

CPG16, a Novel Protein Serine/Threonine Kinase Downstream of cAMP-dependent Protein Kinase*

(Received for publication, August 18, 1998, and in revised form, November 12, 1998)

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Gene expression is necessary for the formation and consolidation of long term memory in both invertebrates and vertebrates. Here, we describe the expression and characterization of candidate plasticity gene 16 (*cpg16*), a protein serine/threonine kinase that was previously isolated from rat hippocampus as a plasticity-related gene. CPG16, when expressed in and purified from bacteria and COS7 cells, was only capable of autophosphorylation and phosphorylation of myelin basic protein but failed to phosphorylate many other peptides and proteins in *in vitro* phosphorylation assays. Recombinant CPG16, when overexpressed and purified from COS7 cells, had a relatively low level of autophosphorylation activity. This activity was significantly stimulated when cAMP-elevating agents (forskolin, 8-bromo-cAMP) were added to the cells but not by any other extracellular stimuli tested, e.g. serum, phorbol esters, and a calcium ionophore. Although the stimulation of CPG16 activity was inhibited by the cAMP-dependent protein kinase inhibitor H-89, it did not serve as a direct substrate for this kinase. This suggests that CPG16 may be activated by a cAMP-stimulated protein kinase cascade. Immunolocalization studies in COS7 and NIH-3T3 cells showed mostly cytoplasmic localization of CPG16 that turned partially nuclear upon stimulation with 8-bromo-cAMP. Moreover, overexpression of CPG16 seems to partially inhibit cAMP-stimulated activity of the transcription factor CREB (cAMP response element-binding protein), suggesting its involvement in the down-regulation of cAMP-induced transcription. Thus, CPG16 is a protein serine/threonine kinase that may be involved in a novel signaling pathway downstream of cAMP-dependent protein kinase.

Learning and memory processes in the brain are characterized by plasticity changes in central nervous system neurons. In a comprehensive search for candidate plasticity-related genes (CPGs¹ (1, 2)), a novel cDNA encoding for a putative

protein kinase, termed *cpg16*, has been isolated from kainate-treated rat hippocampus. Here we show that CPG16 is a protein serine/threonine kinase that, when expressed in COS7 cells, is activated by cAMP via a cAMP-dependent protein kinase (PKA)-induced mechanism. Studies on the effect of CPG16 on transcription have revealed that it may be involved in the down-regulation of cAMP response element-binding protein (CREB) activity. Thus, it is possible that CPG16 participates in the regulation of neuronal plasticity by down-regulating PKA-stimulated transcription.

EXPERIMENTAL PROCEDURES

Northern Blot Analysis—Northern blotting was performed as described previously (3) with 5 µg of RNA/lane. The probe used for detection was the 1600-base pair *cpg16* cDNA (2), which was labeled by the random priming technique (U. S. Biochemical Corp.) using [α -³²P]dCTP according to the manufacturer's instructions.

Construction of Expression Vectors for CPG16—The polymerase chain reaction method was used to clone the cDNA encoding the open reading frame for *cpg16* (2) in-frame with glutathione S-transferase (GST) into the pGEX2T expression vector and purified as described (4). To construct a hemagglutinin (HA)-*cpg16* plasmid, polymerase chain reaction was used to clone the *cpg16* in-frame with the HA epitope tag in a pCGN vector (5). The HA-CPG16 open reading frame was sequenced to ensure its composition.

Transfection of COS7 and NIH-3T3 Cells—COS-7 or NIH-3T3 cells were grown in 6-cm tissue culture plates containing Dulbecco's modified Eagle's medium and 10% fetal calf serum (FCS) to approximately 60% confluence. The cells were then transfected with plasmid DNA, prepared using Qiagen columns (Qiagen). Subsequently, the COS7 cells were transfected by the DEAE-dextran-mediated method as described previously (6) using 2.5 µg/ml DNA. After 10% Me₂SO shock, cells were cultured in normal growth media and maintained in a humidified incubator (5% CO₂) at 37 °C. NIH-3T3 cells were transfected by the calcium phosphate method using 5 µg of DNA (7). After the transfection, the cells were cultured in normal growth media and maintained in a humidified incubator (5% CO₂) at 37 °C.

Activation of Transfected COS7 and Immunoprecipitation of HA-CPG16—Control and HA-*cpg16*-transfected COS-7 cells in 6-cm tissue culture plates were starved for 16 h in 0.1% FCS in Dulbecco's modified Eagle's medium. The cells were then treated with either 10 µM forskolin (Sigma), 10 µM 8-bromo-cAMP (8-Br-cAMP, Sigma), 10% FCS, or other stimuli for various times. If isobutylmethylxanthine (Sigma, 10 µM) was included in an experiment, it was added 5 min before other treatments. After stimulation, the cells were washed once with ice-cold phosphate-buffered saline (PBS) and lysed (10 min on ice) in Buffer H (50 mM β -glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM sodium vanadate, 1 mM benzamidine, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 2 µg/ml pepstatin-A (8)). The cells were then scraped from the plate and centrifuged (14,000 \times g, 20 min, 4 °C). The supernatant containing HA-CPG16 was collected and incubated

* This work was supported by grants from MINERVA and from the Kekst Family Foundation for Molecular Biology. 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.

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‡ This paper is dedicated to the memory of Dr. Yoav Citri, who was tragically killed in a car accident in December, 1995.

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¹ The abbreviations used are: CPG, candidate plasticity-related gene;

8-Br-cAMP, 8-bromo-cAMP; PKA, cAMP-dependent protein kinase; MAPK, mitogen-activated protein kinase; CaMKII, Ca²⁺/calmodulin-dependent protein kinase II; CREB, cAMP response element-binding protein; ERK, external signal-responsive kinase; GST, glutathione S-transferase; HA, hemagglutinin; PBS, phosphate-buffered saline; WIS, Weizmann Institute of Science, Israel; FCS, fetal calf serum; MBP, myelin basic protein.

with 2 μ g of anti-HA antibody (4 °C) for 1 h, then precipitated with protein A-agarose (Sigma) for 1 h. The complexes were washed (4 °C) three times with PBS + 0.1% Nonidet P-40, once with 0.5 M LiCl in PBS, and twice with buffer A ((50 mM β -glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM Na₃VO₄) (8)).

Autophosphorylation and in Vitro Kinase Assays—GST-CPG16 or HA-CPG16 autophosphorylation was performed at 30 °C in a buffer containing 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 20 μ M ATP (20 cpm/fmol), 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 50 mM β -glycerophosphate, 100 μ M orthovanadate in a final volume of 30 μ l. The reactions were terminated by adding SDS-polyacrylamide gel electrophoresis sample buffer and boiled for 5 min. *In vitro* kinase assays were performed using the same reaction mixture with the following substrates or protein kinases. Recombinant Jun N-terminal kinase, recombinant external-signal responsive kinase (ERK), recombinant p38 mitogen-activated protein kinase (p38MAPK), recombinant MAPK/ERK kinase, and recombinant AN-EE-MAPK/ERK kinase were prepared as described (7). Activated ERK was prepared by phosphorylation with active MAPK/ERK kinase (7). Nuclear K-7 was the kind gift from Dr. K. Bomsztyk, University of Washington, Seattle. Purified vitronectin and PKA (catalytic subunit) were gifts from Dr. S. Shaltiel, The Weizmann Institute of Science, Israel (WIS). GST-Jun 1-74 and GST-ATF2 16-96 as well as GST-Rho and GST-ARF were expressed in bacteria and purified as described (10). MAP2 was a gift from Dr. Z. Eleazar (WIS). Peptides based on the following sequences were synthesized by the biological services at the WIS: epidermal growth factor receptor 661-675, glycogen synthase kinase 6-20, Jun 60-78, and Far1 134-150. Myosin light chain, histone III α , myelin basic protein (MBP), β -casein, and protamine were purchased from Sigma.

Phosphoamino Acid Analysis—³²P-Labeled proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (Immobilon-P; Millipore Corp.). Membrane slices containing the proteins were hydrolyzed in constant boiling 5.7 N HCl and analyzed by two-dimensional electrophoresis as described (11).

Immunostaining—NIH-3T3 cells were transfected using the calcium phosphate method using 5 μ g of DNA. After transfection, the cells were replated on glass coverslips contained in a 6-well tissue culture dish and allowed to grow for 36 h after which the cells were starved in Dulbecco's modified Eagle's medium and 0.1% calf serum for 16 h. Cells were activated with the appropriate compounds and then fixed with 3% paraformaldehyde for 20 min. After one wash with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min and blocked in 3% bovine serum albumin in PBS for 30 min. The primary monoclonal antibody, anti-HA antibody (antibody unit, WIS) was applied to the cells at a final concentration of 1 μ g/ml in PBS for 45 min, washed with PBS, and incubated again with lissamine rhodamine-conjugated goat anti-mouse IgG and IgM (Jackson ImmunoResearch Laboratories) 1:200 in PBS for 45 min. After washing with PBS, coverslips were mounted, analyzed, and photographed using a Zeiss Axiohot camera.

CREB-Luciferase Assay—The assay was performed as described by Spengler *et al.* (12). Briefly, Madin-Darby canine kidney cells expressing a stable luciferase reporter construct containing five copies of the CRE element were transfected by electroporation with an expression vector (1 μ g) containing HA-*cpg16*. Six h after transfection, serum was removed from the cells, and after a total of 24 h, stimulants were added. Four h after stimulation, the medium was discarded, and the cells were washed with PBS. The cells were then lysed by adding luciferase lysis buffer (Labsystems, Inc.). Cell extracts were transferred to the luminometer (Dynatech Laboratories, Inc.), and luciferase activity was recorded. The resulting data were analyzed using Excel software (Microsoft Corp.).

RESULTS

Northern Blot Analysis of *cpg16*—*cpg16* was recently identified (2) in a differential cDNA screen from kainate-stimulated rat hippocampus dentate gyrus. We performed Northern blot analysis of poly(A)-selected mRNA from total rat brain, rat dentate gyrus, kainate-activated rat dentate gyrus, heart, liver, spleen, and kidney with a ³²P-labeled probe of the 1600-base pair cDNA of *cpg16* as described above. Hybridization was found in the rat brain and rat dentate gyrus, with strong up-regulation of the *cpg16* message after treatment with kainic acid. In most of the other tissues examined, no expression of *cpg16* was detected, although a weak signal could be seen in the kidney (Fig. 1). Because a similar (but not identical) sequence was recently detected in a mouse skin library, the expression of

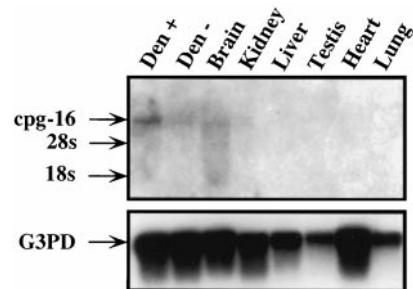


FIG. 1. Tissue distribution Northern blot of *cpg16*. A Northern blot containing 5 μ g of poly(A⁺) RNA from kainate-treated dentate gyrus (*Den* +), untreated dentate (*Den* -), total brain, kidney, liver, testis, heart, and lung was probed with the *cpg16* cDNA clone (*upper panel*) and with a glyceraldehyde 3-phosphate dehydrogenase (*G3PD*) probe as a control (*lower panel*).

this gene may not be limited to the brain, and it could play a role in additional tissues.

Expression of *cpg16* in Bacteria and in Mammalian Cells—The predicted open reading frame of *cpg16* encodes a putative protein of approximately 50 kDa. Based on sequence homology, the primary structure of CPG16 predicted a protein serine/threonine kinase containing all 15 characteristic amino acids of these kinases (13). To determine whether CPG16 is indeed a 50-kDa functional protein kinase and to characterize its enzymatic properties, *cpg16* was fused in-frame with cDNA of GST, expressed in *Escherichia coli* and purified using glutathione beads. Coomassie Blue staining of the purified preparation revealed one main protein at 75 kDa (Fig. 2), which corresponded to the expected molecular mass for the GST-CPG16 fusion protein. Another band that could be detected in the purified fraction at the molecular mass of 27 kDa is probably the GST protein alone. In the presence of Mg²⁺ and ATP, the purified GST fusion underwent autophosphorylation in a time-dependent manner, indicating that the *cpg16* cDNA indeed encodes for a functional kinase (Fig. 2). A lower molecular weight band (35 kDa) that was detected upon autophosphorylation most likely represents a degradation product of the GST-CPG16 itself, as judged by its increase when protease inhibitors were omitted from the bacterial extraction buffer in the course of purification (data not shown).

To allow for studies of CPG16 in mammalian cells, the cDNA of *cpg16* was inserted into a mammalian expression vector containing an HA tag. The HA-*cpg16* was transfected into COS7 cells followed by starvation of the cells, lysis, and immunoprecipitation of the HA-containing proteins. One band was detected when the purified preparation was subjected to Western blot analysis with the anti-HA antibody (52 kDa, Fig. 3A), which corresponded to the expected molecular mass of CPG16 fused to HA. Because a band with an identical molecular mass was autophosphorylated upon incubation of the purified proteins with ATP, the identified band is most likely the autophosphorylated HA-CPG16.

Substrate Specificity of CPG16—CPG16 with both HA and GST tags showed low (8 nmol/min/mg) autophosphorylation activity. For a clue regarding a possible physiological substrate(s) for CPG16, we performed an *in vitro* substrate specificity assay in which we tested a group of peptides, proteins, and cytosolic extracts for their ability to be phosphorylated by CPG16. Using Mg²⁺ and ATP as cofactors, CPG16 appeared to have a restricted specificity, as it showed activity only toward MBP at a low rate of 12 nmol/min/mg (Table I and Fig. 3). This specificity of CPG16 was most apparent when denatured brain extract, which contains thousands of proteins, was used as a substrate. In most of the experiments performed, CPG16 failed to phosphorylate any of the proteins in the tested preparation

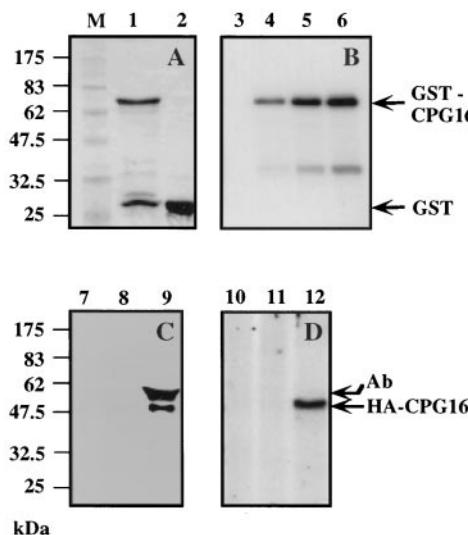


FIG. 2. Expression and autophosphorylation of GST-CPG16 and HA-CPG16. *A*, purification of GST-CPG16 from BL21(DE3) bacteria was as described under “Experimental Procedures.” The following samples were loaded on a 10% SDS-polyacrylamide gel electrophoresis, which was stained with Coomassie Blue: *lane 1*, purified GST-CPG16; *lane 2*, purified GST. *B*, time course of GST-CPG16 autophosphorylation. Autophosphorylation reaction was carried out as described under “Experimental Procedures,” and aliquots from the reaction were sampled at the following time intervals and examined by SDS-polyacrylamide gel electrophoresis and autoradiography: *lane 3*, time 0; *lane 4*, 5 min; *lane 5*, 15 min; and *lane 6*, 40 min. *C*, immunoprecipitation and Western blot detection of either HA-cpg16 or vector control-transfected COS-7 cells. Transfection, immunoprecipitation with anti-HA monoclonal antibody, and immunodetection with anti-HA polyclonal antibody were carried out as described under “Experimental Procedures.” Samples from the following treatments of COS7 cells were loaded: *lane 7*, nontransfected cells; *lane 8*, vector control-transfected cells; *lane 9*, HA-cpg16-transfected cells. *D*, autophosphorylation of HA-cpg16 immunoprecipitated from transfected COS-7 cells. HA-CPG16 was immunoprecipitated using anti-HA monoclonal antibody from the following COS7 cells and subjected to autophosphorylation reaction as described under “Experimental Procedures.” *Lane 10*, nontransfected cells; *lane 11*, vector control-transfected cells; and *lane 12*, HA-cpg16-transfected cells. The location of GST-CPG16, GST alone (GST), HA-CPG16, and the HA antibodies (Ab) are indicated.

(Fig. 3), although in some of the experiments a phosphorylated 45-kDa band could be detected upon phosphorylation with CPG16 (data not shown). Phosphoamino acid analysis of the autophosphorylated GST-CPG16 and of CPG16-phosphorylated MBP revealed that the phosphorylation occurred only on serine and threonine (Fig. 3B). This is consistent with the predicted protein serine/threonine kinase sequence of *cpg16* (2, 13).

When compared with other protein kinases, CPG16 shows the highest homology to members of the Ca^{2+} /calmodulin-dependent protein kinases and in particular to Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII). Interestingly, CaMKII was shown to be a key player in hippocampal plasticity (14, 15), mainly by affecting the transcription factor CREB (16). We therefore examined whether Ca^{2+} and calmodulin are capable of modulating the autophosphorylation activity of CPG16. We found that neither Ca^{2+} and calmodulin nor the CaM kinase inhibitors EGTA, calmidazolium, and W7 had any effect on the autophosphorylation of the bacterially expressed GST-CPG16 (Fig. 4 and data not shown). Similarly, there was no effect of Ca^{2+} or calmodulin on GST-CPG16 activity toward any of the substrates examined in *in vitro* phosphorylation assays (data not shown). Moreover, when the sequence of *cpg16* was aligned with the sequence of CaMKII, the calmodulin binding region of CaMKII differed from the corresponding region in CPG16 and predicted a cryptic calmodulin-binding site. Indeed, GST-CPG16 did not bind to a calmodulin-agarose column under any

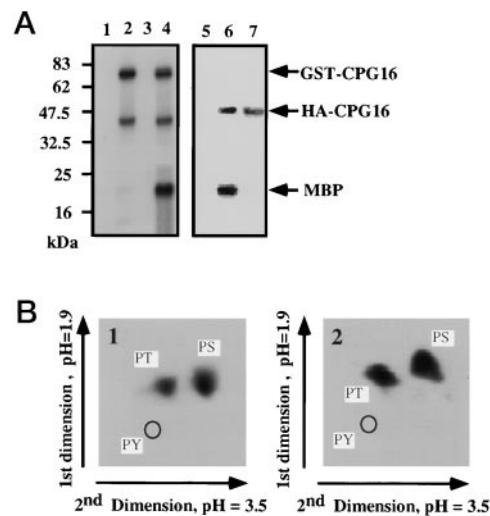


FIG. 3. In vitro phosphorylation by CPG16 and phosphoamino acid analysis. *A*, autophosphorylation, phosphorylation of MBP, and of total denatured brain extract. *In vitro* kinase assay is shown with buffer control (*lanes 1* and *3*), bead-conjugated GST-CPG16 (*lanes 2* and *4*), bead-conjugated HA-CPG16 (*lanes 6* and *7*), and control immunoprecipitation (no HA antibodies, *lane 5*) from HA-cpg16-transfected COS7 cells. The following substrates were incorporated in the assays: *lanes 4* and *6*, MBP (10 μg); *lane 7*, denatured (65 °C, 10 min) brain extract. The reaction was performed as described under “Experimental Procedures.” *B*, 1, phosphoamino acid analysis of autophosphorylated GST-CPG16; 2, phosphoamino acid analysis of MBP phosphorylated by HA-CPG16. PT, phosphothreonine; PS, phosphoserine; PY, phosphotyrosine).

TABLE I
Substrate specificity for CPG-16

In vitro phosphorylation assays were performed as described under “Experimental Procedures.”

Substrate	Activity $\text{nmol}/\text{min}/\text{mg}$
Myelin basic protein (0.33 mg/ml)	12
Hippocampal extracts (denatured)	<0.05
Recombinant Jun N-terminal kinase (12 $\mu\text{g}/\text{ml}$)	<0.05
Recombinant ERK2 (40 $\mu\text{g}/\text{ml}$)	<0.05
Recombinant MAPK/ERK kinase (40 $\mu\text{g}/\text{ml}$)	<0.05
Recombinant p38MAPK (60 $\mu\text{g}/\text{ml}$)	<0.05
Histone III (2 mg/ml)	0.08
β -Casein (2 mg/ml)	<0.05
Protamine (2 mg/ml)	0.08
K-7 (80 $\mu\text{g}/\text{ml}$)	<0.05
Vitronectin (40 $\mu\text{g}/\text{ml}$)	<0.05
Myosin light chain (0.33 mg/ml)	<0.05
GST-Jun1-91	<0.05
GST-ATF2 16-96	<0.05
GST-Rho (0.2 mg/ml)	<0.05
GST-ARF (60 $\mu\text{g}/\text{ml}$)	<0.05
MAP2 preparation (0.80 mg/ml)	0.1
EGF receptor peptide (0.66 mg/ml)	<0.05
GSK3 peptide (0.80 mg/ml)	<0.05
Jun peptide (0.80 mg/ml)	<0.05
Far1 peptide (0.33 mg/ml)	<0.05
PLA2 peptide (0.66 mg/ml)	<0.05

condition tested (data not shown), strongly suggesting that CPG16 is not dependent on Ca^{2+} or calmodulin for its direct enzymatic activity.

CPG16 Is Stimulated by cAMP in COS7 Cells—Because the activity of many protein serine/threonine kinases can be increased in response to extracellular agents, we undertook to determine whether the low basal activity of CPG16 can be modulated by any extracellular stimuli. Thus, COS7 cells were transiently transfected with HA-CPG16, serum-starved, and treated with agents that activate intracellular signaling pathways. HA-CPG16 was then immunoprecipitated using anti-HA

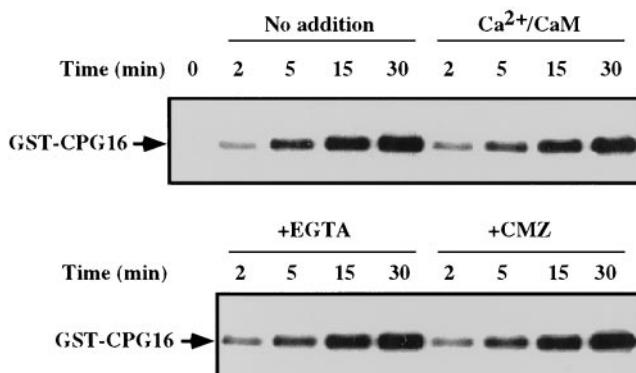


FIG. 4. Effect of Ca^{2+} and calmodulin on the autophosphorylation of CPG16. Autophosphorylation of GST-CPG16 was carried out as described under “Experimental Procedures” with no addition (upper left), with $10 \mu\text{M}$ CaCl_2 and $30 \mu\text{M}$ calmodulin (upper right), with $10 \mu\text{M}$ CaCl_2 , $5 \mu\text{M}$ calmodulin, and $100 \mu\text{M}$ EGTA (lower left), and with $10 \mu\text{M}$ CaCl_2 , $30 \mu\text{M}$ calmodulin, and $125 \mu\text{M}$ calmidazolium (CMZ, lower right).

antibodies and subjected to *in vitro* kinase assays. Interestingly, both forskolin and 8-Br-cAMP agents, which increase the intracellular levels of cAMP, caused a substantial transient activation of CPG16. Specifically, HA-CPG16 purified from forskolin-treated cells exhibited a 6–8-fold increase in its autophosphorylation activity, which peaked at 30 min after treatment and decreased again to basal levels within 60–90 min after stimulation (Fig. 5). 8-Br-cAMP and dibutyryl cAMP yielded similar results (Fig. 5 and data not shown), although the time course of activation in response to 8-Br-cAMP was somewhat slower and peaked at around 45 min after treatment. The addition of isobutylmethylxanthine to inhibit phosphodiesterases and further increase cAMP had only a small effect on the kinetics of the phosphorylation of HA-CPG16.

Other extracellular agents that were used to identify stimulators or activators of HA-CPG16 included the Ca^{2+} ionophore, A23187, the protein kinase C activator phorbol-12-myristate-13-acetate, the protein-tyrosine phosphatase inhibitor vanadate, epidermal growth factor, and FCS. All these compounds are known to activate several downstream signaling pathways including MAPK cascades and others but had no detectable effect on the autophosphorylation activity of CPG16 (Fig. 5 and data not shown). Moreover, even after purification from stimulated cells, there was no increase of CPG16 activity toward any of the *in vitro* substrates examined besides MBP (data not shown).

CPG16 Is Activated Downstream of PKA—From the above experiments performed on COS7 cells, it appears that CPG16 is exclusively activated by cAMP-elevating agents, which transmit their signals primarily through the activation of the protein kinase PKA. Therefore, we used the specific PKA inhibitor H89 to examine whether PKA is indeed involved in CPG16 activation. Thus, H89 was added to the examined cells before stimulation by forskolin or 8-Br-cAMP (17), and the autophosphorylation activity of CPG16 was determined as described above. Our results show that H89 significantly inhibited the forskolin stimulation of CPG16 when added to the transfected COS7 cells 15 min before stimulation (Fig. 6). Therefore, these results clearly indicate the involvement of PKA in the activation process of CPG16.

We then examined whether CPG16 can be a direct substrate of either PKA or ERK/MAPK that was suspected to phosphorylate a PXSP consensus sequence in the N terminus of CPG16 (18, 19). Although both the catalytic subunit of PKA and activated ERK2 were very active in phosphorylating vitronectin and MBP, respectively, no phosphorylation of purified HA-

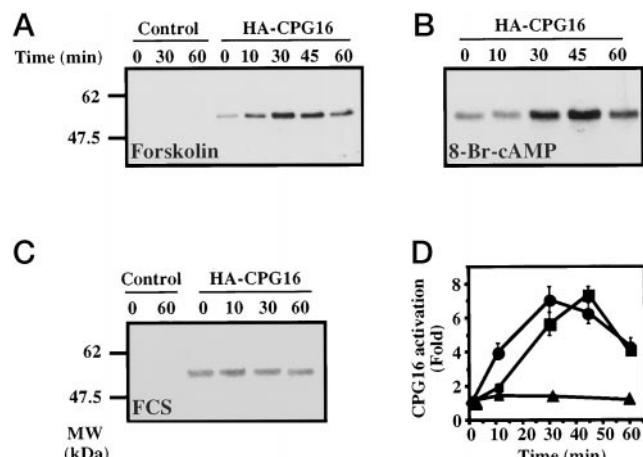


FIG. 5. Effect of cAMP on CPG16. COS7 cells were transfected with either HA-cpg16 (panels A, B, and C, HA-CPG16) or vector control (A and C, Control), allowed to grow for 36 h, and then serum-starved for an additional 16 h. Then the cells were treated for the indicated times with Me_2SO (panels A and B, time 0), with forskolin ($10 \mu\text{M}$, panel A, both in Control and HA-CPG16), with 8-Br-cAMP (panel B), with FCS (10%, panel C, 10, 30, and 60 min, both in Control and HA-CPG16), or left untreated (panel C, time 0). The cells were then harvested, and the autophosphorylation activity of CPG16 was determined as described under “Experimental Procedures.” The amount of HA-CPG16 precipitated was determined in some of the experiments by Western blot with anti-HA polyclonal antibody and was found to be equal among the different points (data not shown). The graphs (panel D) represent the average of the densitometric readings of the autophosphorylated HA-CPG16 from three similar experiments. MW, molecular mass.

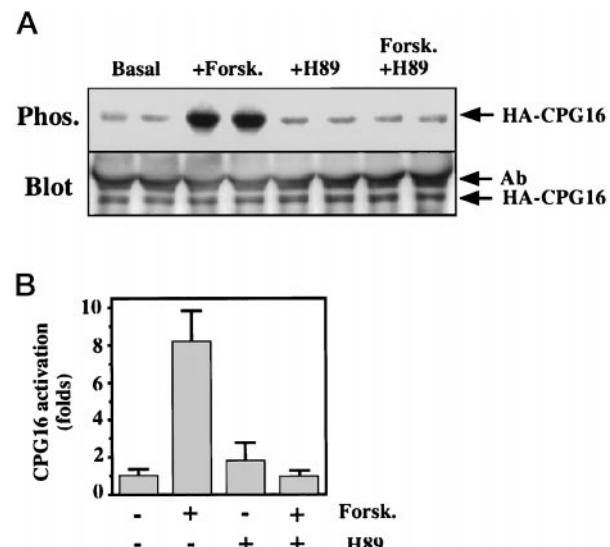


FIG. 6. Inhibition of forskolin activation of CPG16 by H89. A, COS7 cells were transfected with HA-cpg16, then allowed to grow for 36 h and serum-starved for an additional 16 h. Then, the cells were treated with $10 \mu\text{M}$ forskolin for 30 min (+Forsk.) or pretreated with $4 \mu\text{M}$ H89 (CalBiochem) for 15 min and then treated with forskolin (Forsk. +H89) or left untreated (+H89). Autophosphorylation activity of HA-CPG16 was determined as described under “Experimental Procedures.” The results of the autophosphorylation reaction (Phos.) are shown in the upper panel. The amount of immunoprecipitated HA-CPG16 was determined by Western blotting (Blot) using anti-HA polyclonal antibody (Ab). These results are shown in the lower panel, two lanes for each treatment. B, quantitation of the experiment. The results are the mean of two distinct experiments that were done in duplicate.

CPG16 by these kinases could be detected even after a prolonged phosphorylation reaction (Fig. 7). Recombinant active MAPK/ERK kinase (7) and casein kinase II also failed to phosphorylate HA-CPG16 (data not shown), and CPG16 had no apparent effect on all four tested kinases. Therefore, the acti-

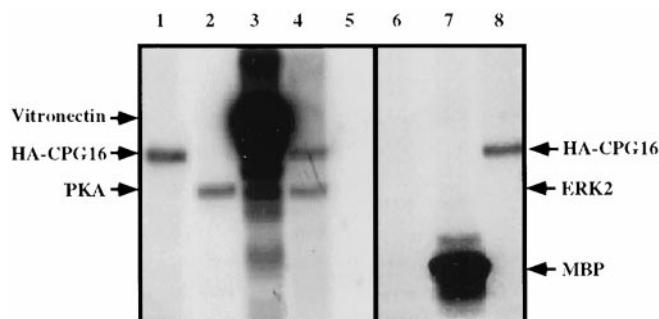


FIG. 7. **Phosphorylation of CPG16 by PKA and ERK2.** Phosphorylation reactions were performed as described under "Experimental Procedures" with the following components: 1, immunoprecipitated HA-CPG16 ($\sim 0.2 \mu\text{g}$); 2, PKA ($0.1 \mu\text{g}$); 3, PKA + vitronectin ($1 \mu\text{g}$); 4, HA-CPG16 + PKA; 5, buffer alone; 6, active ERK ($0.05 \mu\text{g}$); 7, active ERK + MBP ($5 \mu\text{g}$); 8, HA-CPG16 + active ERK. Reactions proceeded for 30 min before inactivation by boiling in a sample buffer.

vation of CPG16 may occur by a cAMP-dependent PKA-induced mechanism (probably a kinase cascade) similar to the activation of phosphorylase kinase downstream of PKA (20).

Studies on a Possible Role of CPG16 in the Nucleus—To obtain information on the subcellular localization of CPG16, we performed immunolocalization studies in COS7 and in NIH-3T3 cells transiently transfected with HA-*cpg16*. In both cell types, most of the HA staining was detected in the cytosol, although some enrichment in nuclear staining could be observed after 8-Br-cAMP and forskolin stimulation of the cells (Fig. 8 and data not shown). This nuclear enrichment could not be detected when the cells were stimulated with FCS under conditions in which ERKs are translocated to the nucleus (7). Although mostly cytosolic, the limited translocation indicated that CPG16 may have a nuclear role upon cAMP elevation.

The translocation of CPG16 to the nucleus prompted the notion that it might participate in the regulation of transcription. Because the phosphorylation of CPG16 is affected by cAMP, we tested the possibility that overexpression of CPG16 would influence gene expression through the transcription factor CREB. Madin-Darby canine kidney cells expressing a stable luciferase reporter construct containing five copies of the CRE element were stimulated with various cAMP-elevating agents, and their luciferase reporter activity was determined (12). As expected, activation of these cells with forskolin, 8-Br-cAMP, epidermal growth factor, and isoproterenol but not calcium ionophore, peroxovanadate, and phorbol ester (phorbol-12-myristate-13-acetate) increased the activity of the luciferase reporter gene (Fig. 9). However, when these cells were transfected with a large amount of expression vector containing the cDNA for CPG16 ($5 \mu\text{g}/\text{plate}$), a moderate inhibition compared with the control vector was detected in the luciferase activity (Fig. 9). Lesser amounts of the vector containing the CPG16 did not have a significant effect on this activity. Thus, it is possible that CPG16 participates in the down-regulation of cAMP-induced transcription, although the small amount of inhibition may suggest that this inhibition might be an indirect process.

DISCUSSION

Glutamate receptors produce long term plasticity changes in central nervous system neurons, at least in part by induction of transcription cascades (21, 22). Glutamate-activated transcription factors induce a first wave of gene expression, the immediate-early gene, many of which encode transcription factors, for example, *c-fos* (23). The immediate-early gene products in turn induce hundreds of downstream genes, many of which are thought to produce plastic changes in brain neurons and therefore are known as candidate plasticity-related genes (CPGs)

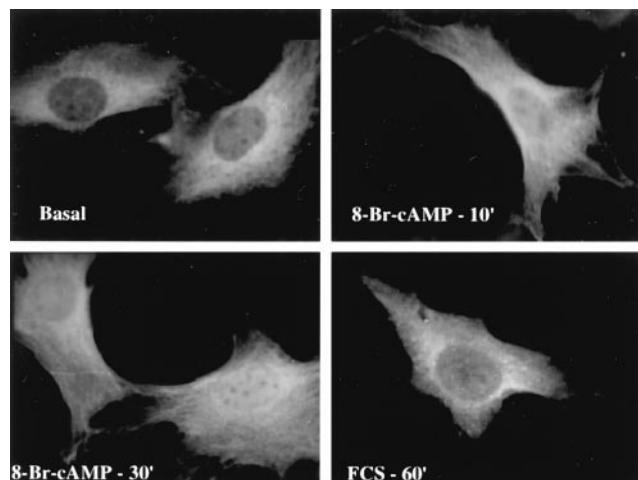


FIG. 8. **Localization of HA-CPG16 in NIH-3T3.** NIH-3T3 cells transfected with HA-*cpg16* were grown on coverslips for 36 h after transfection and serum-starved for an additional 16 h. Then the cells were stimulated with 8-Br-cAMP ($10 \mu\text{M}$, 10 and 30 min), with FCS (10%, 60 min), or left untreated (control). The cells were fixed and stained with anti-HA monoclonal antibody as described under "Experimental Procedures." Staining of HA-CPG16-transfected cells with secondary antibody alone or staining of untransfected cells did not result in any significant fluorescence (data not shown).

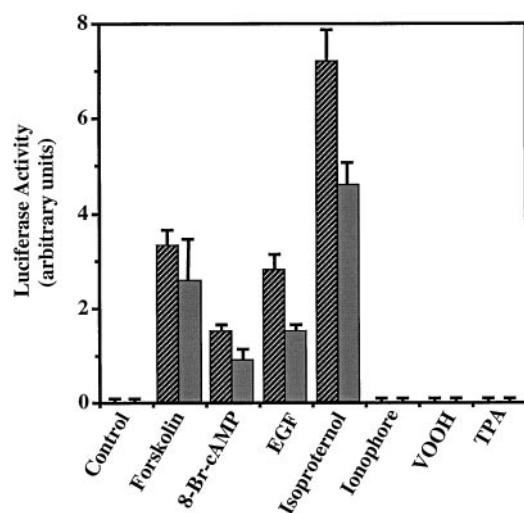


FIG. 9. **Effect of CPG16 on cAMP-induced activation of CREB.** Madin-Darby canine kidney cells expressing a stable luciferase reporter construct containing five copies of the CREB element were transfected by electroporation with an expression vector ($5 \mu\text{g}$) containing HA-*cpg16*. Six h after transfection, the cells were serum-starved, and after a total of 24 h, the following stimulants were added: forskolin ($10 \mu\text{M}$), 8-Br-cAMP ($10 \mu\text{M}$), epidermal growth factor (EGF, 50 ng/ml), isoproterenol ($10 \mu\text{M}$), A23187 (Ionophore, 20 μM), peroxovanadate (VOOH, 100 μM), and 12-O-tetradecanoylphorbol-13-acetate (TPA) ($100 \mu\text{M}$). Luciferase activity was determined as described under "Experimental Procedures."

(1)). Recently, Hevroni *et al.* (2) identified about 400 genes that are either induced or down-regulated upon kainate (glutamate analog) induction. One of the most intriguing genes identified was CPG16, which demonstrated sequence homology with the plasticity-related CaMKII.

In this study we tried first to check whether or not CPG16 is indeed a Ca^{2+} /calmodulin-dependent kinase. A careful analysis of the C-terminal region of CPG16 revealed that six amino acids essential for calmodulin binding are missing. Indeed, we found that CPG16 failed to bind to a calmodulin-agarose column, indicating that the cryptic site was not sufficient to promote calmodulin binding. Moreover, no effect of Ca^{2+} or cal-

modulin on the *in vitro* or *in vivo* activity of CPG16 could be detected (Fig. 4). Therefore, our results clearly indicate that CPG16 is regulated by a different mechanism than that involved in the activation of Ca^{2+} /calmodulin-regulated kinases.

Although not influenced by calmodulin and Ca^{2+} , a different regulator was identified when COS7 cells were transiently transfected with CPG16. Of several stimuli that were tested for their ability to modulate CPG16 activity immunoprecipitation, cAMP-elevating agents were shown to have a stimulatory effect. Thus, forskolin or 8-Br-cAMP increased CPG16 autophosphorylation activity by about 6–8-fold over its basal activity, whereas other stimuli did not have any effect. The H89 inhibitor, which selectively inhibits PKA activity, abolished the cAMP-dependent stimulation, indicating that CPG16 acts downstream of PKA. However, CPG16 could not be phosphorylated or directly activated by PKA *in vitro*. Moreover, the activation observed was much smaller than the activation of PKA itself, which under similar conditions may usually reach more than 50-fold. Taken together, these data suggest that CPG16 may be activated by a PKA-induced mechanism that could be a PKA-induced kinase cascade. Activation because of phosphorylation by an intermediate kinase is likely also because of the existence of threonine residue in the activation loop of CPG16 that is analogous to the activation sites of many activated protein kinases such as ERK and MAPK/ERK kinase (8). However, at this stage, the direct mechanism of activation is not clear. The relatively small activation of 6–8-fold could be explained by higher susceptibility to modulating enzymes such as phosphatases. An alternative explanation could be a possible dependence on cofactors or intermediate molecules that may be essential to secure full activation but are not expressed in high quantities in the COS7 cells. It is also possible that the activation toward the real substrate of CPG16 is higher than the activation of its autophosphorylation or activity toward MBP.

The involvement of cAMP in neuronal plasticity is well established, and many studies have documented the involvement of this second messenger in the synaptic plasticity of memory and behavior (25). cAMP is known to transmit its signals primarily through several PKA isoforms that may function either in the cytosol or translocate to the nucleus and regulate processes there (26). In the cytosol, the PKAs may regulate metabolic as well as protein synthesis processes (26). However, the main function of PKA in the nucleus is to regulate the transcription of cAMP-dependent genes, which is mediated primarily by phosphorylation of the transcription factor CREB (16). In this regard, the induction of CPG16 by kainate and its activation by cAMP may indicate that this kinase is one of the components that participates in plasticity determination. However, unlike overexpression of the catalytic subunit of PKA, the overexpression of CPG16 seems to slightly inhibit the cAMP-stimulated CREB activity. Therefore, unlike the stimulatory effect of PKA, CPG16 is likely to participate in the down-regulation of cAMP signals in the nucleus. Inhibitory processes have been shown in all levels of the PKA cascades, including the down-regulation of receptors by phosphorylation, hydrolysis of cAMP by phosphodiesterases, or phosphorylation/dephosphorylation inhibition of PKA targets. Interestingly, cAMP-regulated protein phosphatase activity has been shown recently to down-regulate CaMKII activity during long-term potentiation (27). Therefore, CPG16 may participate in the regulation of CREB either by phosphorylating its inhibitory residues or alternatively, via the activation of phosphatases that are involved in the inactivation of CREB.

Although the role of CPG16 in the nucleus is intriguing, it should be noted that its localization is primarily cytosolic, and only a small amount of nuclear translocation was observed

upon 8-Br-cAMP treatment of cells. Therefore, CPG16 probably phosphorylates and modulates the activity of other downstream targets mainly in the cytosol; however, these targets have yet to be identified. An intriguing clue regarding a possible substrate for CPG16 came from the recent identification of KIAA0369 (28). This cDNA was found to have a high degree of identity to both CPG16 and doublecortin (9, 24), the latter is linked to the genetic disease x-linked lissencephaly and double cortex syndrome. It is therefore possible that CPG16 is involved in the regulation of doublecortin itself or to one of its related proteins; however, more work is needed to confirm this hypothesis.

In summary, we have characterized a neuronal plasticity-related protein kinase, CPG16. Although it shows high sequence homology to Ca^{2+} /calmodulin protein kinases, we have found that it is not influenced by Ca^{2+} or calmodulin but is activated by cAMP-elevating agents. However, CPG16 is not an *in vitro* substrate for PKA, suggesting that it may be activated by a PKA-dependent cascade. CPG16 was also shown to translocate into the nucleus upon stimulation and to have a weak inhibitory effect on cAMP-stimulated transcriptional activity of CREB. Therefore, CPG16 may participate in the down-regulation of cAMP signals toward plasticity-related genes.

Acknowledgments—We thank Drs. Muriel Zohar, Zeev Gechtman, and Elly Nedivi for valuable discussions and comments on the manuscript as well as Tami Hanoch for her skillful technical assistance and Drs. Uri Gat and David De graaf for their assistance with the CREB-luciferase assays.

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