A cDNA Encoding Protein Kinase C Identifies Two Species of mRNA in Brain and GH₃ Cells*

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Antiserum raised against purified protein kinase C (the Ca²⁺/phospholipid-dependent enzyme) (Ballester, R., and Rosen, O. M. (1985) J. Biol. Chem. 260, 15194–15199) was used to screen a rat brain cDNA library in the prokaryotic expression vector λgt11. Three positive clones were isolated and shown to have overlapping restriction endonuclease maps. The positive recombinant phage with the longest cDNA insert (1.4 kilobases (kb)) was used for production of a β-galactosidase fusion protein. Rabbit antiserum raised against the fusion protein recognized a single rat brain polypeptide of Mr 80,000 which was identified as protein kinase C by the following criteria: (i) electrophoretic co-migration with purified protein kinase C, (ii) partial co-purification with protein kinase C, and (iii) disappearance from the cytosol of phorbol 12-myristate 13-acetate-treated GH₃ cells. The nick-translated cDNA hybridized with two mRNAs, 8 kb and 3.5 kb, whose tissue distribution was in agreement with that reported for protein kinase C activity. Hybrid selection with immobilized cDNA identified mRNA encoding a protein of Mr 80,000 that could be precipitated by antibody to purified protein kinase C. Treatment of GH₃ cells with phorbol 12-myristate 13-acetate, which promotes translocation and subsequent degradation of protein kinase C, did not alter the level of either message.

Protein kinase C, a Ca²⁺/phospholipid-dependent enzyme activated by phosphatidylserine, diacylglycerol, and Ca²⁺, is the cellular receptor for tumor-promoting phorbol esters (1–5). The biologically active ester, phorbol 12-myristate 13-acetate (PMA), and certain hormones that stimulate the release of diacylglycerol from phosphatidylinositol (6,7) trigger association of the predominantly cytosolic kinase with the plasma membrane. The translocation, activation, and subsequent turnover of the enzyme have been assessed by measuring enzyme activity (8–10), phorbol ester binding (see Ref. 11 for review), and, most recently, by immunoprecipitation of metabolically labeled enzyme with an antibody elicited to purified rat brain protein kinase C (12). Since all studies of the modulation of protein kinase C have of necessity analyzed only biochemical and immunological properties of the enzyme, it was important to develop probes to evaluate potential transcriptional regulation. For this reason, the antiserum prepared to purified protein kinase C (12), which recognizes only protein kinase C in an immunoblot of rat brain cytosol, was used to screen a rat brain cDNA library in the prokaryotic expression vector λgt11. We now report isolation of a cDNA clone encoding a portion of protein kinase C and the use of this probe to detect two mRNAs whose cellular distribution is consistent with that of protein kinase C activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—RNasin, λgt11 bacteriophage and Escherichia coli host strains Y1090 and Y1089 were purchased from Promega Biotech. Nucleoside triphosphates and PMA were from P-L Biochemicals. DNA polymerase I and restriction enzymes were obtained from New England Biolabs. International Biotechnologies, Inc. was the source of cesium chloride and IPTG. Guanidine isothiocyanate was purchased from Fluka and oligo(dT)-cellulose was from Collaborative Research. Proteinase K was from Bethesda Research Laboratories. Nitrocellulose sheets (BA 85, 0.45 μm) were obtained from Schleicher & Schuell. All radiomonomoieties were from Amersham Corp. Grand Island Biological Co. was the source of RPMI 1640 media. Protein A-Sepharose CL-4B, bovine serum albumin (fraction V), Nonidet P-40, pepstatin, leupeptin, and aprotinin were all from Sigma. Soybean trypsin inhibitor was supplied by Boehringer Mannheim.

**Preparation of β-Galactosidase Fusion Proteins and Fusion Protein Antiserum**—E. coli strain Y1089 was infected for 20 min at 32°C with recombinants immunoselected with antibody to either protein kinase C or, as a control, to the human erythrocyte glucose transporter. Infected cells were plated at low density and incubated 16 h at 32°C. Single colonies were replica-plated, and lysogens that grew at 32°C but not 42°C were selected and grown to an OD₆₅₀ of 0.5. Lysogens were induced at 45°C for 20 min, followed by incubation for 1 h at 37°C in the absence or presence of 10 mM IPTG (15).

Rabbits were immunized perilymphnodally with SDS-polyacrylamide gel pieces containing 40 μg of fusion protein homogenized in complete Freund's adjuvant. Subsequent injections of either purified fusion protein or E. coli lysate were delivered intramuscularly with incomplete adjuvant. Data shown here were obtained using antiserum from one rabbit following the third boost.

For immunoblotting, proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper (16). The blots were incubated for 2 h with antiserum directed against either purified protein kinase C (1:100 dilution), human glucose transporter (1:75 dilution), fusion protein (1:25 dilution), or preimmune serum of appropriate dilution. Following incubation with IgG-protein A for 30 min, the blots were dried and subjected to radioautography at −70°C with an intensifying screen.

**Cell Culture and Preparation of Cell Extracts**—GH₃ cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum, 2 mM glutamine, and 7.5 mM Hepes buffer, pH 7.5. Cells were main-
tained in monolayer culture for 5-10 days before use. Prior to PMA treatment, cells were incubated with fresh media for 18 h. Either 400 nM PMA or dimethyl sulfoxide (the vehicle for PMA) was then added directly to the culture medium. Cell extracts were prepared by scraping the monolayers into homogenization buffer (5 mM NaCl, 5 mM Tris buffer (pH 7.5), 10 mM EGTA, 5 mM MgCl₂, 2 mM dithiothreitol) in the presence of protease inhibitors, aprotinin, leupeptin, pepstatin, and soybean trypsin inhibitor) as previously described (15). Cells were disrupted by repeated passage through a 26-gauge needle. Lysates were centrifuged at 100,000 × g for 1 h to obtain the high speed supernatant fluid. Partial purification of protein kinase C was achieved by applying this fraction to a DEAE-52 column (equilibrated with 20 mM Heps buffer (pH 7.5), 5 mM EGTA, 2 mM EDTA, and 2 mM dithiothreitol) and collecting the 0.1 M NaCl eluate as previously described (17).

Analysis of RNA and DNA—RNA was isolated from tissues by homogenization in guanidine isothiocyanate and centrifugation through 5.7 M cesium chloride (18) or from GH3 cells by Nonidet P-40 lysis in the presence of RNasIn, followed by proteinase K digestion and phenol/chloroform extraction (19). Further purification was achieved by oligo(dT)-cellulose chromatography (20).

RNA was denatured, subjected to electrophoresis in a formaldehyde-agarose gel, transferred to nitrocellulose, and incubated with ³²P-labeled nick-translated probe (21) at 42 °C in 50% formamide, 2 × Denhardt’s solution, 5 × SSC (SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), and 10% dextran sulfate. The nitrocellulose filter was washed at 50 °C with 0.1% SDS and 0.1 × SSC.

For the hybridization selection, DNA was denatured, immobilized on nitrocellulose filters, and hybridized to poly(A)⁺ RNA at 50 °C for 3 h (22). The selected RNA was eluted and translated with a rabbit reticulocyte lysate kit from Promega Biotec. Immunoprecipitation of the translated protein was performed as previously described (12).

For the Southern hybridization, high molecular weight DNA (23) was digested with the indicated restriction endonuclease, subjected to electrophoresis in 1% agarose, transferred to nitrocellulose, and hybridized to the nick-translated probe (24). Hybridization was carried out at 42 °C, and the filter was washed at 50 °C as described above.

RESULTS AND DISCUSSION

Ten positive clones were identified by screening 1.5 × 10⁵ plaques with the second antibody-biotin system of Vector Laboratories. DNA was prepared from three of the bacteriophage, and the inserts were shown to have overlapping restriction endonuclease maps. To confirm the identity of the positive clones, a β-galactosidase fusion protein was prepared by infecting E. coli Y1089 with the Xgt11 bacteriophage containing the largest insert (1.4 kb) that produced immunoreactive protein. The Coomassie Blue staining pattern of the fusion protein in a lysate from E. coli induced by IPTG is shown in Fig. 1a. The corresponding immunoblot with antibody raised against purified protein kinase C (which does not recognize β-galactosidase) is shown in Fig. 1b. The largest fusion protein synthesized had an M₀ of 150,000 and is indicated by the arrow. Immunoreactive bands of lower M₀ are probably degradation products. A similar radioautogram was obtained when the blot was incubated with antibody directed against β-galactosidase (data not shown). Fig. 1c shows the immunoblot of a fusion protein prepared with a phage containing the C-terminal 10 kilodaltons of the rat brain glucose transporter and probed with antiserum against the human erythrocyte transporter (25). The Coomassie Blue staining pattern of this lysogen lacked the major immunoreactive protein of M₀, 150,000 shown in Fig. 1a. Thus the M₀, 150,000 fusion protein appears unique to the putative protein kinase C clone. The transporter fusion protein was used as a negative control throughout the present study.

Antibodies were prepared to the 150,000 kilodalton fusion protein as well as to the glucose transporter fusion protein by immunizing rabbits with either E. coli lysate or fusion protein purified by SDS-polyacrylamide gel electrophoresis (100–200 μg of fusion protein per injection). An immunoblot of rat brain cytosol (Fig. 2a) showed that a protein of M₀, 80,000 was

![Fig. 1. Preparation of β-galactosidase fusion proteins. E. coli strain Y1089 was infected with the recombinants immunoselected with antibody to either protein kinase C (a and b) or to the human erythrocyte glucose transport (c). Lysogens were induced in the absence (−) or presence (+) of 10 mM IPTG. a shows Coomassie Blue stain of lysogens infected with the protein kinase C–immunoselected phage containing the 1.4-kb insert. Lysates were subjected to electrophoresis in a 7.5% SDS-polyacrylamide gel. b is a radioautogram of an immunoblot of lysogens from panel a. Proteins were transferred to nitrocellulose (16), and the blot was incubated with antibody to purified protein kinase C (12) followed by ¹²⁵I-protein A. c is a radioautogram of an immunoblot of a β-galactosidase fusion protein containing the C-terminal region of the rat brain glucose transporter. The blot was incubated with an antibody to human erythrocyte glucose transporter (25). Each lane in panels a–c contains 100 μg of E. coli lysate. The arrow indicates the largest fusion protein synthesized by each culture.

![Fig. 2. Immunoblot of rat brain proteins with antibody raised against the protein kinase C-β-galactosidase fusion protein. a, immunoblot of a 100,000 × g supernatant fluid from rat brain homogenate (100 μg of protein per lane). b, immunoblot of partially purified rat brain protein kinase C (30 μg of protein per lane). The protein kinase C was purified by chromatography on DEAE-cellulose, gel filtration and DEAE-trisacryl ion-exchange chromatography as previously described (17). Lanes 1 and 3, preimmune and immune serum, respectively, from a rabbit immunized with the β-galactosidase fusion protein; lane 2, antibody raised against purified protein kinase C.](image-url)
recognized by immune serum (lane 3), but not by preimmune serum from the same rabbit (lane 1) or antisera raised against the transporter fusion product (data not shown). The protein of M, 80,000 co-migrated with the protein recognized by the antibody prepared to purified protein kinase C (lane 2). Several minor bands (including one of M, 44,000) recognized by immune but not preimmune serum were also detected with sera from rabbits immunized against the glucose transporter fusion protein. Immune but not preimmune serum continued to recognize a protein of M, 80,000 in a 10-fold purified protein kinase C preparation (Fig. 2b, lanes 1 and 3). Antisera from rabbits immunized with the glucose transporter fusion protein did not cross-react.

To confirm the identification of the immunoreactive 80-kilodalton protein, GH3 cells were treated with 400 nM PMA, a condition which drastically and as far as is known, specifically reduces cellular protein kinase C (12). Cytosolic extracts were immunoblotted with antibody to either purified protein kinase C (Fig. 3a) or the 150-kilodalton fusion protein (Fig. 3b). Both antisera detected the loss of an immunoreactive species, M, 80,000, in response to PMA treatment.

The identification of the cDNA was further supported by Northern blot hybridizations using a nick-translated probe prepared from the 1.4-kb insert that had been subcloned into the EcoRI site of pUC19 (14). The probe hybridized with two RNA species of 3.5 and 8 kb in rat brain poly(A+) RNA (Fig. 4A). The 3.5-kb species, which reveals message levels intermediate between those of brain and the other tissues, is of interest. A message of this size is consistent with that of protein kinase C activity (26–28). Message levels were higher in brain (lane 5) and 5–10-fold lower in the other tissues surveyed (lung, adipose, kidney, and liver, lanes 3, 4, 6, and 7, respectively). The protein kinase C activity of GH3 cells (12) is about 3–5-fold lower than rat brain (17). This is consistent with the Northern blot of GH3 cells (lane 2) which reveals message levels intermediate between those of brain and the other tissues. Using RNA prepared from PMA-treated (Fig. 4b, lanes 2–4, 6) and untreated (Fig. 4b, lanes 1 and 5) GH3 cells, it was evident that treatment with 400 nM PMA did not significantly affect protein kinase C mRNA levels following exposures of up to 1 h (lane 4) or as long as 24 h (lanes 5 and 6). This conclusion is in agreement with the report that synthesis of protein kinase C (as judged by pulse labeling with [35S]methionine) is unchanged in PMA-treated cells (12). The observation that there appear to be two mRNAs is of interest. A message of 3.5 kb would be sufficient to encode a protein of M, 80,000. The two messages might be alternately spliced forms of the same primary transcript or messages from two closely homologous genes. The relative abundance of the two messages appears similar in brain and GH3 cells; it remains to be determined whether this is the case for other tissues in which the level of protein kinase C mRNA is low.

Hybrid selection was performed to show that the mRNA identified by the 1.4-kb insert in fact encodes an 80-kilodalton...
protein (Fig. 5). pUC19 DNA into which the 1.4-kb insert had been subcloned, pUC19 DNA alone, and a control plasmid into which the full-length rat brain glucose transporter cDNA had been subcloned were immobilized on nitrocellulose and hybridized with rat brain poly(A)+ RNA. The RNA thus selected was eluted, translated, and immunoprecipitated with the antibody raised against purified protein kinase C. As shown in Fig. 5, the plasmid containing the 1.4-kb insert selected mRNA encoding an M, 80,000 protein identified as protein kinase C by immunoprecipitation. The M, 80,000 protein was not observed when either the control plasmid or the plasmid containing the glucose transporter cDNA was used for hybrid selection. Thus one or both of the messages observed on Northern blot analysis encode protein kinase C.

Southern analysis of high molecular weight DNA prepared from rat brain using the 1.4-kb cDNA indicates a simple pattern but one with multiple large hybridizing fragments (Fig. 6). This is consistent with either a single large gene or several closely related homologous sequences in the rat genome.

Although the portion of the kinase molecule encoded by the 1.4-kb insert is not known with certainty, it is likely that it includes the phospholipid- and Ca2+-binding domain rather than the kinase domain, since the antibody used to identify the clone recognizes only the intact M, 80,000 species and not the M, 50,000 phospholipid- and Ca2+-independent trypsin-generated fragment of protein kinase C, designated protein kinase M (29–31).

In conclusion, we have isolated a clone for protein kinase C in a Agt11 cDNA library from rat brain. Initial studies indicate that there are two predominant messages that hybridize with this cDNA (3.5 and 8 kb) and that both are more prevalent in brain and a neural-derived cultured cell line than in other tissues examined. This distribution is consistent with that reported for protein kinase C activity. The content of both mRNAs remained unaffected by phorbol ester-induced down-regulation of the enzyme in GH3 cells.