Assessment of Protein Kinase C Isozymes by Enzyme Immunoassay and Overexpression of Type II in Thyroid Adenocarcinoma

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ABSTRACT

A two site enzyme immunoassay which quantitatively identifies types I, II, and III of protein kinase C isozymes has been designed. The soluble protein kinase C isozymes were selectively immobilized by type-specific monoclonal antibodies, MC-1a, -2a, and -3a (H. Hidaka et al., J Biol. Chem., 263: 4523-4526, 1988) which bind to the regulatory domain (NH₂-terminal side) of protein kinase C. The amount of each isozyme was then determined using a horseradish peroxidase-conjugated polyclonal antibody raised against the COOH-terminal peptide of protein kinase C. By adding increasing concentrations of the antigen, the range of the assay proved to be 0.51-51.1, 0.881-8.1, and 0.31-31 nm for types I, II, and III, respectively. This sandwich method was used to determine the level of protein kinase C isozymes in rabbit tissues. Type I was mainly present in the cerebrum and cerebellum; the highest amount of type II isozyme was present in blood platelets [26.0 ± 3.8 (SE) µg/g wet tissue]. We compared the protein kinase C isozyme levels in human normal thyroid gland and thyroid cancer tissues and found that type II protein kinase C specifically increased in thyroid cancer tissues. Immunocytochemical examination using MC-2a revealed that the cytoplasm of the cancer cells showed prominent immunoreactivity for type II isozyme.

INTRODUCTION

Protein kinase C is a widely distributed Ca²⁺- and phospholipid-dependent serine/threonine protein kinase originally purified from rat brain cytosol as a monometric protein with an apparent molecular mass of 80 kDa (1). Protein kinase C has been proposed to play a key role in the complex processes of neoplastic transformation based on the following evidence: (a) protein kinase C is specifically bound and activated by phorbol ester (2); (b) protein kinase C phosphorylates ras oncogene product (3); (c) the specific introduction of cDNAs of protein kinase C isozymes into cultured cells causes overproduction of protein kinase C and disordered growth control (4, 5). Moreover, the significant elevation of protein kinase C activity was observed in human breast tumors (6).

Several laboratories including our own succeeded in cloning protein kinase C and the complete structures of three species, having α, β, and γ sequences, have been clarified by analysis of the cDNA clones obtained from brain and spleen libraries of several mammalian species (7–10). Independent of the molecular cloning, brain protein kinase C was further separated into three immunologically distinguishable fractions, types I, II, and III, on a hydroxyapatite column (11). The biochemical properties of the three fractions, albeit similar, do differ in cofactor dependencies for kinase activity (11, 12). It now seems certain that type I, II, and III protein kinase C isozymes are products of γ-, β-, and α-type genes, respectively, as based on identification of immunocytochemical and in situ hybridization histochemical localization and the expression in COS cells transfected with three types of cDNAs (13–15).

To correlate the expression of protein kinase C isozymes with neoplastic transformation of cells, we attempted to determine the quantity of the three types of isozymes, in various tissues by a two-site EIA method for detecting type I, II, and III protein kinase C. The assay is simple and quantitative, and the sensitivity in detecting each isozyme is high. Evaluation of the levels of protein kinase C isozymes in organ/tissues of rabbit and in normal/malignant tissue of human thyroid gland was made with this EIA system.

MATERIALS AND METHODS

Principle of Immunoassay. Use was made of protein A purified monoclonal antibodies (purified from ascites fluid), MC-1a, -2a, and -3a, which specifically bind to NH₂ terminals of type I, II, and III protein kinase C, respectively, as the capture (plate) antibodies and the anti-COOH-terminal peptide polyclonal antibody, PC-40 (horseradish peroxidase conjugated), as the sandwich cover; each involved use of quantities in excess of antigen.

Preparation of Antibodies. Monoclonal antibodies, MC-1a, -2a, and -3a, were prepared by injecting the purified protein kinase C into mice and the hybridomas were isolated by the standard method of Kohler and Milstein (16) and characterized as described (1). Antiserum against the COOH-terminal peptide of protein kinase C was raised in Japanese White rabbits as follows. The polypeptide, TRHPVPVTTPDQEVIR-NIDQSEFGSFVNSEFLVEYKSV, corresponding to the 40-amino acid sequence of COOH-terminal type II rabbit protein kinase C(10) synthesized by an Applied Biosystem peptide synthesizer model 430A and conjugated with keyhole limpet hemocyanin, was emulsified with an equal volume of Freund's complete adjuvant and injected s.c. at 50–100 sites on the shaven dorsum of rabbits (30 µg antigen/kg body weight). This was repeated at the rate of one injection/week for 6 weeks and the rabbits were bled 10 days after the last injection. The IgG fraction of the antisera was separated by 38% SO₄(NH₄)₂ precipitation and DEAE-cellulose chromatography and was named PC-40. Horseradish peroxidase labeling of PC-40 was performed according to the method of Nakane and Kawaoli (17).

Immunooassay Procedure. Monoclonal antibodies, 0.5 µg in 0.1 ml of buffer A [0.1 M sodium phosphate (pH 7.4), 0.1% NaN₃] were placed in wells of microtiter plates. After overnight incubation at 4°C, excess monoclonal antibody solutions were removed and the wells were washed three times with 0.35 ml of PBS. Each of the monoclonal antibody-coated microtiter plate wells were incubated with 0.35 ml of blocking buffer (5% BSA in buffer A) overnight at 4°C. After three washings with PBS, 0.1 ml of tissue extract sample solutions or various amounts of purified protein kinase C isozymes were added. The wells were incubated for 2 h at 4°C and washed three times with PBS. PC-40 conjugated with horseradish peroxidase (100 ng in 0.1 ml), 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl, and 1% BSA was added to each well and the preparations were incubated for 2 h at 4°C. Each well was washed as described and horseradish peroxidase

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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. 1 Supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan. 2 To whom requests for reprints should be addressed, at Department of Pharmacology, Nagoya University School of Medicine, Showa-ku, Nagoya 466, Japan. 3 The abbreviations used are: cDNA, complementary DNA; EIA, enzyme immunoassay; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
activity bound to the well was assayed. The enzyme reaction was initiated by the addition of 0.1 ml of 0.1 M citrate-phosphate buffer (pH 5.1) containing 3 mM H$_2$O$_2$ and 1 mg/ml of o-phenylenediamine. After 10 min incubation at room temperature, the enzyme reaction was halted by adding 0.1 ml of 2 N H$_2$SO$_4$. The amounts of oxidation of o-phenylenediamine were measured by absorption at a wavelength of 492 nm.

Preparation of Tissue Samples. From the hearts of anesthetized Japanese White rabbits (2.5-3.0 kg), blood was withdrawn and platelets were separated, as described (18). Other tissues were homogenized in cold homogenate buffer [0.05 M sodium phosphate buffer [pH 7.4] containing 1% BSA, 0.1 M NaCl, 20 mM EDTA, 1 mM MgCl$_2$, and 0.001% leupeptin] at 10% wet tissue per volume, using a Teflon homogenizer.

Immunohistochemistry. For light microscopic immunohistochemistry, peroxidase-antiperoxidase staining was performed according to the method of Sternberger (19). Surgically obtained thyroid tissues were fixed in neutralized formaldehyde solution and embedded in paraffin. Sections (4 µm) were made on a sliding microtome and collected on glass slides. After deparaffinization, the sections were stained in the sequence of 2% normal rabbit serum for 30 min; primary antibodies (10 µg/ml of protein concentration) for 2 h; rabbit anti-mouse IgG (DAKO, Copenhagen, Denmark) diluted 1:50 for 1 h; mouse peroxidase-antiperoxidase complex (DAKO) diluted 1:250 for 1 h. All three antibody solutions were made with 50 mM Tris-HCl, pH 7.5-150 mM NaCl containing 1% normal rabbit serum. All the sections were then incubated in Graham Karnovsky's medium for peroxidase which contained 0.05% 3,3′-diaminobenzidine tetrachloride and H$_2$O$_2$ in Tris-HCl buffer, pH 7.2, for 15 min and counterstained with 1% methyl green solution. They were examined and photomicrographed with an Olympus AHB-LB microscope.

RESULTS

EIA Sensitivity and Specificity. The monoclonal and polyclonal antibodies described in “Materials and Methods” exhibited a single band (80 kDa) with the extract of the rabbit brain, by immunoblot analysis (Fig. 1). The polyclonal antibody (PC-40) cross-reacted with all three types of protein kinase C, and monoclonal antibodies MC-1a, -2a, and -3a showed a selective immunoreactivity against each respective enzyme (Fig. 2). Immunoblot analysis of the trypsin-digested protein kinase C revealed that all the monoclonal antibodies bound to the regulatory domain (33 or 35 kDa) of protein kinase C. On the contrary PC-40, an anti-COOH-terminal peptide polyclonal antibody recognized the catalytic domain (48 kDa) of the enzyme (Fig. 3). Using these antibodies, the two site EIA could detect type I, II, and III protein kinase C down to 4.1, 0.65, and 2.5 ng/100 µl of assay, respectively, corresponding to 510, 81, and 310 pmol of protein kinase C, with a minimal cross-reaction with different types of protein kinase C (Fig. 4). The binding of PC-40 to protein kinase C immobilized on a microtiter plate was specifically inhibited by the COOH-terminal peptide of protein kinase C (Fig. 5). Several protein kinases (cyclic AMP-dependent protein kinase, myosin light chain kinase, calmodulin-dependent protein kinase II, casein kinases I and II) gave no signal when tested in the EIA at a concentration of 10 µg/ml (data not shown).

Protein Kinase C Isozyme Contents of Rabbit Tissues. Quantitative estimation of the levels of protein kinase C isozymes in rabbit tissues was carried out by EIA, based on the standard curves of Fig. 4. The detectable level of type I protein kinase C was observed in the cerebrum [14.5 ± 1.1 (SE) µg/g wet tissue] and in the cerebellum [3.5 ± 0.9 µg/g wet tissue]. The contents of type II and III isozymes in the cerebrum were practically equivalent to that of type I (20.2 ± 1.5 and 13.7 ± 1.3 µg/g wet tissue).
EIA OF PROTEIN KINASE C ISOZYMES

1234

80K

48K

45K

35K

33K

dye front

Fig. 3. Epitope mapping of MC-la, -2a, and -3a, and PC-40. Protein kinase C isoforms (25 µg/ml) was digested with trypsin (1 µg/ml) at 30°C for 3 min. Trypsin-treated protein kinase C was electrophoretically transferred to a nitrocellulose membrane after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel). Immunoblot analysis was done with 1 ng/ml monoclonal antibody MC-la (Lane 1), MC-2a (Lane 2), MC-3a (Lane 3), and polyclonal antibody PC-40 (Lane 4). k, molecular mass in thousands.

tissue, respectively). As for type II protein kinase C, blood platelets contained the highest amount (26.0 ± 3.8 µg/g wet tissue) and much lesser amounts of type I and III were present. There were considerable amounts of types II and III in the spleen (7.6 ± 1.0 and 5.8 ± 0.5 µg/g wet tissue, respectively). All these results are summarized in Table 1.

Protein Kinase C Isozyme Contents of Human Thyroid Gland. The amounts of protein kinase C isoforms in normal and malignant tissue samples of human thyroid gland were examined by EIA. Results were shown in Fig. 6. Normal thyroid gland contained 0.63 ± 0.15 µg/g wet tissue of type III protein kinase C and 0.11 ± 0.05 µg/g wet tissue of type II. In contrast with this, the higher amount of type II enzyme was expressed in thyroid cancer tissue, while no significant change of type I and III levels was observed. To confirm this, immunocytochemical investigation was performed. In the histologically normal portion of the thyroid tissue, the epithelial cells of the thyroid follicles were negative for isozyme I and negative or faintly positive for isozyme II. In thyroid papillary adenocarcinoma, the cancer cells were negative for isozyme I, but they showed strong immunoreactivity for isozyme II. In the cancer cells, the reaction product was located in the cytoplasm and the nucleus was mostly negative for it. The difference of the intensity of the immunoreactivity for isozyme II was prominent as shown in Fig. 7.

DISCUSSION

Since protein kinase C was found to be encoded by a multiple gene family, the distinct distribution of protein kinase C isoforms was noted using in situ hybridization techniques (13), Northern blot analysis (9, 10), immunocytochemistry, immunoblot analysis (1), and hydroxylapatite column chromatography (12, 14). However, quantification of isoforms by prescribed methods appears not to be suitable for routine analysis of a limited amount of samples. Such being the case, we developed a two-site EIA method for detecting each protein kinase C isozyme. This EIA method is sensitive to the extent that we...
Table 1  Distribution of cytosolic (A) and membranous (B) protein kinase C isozymes in rabbit tissues

Contents of protein kinase C isozymes in the various tissues were determined by enzyme immunoassay. The soluble protein kinase C fraction (A) was extracted in 50 mM sodium phosphate buffer (pH 7.4) containing 1% BSA, 0.1 M NaCl, 20 mM EDTA, 1 mM MgCl2, and 0.001% leupeptin; then 0.1 ml of the extract (100-500 µg protein) was used for the enzyme immunoassay. The insoluble protein kinase C fraction (B) was extracted from the residue in the extraction buffer containing 0.5% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate. Results are expressed as µg of rabbit brain protein kinase C isozyme equivalents/g wet tissue. Each value represents the mean ± SE of duplicate determinations of four preparations.

<table>
<thead>
<tr>
<th>Type</th>
<th>Cytosol</th>
<th>Membrane</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebrum</td>
<td>10.2 ± 1.1</td>
<td>4.3 ± 0.9</td>
<td>14.5 ± 1.1</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>2.1 ± 1.5</td>
<td>1.4 ± 1.1</td>
<td>3.5 ± 0.9</td>
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<tr>
<td>Lung</td>
<td>–</td>
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<tr>
<td>Heart</td>
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<tr>
<td>Kidney</td>
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<td>Testis</td>
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<td>Skeletal muscle</td>
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<td>Platelet</td>
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* –, kinase level too low to be accurately determined.

**Fig. 6.** The amounts of protein kinase C isozymes in normal thyroid gland and papillary adenocarcinoma. The extraction and determination of protein kinase C isozymes were performed as described in the legend to Fig. 4. Each value represents the mean ± SE (bars) of duplicate determinations of four patients' species. *, P < 0.05.

**Fig. 7.** Immunocytochemistry of human thyroid gland and papillary adenocarcinoma. Light microscopic photographs of human thyroid tissues stained by the peroxidase-antiperoxidase method using the antibody (MC-2a) for protein kinase C (isozyme II). A, histologically normal portion, which is adjacent to the cancer tissue shown in B, of the surgically obtained thyroid gland. Note strong immunoreactivity in the cancer tissue. × 250.
The overproduction of type II isozyme in thyroid cancer cells was confirmed by immunocytochemical examination using MC-2a. Our EIA system is expected to make feasible quantitative analyses of protein kinase C isozymes with even limited amounts of cultured cells and biopsied tissues.

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REFERENCES

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