the data shown are representative of at least three independent experiments with each assay done in triplicate. The error bars indicate the S.D. values. Luciferase activities are expressed as fold induction, after correction for β-galactosidase activities. B, NIH3T3 cells were transfected with the pG5-luciferase reporter plasmid (1 μg) and the pGAL4-c-Jun plasmid (50 ng), together with either pReCMV-Δ93GK (2 μg), pCEP4-HA-MEKK1-D1369A (+, 2 μg; ++, 5 μg), or both of the latter plasmids, as indicated. The MEKK1-D1369A plasmid encodes a dominant negative mutant of MEKK1. The pcDNA3 plasmid DNA was used as a control (−). Twenty-four hours after transfection, cell extracts were prepared, and luciferase activities were measured and normalized with respect to β-galactosidase activities. C, NIH3T3 cells were transfected with the pAP1-luciferase reporter plasmid (1 μg), which has three copies of AP-1 sites, together with increasing amounts (0, 0.1, 0.5, or 2 μg) of the pReCMV-Δ93GK plasmid or the pCEP4-HA-MEKK1 plasmid. The cells were also cotransfected with the pCMV-β-gal plasmid. The pcDNA3 plasmid DNA was used as a control (−). Twenty-four hours after transfection, cell extracts were prepared, and luciferase activities were measured and normalized with respect to β-galactosidase activities.

Fig. 4. JNK1 signal transduction pathway activated by PKG. Intracellular levels of cGMP can increase through either activation of guanylate cyclase or through inhibition of PDEs 2 and 5. This activates PKG and then PKG directly phosphorylates and activates MEKK1. MEKK1 then phosphorylates and activates SEK1, which in turn phosphorylates and activates JNK1. Activation of JNK1 then plays a critical role, together with other signals, in activation of c-Jun, gene transcription, and the induction of apoptosis by mechanisms yet to be determined.

It is known that MEKK1 can play an important role in various stress responses and in apoptosis, through activation of the downstream kinases SEK1 and JNK1 (20). However, the precise proteins that act upstream of MEKK1 to cause its activation are not clearly defined. Deak et al. (15) identified a major site of autophosphorylation (Thr575) within the “activation loop” of MEKK1. Phosphatase treatment of a constitutively active MEKK1 or mutation of Thr575 to alanine decreased the kinase activity of MEKK1 (15). PAK3 and PKC were found that Δ93GK also activated the AP-1 reporter, in a dose-dependent manner (Fig. 3C). Therefore, a constitutively active mutant of PKG is sufficient to lead to activation of the AP-1 enhancer element.

We reported previously that sulindac sulfone (exisulind) and related compounds that inhibit PDEs 2 and 5 and, therefore, increase cellular levels of cGMP, can lead to activation of the MEKK1-SEK1-JNK1 pathway in SW480 human colon cancer cells (8). The present studies provide evidence that PKG can directly phosphorylate and activate MEKK1. A hypothetical signaling pathway based on these results is shown in Fig. 4. Intracellular levels of cGMP can increase, either through activation of guanylate cyclase or through inhibition of PDEs 2 and 5. This leads to activation of PKG. Activated PKG can then directly phosphorylate and activate MEKK1. The activated MEKK1 then phosphorylates and activates SEK1, which in turn phosphorylates and activates JNK1. Previous studies indicate that JNK1 activation can play a critical role in the activation of c-Jun, gene transcription, and the induction of apoptosis (8, 19).

Although the present studies were done mainly with NIH3T3 cells, in unpublished studies we found that transfection of SW480 human colon cancer cells with the constitutively active mutant of PKG also leads to JNK1 activation. Therefore, this signaling pathway probably applies to a variety of mam-
malian cells and may have more general relevance with respect to growth control and apoptosis.

Acknowledgment—We are grateful to Wang-Qui Xing for valuable technical assistance.

REFERENCES

1. Eigenthaler, M., Lohmann, S. M., Walter, U., and Pilz, R. B. (1999) Rev. Physiol. Biochem. Pharmacol. 135, 173–209
2. Vaandrager, A. B., and de Jonge, H. R. (1996) Mol. Cell. Biochem. 157, 23–30
3. Pfeifer, A., Klatt, P., Massberg, S., Ny, L., Saubier, M., Hirneise, C., Wang, G. X., Korth, M., Aasodi, A., Andersson, K. E., Krombach, F., Mayerhofer, A., Ruth, P., Fassler, R., and Hofmann, F. (1998) EMBO J. 17, 3045–3051
4. Denninger, J. W., and Marletta, M. A. (1999) Biochim. Biophys. Acta 1411, 334–350
5. Stacey, P., Rulden, S., Dalpling, A., and Phillips, S. C. (1998) Biochem. Biophys. Res. Commun. 247, 249–254
6. Lincoln, T. M., and Cornwell, T. L. (1993) FASEB J. 7, 328–338
7. Lohmann, S. M., Vaandrager, A. B., Smolenski, A., Walter, U., and De Jonge, H. R. (1997) Trends Biochem. Sci. 22, 307–312
8. Soh, J. W., Mao, Y., Kim, M. G., Pamukcu, R., Li, H., Piazza, G. A., Thompson, W. J., and Weinstein, I. B. (2000) Clin. Cancer Res. 6, 4136–4141
9. Thompson, W. J., Piazza, G. A., Li, H., Liu, L., Fetter, J., Zhou, B., Sperl, G., Ahnen, D., and Pamukcu, R. (2000) Cancer Res. 60, 3338–3342
10. Gudi, T., Lohmann, S. M., and Pilz, R. B. (1997) Mol. Cell. Biol. 17, 5244–5254
11. Li, W., Michieli, P., Alimandi, M., Lorenzi, M. V., Wu, Y., Wang, L. H., Heidar, M. A., and Pierce, J. H. (1996) Oncogene 13, 731–737
12. Xu, S., Robbins, D. J., Christerson, L. B., English, J. M., Vanderbult, C. A., and Cobb, M. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5291–5295
13. Dinerman, J. L., Steiner, J. P., Dawson, T. M., Dawson, V., and Snyder, S. H. (1994) Neuropharmacology 33, 1245–1251
14. Baranick, M., Htun, P., and Schaper, W. (1999) J. Cardiovasc. Pharmacol. 34, 182–189
15. Deak, J. C., and Templeton, D. J. (1997) Biochem. J. 322, 185–192
16. Sadowski, I., and Ptashne, M. (1989) Nucleic Acids Res. 17, 7539
17. Karin, M. (1994) Curr. Opin. Cell Biol. 6, 415–424
18. Karin, M., and Hunter, T. (1995) Curr. Biol. 5, 747–757
19. Basu, S., and Kolesnick, R. (1998) Oncogene 17, 3277–3285
20. Schliesing, T. K., Fanger, G. R., Yujiri, T., and Johnson, G. L. (1998) Front. Biosci. 3, D1181—D1186
21. Siow, Y. L., Kulmar, G. B., Sanghera, J. S., Tai, G., Oh, S. S., and Pelech, S. L. (1997) J. Biol. Chem. 272, 7586–7594
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J. Biol. Chem. 2001, 276:16406-16410.
doi: 10.1074/jbc.C100079200 originally published online March 14, 2001

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