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CHARACTERIZATION OF PROTEIN KINASE C FROM NORMAL AND TRANSFORMED CULTURED MURINE FIBROBLASTS*

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SUMMARY: Protein kinase C of normal and ras-transformed NIH 3T3 cells was purified by chromatography on TSK DEAE-5PW, threonine-Sepharose, and TSK phenyl-5PW columns. Comparison of the fibroblast enzyme with several types of rat brain protein kinase C by chromatography on a hydroxyapatite column and by immunoblotting, indicates that both normal and transformed fibroblasts possess only one of the four subspecies of protein kinase C which have been identified in brain tissues. This subspecies presumably has the structure encoded by α-sequence or a closely related sequence. No significant difference was seen between those enzymes purified from normal and transformed fibroblasts.

Protein kinase C is a ubiquitous serine and threonine-specific protein kinase whose activity is dependent on Ca^{2+} and phospholipids (1). 1,2-sn-Diacylglycerol, which is formed as a result of the

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Abbreviations used are: EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N,'N'-tetraacetic acid; and SDS, sodium dodecyl sulfate.

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receptor-mediated hydrolysis of inositol phospholipids, increases the affinity of this enzyme for Ca^{2+}, rendering it active under physiological conditions. Tumor-promoting phorbol esters can also activate protein kinase C by substituting for diacylglycerol (2) and this enzyme plays important roles in many signaling pathways, including those involved in cell proliferation (3). Recent molecular cloning of the cDNA for protein kinase C has demonstrated the existence of multiple subspecies of protein kinase C in brain tissues (4-11). Biochemical studies (12,13) have revealed heterogeneity in highly purified protein kinase C from rat brain, and at least three distinct forms can be separated by chromatography on a hydroxyapatite column (14-16). Expression of rat brain cDNA clones in COS 7 cells, and comparison of these expressed forms with the purified rat brain enzyme has allowed identification of the primary structure of each form present in brain (15,16).

While many studies on both growth control and cellular transformation have used cultured fibroblasts, protein kinase C has not been well characterized from this source. The studies described here were carried out to identify the species of protein kinase C present in the mouse fibroblast cell line, NIH 3T3, and its *ras*-transformed counterpart, XHT. The results indicate that normal or transformed fibroblasts contain a single subspecies of the four which are expressed in brain tissue. Further, the chromatographic and antigenic properties of the fibroblast enzyme suggest that it corresponds to the α-subspecies of protein kinase C.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture——The *ras*-transformed NIH 3T3 cells, XHT, were kindly provided by Drs. David Stern and Robert Weinberg. Cells were grown in Dulbecco's modication of Eagle's medium (Flow Laboratories) containing 10% fetal bovine serum (Gibco). Cells were harvested by scraping in growth media. After one wash with phosphate-buffered saline containing 0.5 mM EDTA to remove excess media, the cells were lysed in a Dounce homogenizer in 20 mM Tris/HCl at pH 7.5 containing 2 mM EDTA, 10 mM EGTA, 5 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, and 10 μg/ml leupeptin. The resulting cell extract was centifuged at 100,000 x g for 60 min, and the supernatant was used as the source of protein kinase C.

Purification and Assay of Protein Kinase C——Rat brain protein kinase C was purified from the cytosol by DE-52 (Whatman), threonine-Sepharose, and TSK-phenyl 5PW (Toyo Soda) column chromatographies as described by Kikkawa *et al.* (12). The fibroblast enzyme was isolated in a similar manner, except that TSK DEAE-5PW column was used instead of DE-52 column for the initial purification step, and the buffers for the DEAE-5PW and threonine-Sepharose

columns contained 10 µg/ml leupeptin. Protein kinase C was assayed with calf thymus H1 histone as a phosphate acceptor as described (12).

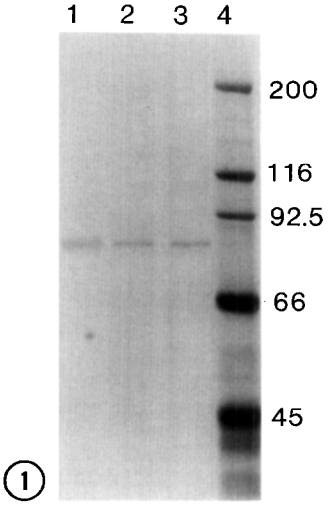
Fractionation on Hydroxyapatite Column———The TSK phenyl-5PW fraction was pooled and loaded onto a hydroxyapatite column (0.6 x 10 cm) connected to a high performance liquid chromatography, Pharmacia FPLC system. The column had been equilibrated with 20 mM potassium phosphate buffer at pH 7.5, containing 0.5 mM EDTA, 0.5 mM EGTA, 10 mM 2-mercaptoethanol, and 10% glycerol. After washing, the column was eluted with a linear concentration gradient of 20 to 280 mM potassium phosphate in a total volume of 48 ml, and fractions of 1 ml each were collected.

Antibodies and Immunoblotting———Three monoclonal antibodies against rat brain protein kinase C were prepared as described previously (17), and the mixture of these antibodies was employed for the present studies. A rabbit antiserum raised to bovine brain protein kinase C was kindly donated by Hans-Peter Biemann and Dr. Ray Erikson. For immunoblotting analysis, samples were fractionated on 7% SDS-polyacrylamide gels and transferred to nitrocellulose. For detection with the monoclonal antibodies, the blots were blocked overnight at 4 °C with 20% normal horse serum and 5% bovine serum albumin in Tris-buffered saline (20 mM Tris/HCl at pH 7.5, 150 mM NaCl). The nitrocellulose was then probed with the antibodies. Immunoreactive bands were visualized using a biotinylated anti-mouse IgG antiserum and avidin-counjugated horseradish peroxidase (Vectastain) employing diaminobenzidine as a substrate. Duplicate gels were fixed and stained with Coomassie blue dye to visualize proteins. For blots using the polyclonal antiserum, gels were run and transferred as above, but were blocked overnight with 30% calf serum in Tris-buffered saline. The blots were incubated with a 1:200 dilution of the antiserum, and bands were visualized as above, except using a biotinylated anti-rabbit IgG antisera and 4-chloro-1-naphtol (Biorad) as substrate in the horseradish peroxidase reaction.

Materials and Other Procedures———TSK DEAE-5PW column was obtained from Toyo Soda, Tokyo, and the hydroxyapatite column (packed hydroxyapatite column, Type S) was purchased from Koken Co. Ltd., Tokyo. SDS-polyacrylamide electrophoresis was done by the method of Laemmli (18), and silver staining was done as described by Merril et al.(19) using a Biorad kit.

RESULTS AND DISCUSSION

Protein kinase C was purified from normal and transformed NIH 3T3 fibroblasts using DEAE-5PW, threonine-Sepharose, and phenyl-5PW column chromatographies. Polyacrylamide gel electrophoresis and silver staining of the eluate from phenyl-5PW revealed that the major protein band migrates with the same apparent molecular weight as rat brain protein kinase C (Fig.1). Although the rat brain enzyme normally showed a diffuse or sometimes double band at this stage of purification (12), the enzymes from normal and transformed fibroblasts gave a single sharp protein band. Further fractionation of protein kinase C by hydroxyapatite column chromatography resolves the rat brain enzyme into three distinct fractions, Type I, II, and III (Fig. 2C) (14-16). Comparison of the chromatographic profile



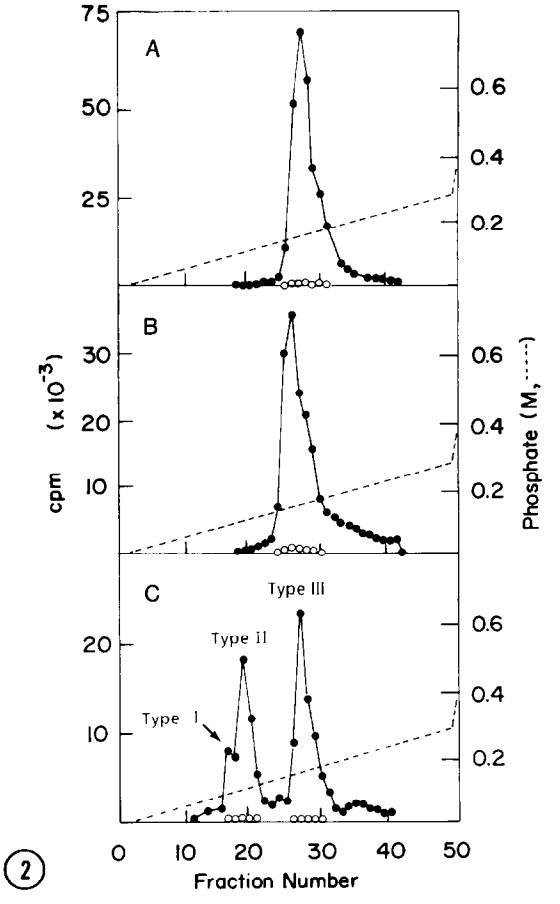


Figure 1. Silver stain of phenyl-5PW-purified protein kinase C. Approximately 100 ng of protein kinase C from rat brain (lane 1), NIH 3T3 cells (lane 2) or ras-transformed XHT cells (lane 3) was fractionated on an 8.5% SDS-polyacrylamide gel and silver stained as described under "EXPERIMENTAL PROCEDURES." Lane 4 shows the migration of standards, with molecular weights indicated in kilodaltons.

Figure 2. Hydroxyapatite fractionation of phenyl-5PW-purified protein kinase C. Phenyl-5PW-purified protein kinase C was fractionated on hydroxyapatite and assayed as described under "EXPERIMENTAL PROCEDURES." A, protein kinase C from NIH 3T3 cells; B, protein kinase C from ras-transformed XHT cells; C, protein kinase C from rat brain. (●), in the presence of $CaCl_2$; (○), in the presence of EGTA.

of the rat brain enzymes with the profiles of the expression products of the four cDNAs for protein kinase C indicates that Type I is encoded by Y-sequence[1/], Type II is a mixture of two subspecies determined by βI- and βII-sequence[2/], and Type III is encoded by

1/ The nomenclature of α, βI, βII, Y has been proposed for the four cDNA clones for protein kinase C isolated from the brain libraries (15,16). The enzymes encoded by these cDNAs consist of 672, 671, 673, and 697 amino acid residues, respectively (4,5,7,8,10).

2/ The two subspecies in Type II fraction are shown to result from alternative splicing from a single gene (7,15).

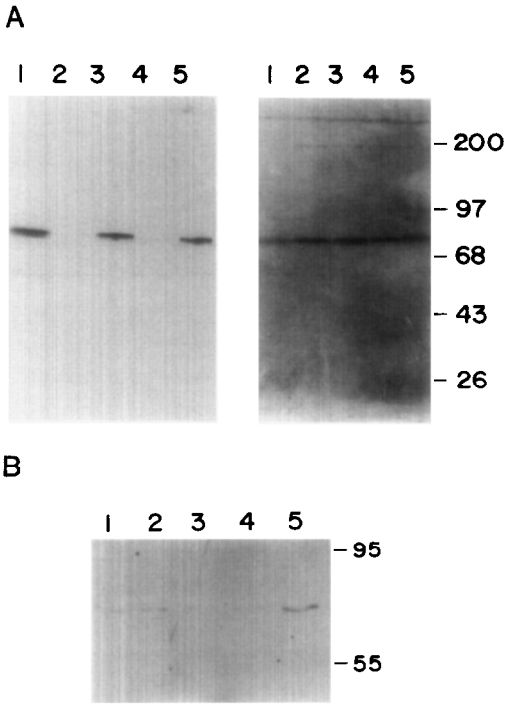


Figure 3. Immunoblotting of phenyl 5PW-purified protein kinase C. Immunoblotting of protein kinase C was carried out as described under "EXPERIMENTAL PROCEDURES." A, left, Immunoblotting with monoclonal antibodies to rat brain enzyme. Enzyme was analyzed as follows: lane 1, rat brain protein kinase C, 0.5 µg; lane 2, NIH 3T3 enzyme, 0.5 µg; lane 3, mixture of rat brain and NIH 3T3 enzymes, 0.25 µg each; lane 4, ras-transformed XHT enzyme, 0.5 µg; lane 5, mixture of rat brain and XHT enzyme, 0.25 µg each. A, right, Coomassie blue stain of duplicate gel to that shown at left with molecular weights indicated in kilodaltons. B, immunoblotting with polyclonal antiserum to bovine brain protein kinase C. Enzyme was analyzed as follows (parentheses indicate approximate amount loaded): lane 1, rat brain protein kinase C, (50 ng); lane 2, NIH 3T3 enzyme, (50 ng); lane 3, mixture of rat brain and NIH 3T3 enzymes (25 ng each); lane 4, mixture of rat brain and XHT enzymes, (25 ng each); lane 5, XHT enzyme, (100 ng). Approximate molecular weights are shown in kilodaltons.

α-sequence (15,16). When enzyme from either NIH 3T3 (Fig. 2A) or ras-transformed XHT (Fig. 2B) was fractionated on hydroxyapatite, a single peak of activity was resolved, which appeared to correspond to Type III enzyme.

Although the subspecies of protein kinase C identified from brain cDNA libraries show closely related sequences, a mixture of three monoclonal antibodies raised to rat brain protein kinase C (17) has recently been shown to preferentially recognize Type I enzyme, and only weakly recognize Types II and III (unpublished). In the immunoblotting analysis shown in Fig. 3, equivalent amounts (by activity) of both rat brain and mouse fibroblast enzymes purified through the phenyl-5PW step were separated on SDS-polyacrylamide gels, transferred onto nitrocellulose, and probed

with the monoclonal antibodies. Coomassie blue staining of a replicate gel confirmed that the amounts of protein kinase loaded were similar (Fig. 3A, right). The monoclonal antibodies recognized a doublet in the rat brain enzyme preparation (Fig. 3A, left, lane 1), while the enzyme from either NIH 3T3 (Fig. 3A, lane 2) or XHT cells (Fig. 3A, lane 4) was only faintly detected. Mixtures of rat brain and fibroblast enzymes were detected by the monoclonal antibodies (Fig. 3A, lanes 3 and 5), indicating that antibody binding to rat brain enzyme is not inhibited by the fibroblast preparations. A polyclonal antiserum raised in rabbit against bovine brain enzyme reacted with both rat brain (Fig. 3B, lane 1) and mouse fibroblast enzyme (Fig.3B, lanes 2 and 5) as well as mixtures (Fig. 3B, lanes 3 and 4) under these blotting conditions. Since the monoclonal antibodies to rat brain enzyme also recognize the enzyme from mouse brain at this purification step (data not shown), the lack of reactivity to the monoclonal antibodies does not appear to be due to a species difference.

The results presented above demonstrate that the pattern of expression of protein kinase C in murine fibroblasts is distinct from that in brain tissues. Both the observed lack of reactivity of the fibroblast enzyme to the monoclonal antibodies, and the elution pattern of the enzyme on hydroxyapatite suggest that fibroblasts express only one type of protein kinase C which apparently corresponds to Type III from rat brain encoded by α-sequence. The transformation of NIH 3T3 cells does not appear to alter the expression of protein kinase C subspecies. More detailed analysis will determine whether protein kinase C from normal or transformed fibroblasts is a single enzyme species encoded by the α-sequence, or is a mixture of subspecies having yet unknown structures. In addition, the expression of a limited number of protein kinase C forms in fibroblasts as compared to brain suggests that there may be cell-specific control of the expression of protein kinase C.

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