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PURIFICATION AND IDENTIFICATION OF CREATINE PHOSPHOKINASE B AS A SUBSTRATE OF PROTEIN KINASE C IN MOUSE SKIN IN VIVO

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Summary We previously described epidermal proteins with molecular weights of 40,000 (p40) and 34,000 (p34) as target proteins of protein kinase C in mouse skin carcinogenesis in vivo. In the present work, p40 was purified from mouse brain by the use of ^{32}P-labeled p40 of BALB/MK-2 cells as a tracer. Following four lines of evidence indicate that p40 is creatine phosphokinase B. 1) The amino acid sequences of all peptide fragments of p40 from mouse brain were located in the primary structure of creatine phosphokinase B. 2) p40 of BALB/MK-2 cells was immunoprecipitated with goat antibody against human creatine phosphokinase B. 3) p40 of BALB/MK-2 cells was absorbed to and eluted from a creatine affinity column. 4) Purified creatine phosphokinase B was phosphorylated in vitro by purified protein kinase C, but not by cAMP-dependent kinase or casein kinase II.

Introduction Protein kinase C (PKC) is a critical component of many signalling pathways of cell growth because it is activated endogenously by a wide variety of growth factors, hormones and neurotransmitters and also exogenously by tumor promoting phorbol esters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (1). Although the presence of isoforms of PKC and their structures are well investigated (1, 2), only a little is known about its substrates and how they are involved in physiological processes and also tumor promotion.

We previously demonstrated that cytosolic proteins of mouse epidermis with molecular weights of 40,000 and 34,000 and pI of 5.2 - 6.2 and 4.7 - 5.1 (p40 and p34, respectively) were specifically phosphorylated at the serine residue by treatment of mouse skin in vivo with TPA and by phosphorylation in vitro with purified PKC (3). There was a good correlation between their extents of phosphorylation and potencies of tumor promoters. In BALB/MK-2 cells derived from epidermal keratinocytes of mice

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The abbreviations used are: PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; CKB, creatine phosphokinase B; EGF, epidermal growth factor; EB, extraction buffer; PMSF, phenylmethylsulfonyl fluoride.

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(4), we found that p40 and p34 along with the other three proteins were also phosphorylated by treatment with TPA (unpublished data). Furthermore, Yamamoto et al. (5) found that p34 was phosphorylated by TPA in primary culture of mouse epidermal keratinocytes. Thus, p40 and p34 seemed common target proteins of PKC in epidermal keratinocytes and to be involved in tumor promotion. We undertook the present study to elucidate the nature of these proteins. This paper describes identification of p40 as creatine phosphokinase B (CKB), and in the accompanied paper, regulation of its enzyme activity by PKC is reported.

MATERIALS AND METHODS

Cells. BALB/MK-2 epidermal keratinocytes, provided by Dr. S. A. Aaronson, NCI, MD, were cultured in low calcium (0.05 mM) Eagle's minimal essential medium supplemented with 10% dialyzed fetal calf serum and 4 ng/ml epidermal growth factor (EGF). The cells were grown at 37°C in an atmosphere of 5% CO_2 in air. A quiescent culture was obtained by culturing the cells for three days in EGF-free medium.

Phosphorylation of BALB/MK-2 Cells and Partial Purification of p40. Quiescent cultures of BALB/MK-2 cells were labeled with carrier-free ^{32}Pi (New England Nuclear, MA) for 2 h in phosphate-depleted medium, and then treated with 100 ng/ml of TPA for 15 min. A cell lysate was prepared by freeze-thawing in extraction buffer (EB) consisting of 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM $NaVO_3$ and centrifugation at 100,000 x g for 1 h. The supernatant was applied to a DE52 column (100 µl bed volume) equilibrated with 0.01% Triton-X 100 in EB, washed with 160 mM NaCl in EB and developed with 220 mM NaCl in EB. p40 recovered in the supernatant with 50% saturated ammonium sulfate was used as a tracer for purification of p40 from mouse brain.

Purification of p40 from Mouse Brain. Brains of 246 mice (CD-1, female, 6 - 8 weeks old) were homogenized in 1 liter of EB in a Dounce homogenizer. The supernatant obtained by centrifugation at 100,000 x g for 1 h was mixed with ^{32}P-labelled p40 of BALB/MK-2 cells (about 5,000 cpm) and applied to a DE 52 column (100 ml bed volume). The column was washed with 100 mM NaCl in EB and developed with a linear gradient of 100 - 400 mM NaCl in EB. After dialysis against 25 mM imidazole-HCl (pH 7.4), the p40 fraction was separated by chromatofocusing on a PBE94 (Pharmacia) column equilibrated with the dialysis buffer, followed by gel-filtration with Sephacryl S300 (Phamacia). Proteins were eluted with one-eighth diluted PB74 (Pharmacia) buffer-HCl (pH 4.0) and fractionated. Ammonium sulfate was added to the radioactive p40 fractions to 80% saturation. The precipitate was dissolved to 2 M ammonium sulfate in EB and applied to a hydrogen bond column HB-1 (Whatman) equilibrated with 2 M ammonium sulfate in EB. p40 was eluted with a linear gradient of decreasing ammonium sulfate concentration of 2 to 0 M. The fraction of p40 was desalted, freeze-dried, and applied to SDS-PAGE in 10% acrylamide gel. The radioactive band of p40 was cut out and again subjected to SDS-PAGE in 10% acrylamide gel. The homogeneous p40 protein was transferred electrically from the gel on to a nitrocellulose filter (Schleicher & Shuell, Dassel, FRG) and digested with trypsin at 1 ng/ml in 100 mM Tris-HCl (pH 8.0) at 37°C for 12 h (6). The resulting peptides were separated by HPLC in an LC-6A apparatus (Shimadzu, Kyoto, Japan) on a reverse phase column (TSK ODS-120T, Toso, Tokyo, Japan) and were analyzed with an automated peptide sequencer (477A, Applied Bio System).

Purification of CKB. CKB was purified from mouse brain by a modification of the method of Roberts (7). Briefly, brains of BALB/c mice were homogenized in buffer consisting of 50 mM Tris-HCl (pH 7.5) and 5 mM

2-mercaptoethanol and then extracted with ethanol. The supernatant containing the enzyme was applied to a column of DEAE-Sephacel (10 ml bed volume) developed with a gradient of 0 - 400 mM NaCl in the above buffer and then to a creatine-conjugated cellulofine column with a gradient of 0 - 800 mM NaCl in the same buffer.

Dephosphorylation and Phosphorylation of CKB in a Cell-free System. Purified CKB was dephosphorylated by alkaline phosphatase conjugated with agarose beads (7.7 unit/ml, Sigma, St. Louis, MO) in a buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM MgCl, 0.1 mM $ZnCl_2$, 200 mM NaCl and 5 mM 2-mercaptoethanol at 37°C for 30 min. Dephosphorylated CKB (1.5 µg) was phosphorylated in vitro by PKC (0.1 µg) purified from mouse brain by the method of Kitano et al. (8) in the reaction mixture (40 µl) containing 20 mM Tris-HCl (pH 7.2), 5 mM $MgSO_4$ and 34 µM of [γ-^{32}P]ATP (0.79 Ci/mmol) in the presence of 1 mM $CaCl_2$, 64 µM phosphatidylserine and 16 nM TPA for 30 min at 37°C. For phosphorylation by cAMP-dependent protein kinase, dephosphorylated CKB (10 µg) and purified catalytic subunit of bovine cAMP-dependent protein kinase (1 µg) were incubated in reaction mixture (50 µl) containing 125 mM Tris-HCl (pH 7.0), 25 mM Mg $(CH_3COO)_2$, 5 mM EGTA and 200 µM of [γ-^{32}P]ATP (0.05 Ci/mmol) for 30 min at 37°C. CKB (10 µg) was also reacted with purified porcine casein kinase II (200 units) in reaction mixture (50 µl) containing 100 mM NaCl, 30 mM Tris-HCl (pH 75), 5 mM $MgCl_2$ and 200 µM of [γ-^{32}P]ATP (0.05 Ci/mmol) for 30 min at 37°C. Phosphorylation was stopped by addition of a solution containing SDS, and subjected to SDS-PAGE and autoradiography.

Immunoprecipitation of CKB. Cell lysates of BALB/MK-2 cells prepared as described above were immunoprecipitated with goat-antibody against human CKB (Chemicon International Inc., Femecula, CA) in the presence of protein G (Sigma, St. Louis, MO) and subjected to SDS-PAGE and autoradiography.

RESULTS

Purification and Identification of p40 as CKB. We first purified p34 and p40 from skin of the backs of CD-1 mice: only 3 µg of p34 and 0.6 µg of p40 were obtained from 640 mice and the amino-terminal of p34 was masked. Because of the difficulty in biochemical manipulation of skin and the low yields of proteins, we screened various other tissues for p34 and p40 by 2 dimensional-gel electrophoresis of partially purified proteins, and found that brain and kidney also contained these proteins. Using ^{32}P-labeled p34 and p40 of TPA-treated BALB/MK-2 cells as tracers, these proteins were purified from mouse brain sequentially by DEAE-cellulose chromatography with DE-52 (Fig. 1A), chromatofocusing with a pH range of 4 to 7 (Fig. 1B), gel-filtration chromatography with Sephacryl S300 (Fig. 1C) and then with hydrogen bond column HB-1 (Fig. 1D). The resulting radioactive fraction was further purified by SDS-PAGE (Fig. 2), followed by analysis of amino acid sequences of tryptic peptides. Informative sequences were obtained for p40 (Fig. 3). By computer-aided homology search, p40 was identified as CKB, because the amino-acid sequences of all peptide fragments of p40 were located in the primary structure of this enzyme, which contains five serine residues as a putative sites for PKC-phosphorylaion according to the positions of neighbouring basic residues.

This identification was confirmed by demonstrating immunoprecipitation of p40 with goat antibody against human CKB. For the results shown in Fig.

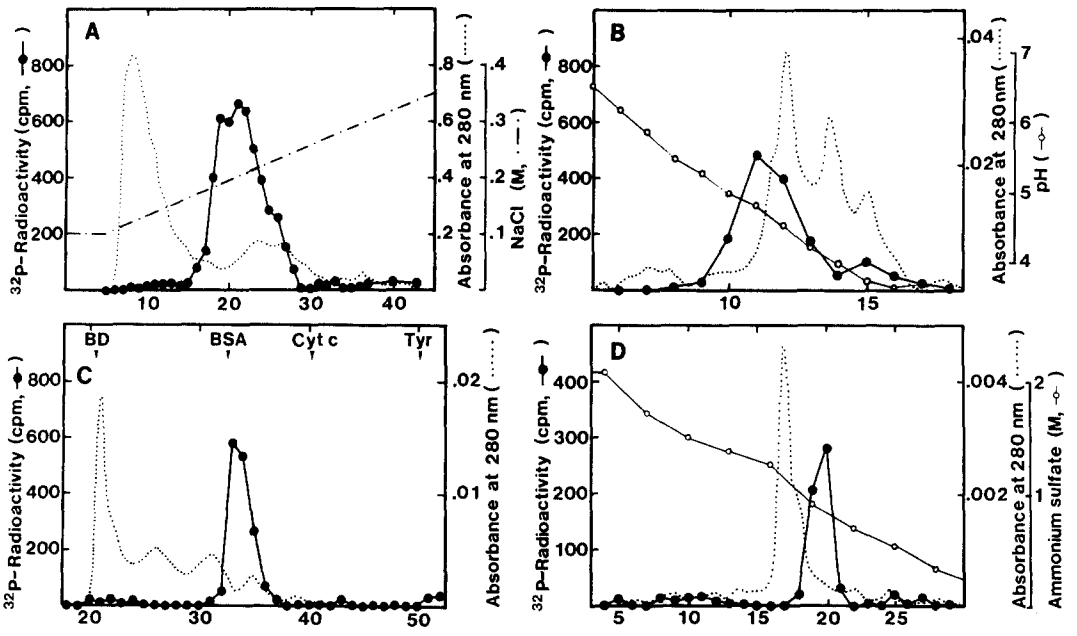

Fig. 1. Purification of p40 from mouse brain by the use of partially purified ^{32}P-labeled p40 of TPA-treated BALB/MK-2 cells as a tracer. ●, Radioactivity of ^{32}P; -----, Absorbance at 280 nm. **(A)** DEAE-cellulose chromatography with DE-52. **(B)** Chromatofocusing on a PBE 94 column. **(C)** Gel-filtration with Sephacryl S300. **(D)** Hydrogen bond column HB-1 (BD, blue dextran; BSA, bovine serum albumin; Cytc, cytochrome C; Tyr, tyrosine).

4A, quiescent cultures of BALB/MK-2 cells were labeled with ^{32}Pi for 2 h, treated with 100 ng/ml of TPA for 15 min, lysed and treated with antibody. p40 was precipitated from the lysate of TPA-treated cells. Furthermore, a

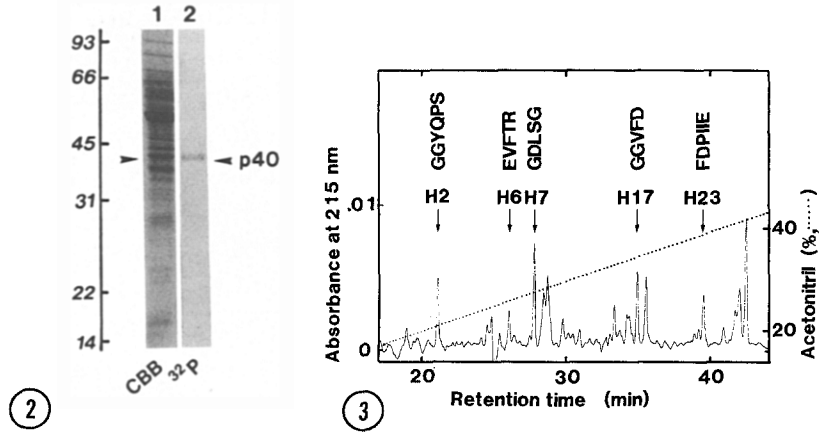

Fig. 2. SDS-PAGE of the partially purified p40 fraction by the procedure shown in Fig. 1. Lane 1, Coomassie brilliant blue (CBB) staining; Lane 2, autoradiography. The radioactive band of p40 (indicated by arrowheads) was cut out and again subjected to SDS-PAGE, followed by transfer to a nitrocellulose filter and analysis of amino acid sequences.

Fig. 3. Amino acid sequences of peptide fragments of p40 purified from mouse brain.

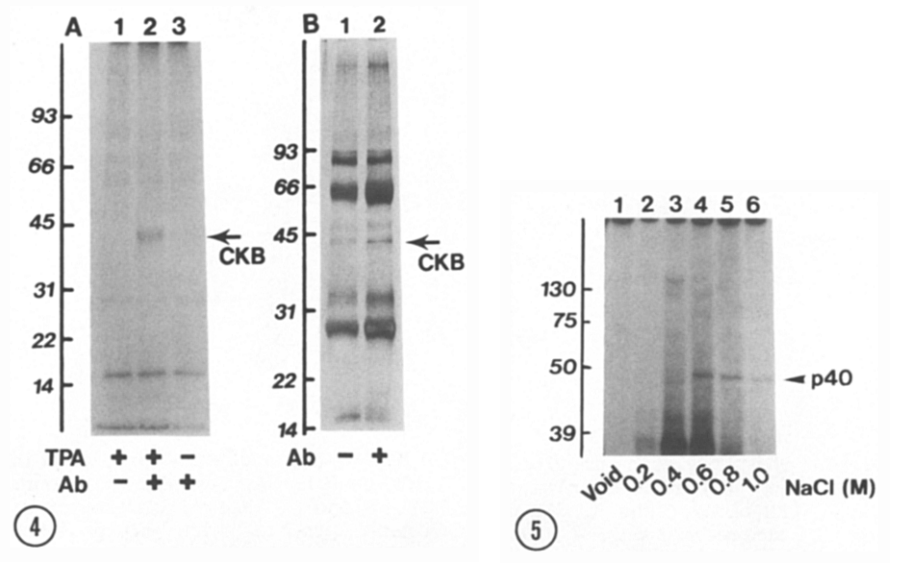

Fig. 4. **(A)** Autoradiography of immunoprecipitated CKB from BALB/MK-2 cells treated with 100 ng/ml of TPA for 15 min. Lane 1, TPA-treated cells precipitated with normal serum; lane 2, TPA-treated cells precipitated with antibody specific to CKB; lane 3, acetone-treated control cells treated with the antibody. **(B)** Immunoprecipitated CKB from untreated BALB/MK-2 cells. Silver-staining of precipitates with normal serum (lane 1) and the antibody (lane 2).

Fig. 5. p40 eluted from a creatine-affinity column chromatography. Partially purified p40 from TPA-treated BALB/MK-2 cells was applied to a creatine-conjugated cellulofine column and eluted with a stepwise gradient of 0-1 M NaCl, followed by SDS-PAGE and autoradiography.

band of Mr 40,000 staining with silver was immunoprecipitated with the antibody from a lysate of untreated BALB/MK-2 cells (Fig. 4B). These results indicate that BALB/MK-2 cells contained CKB and that this enzyme was phosphorylated on treatment with TPA.

Further identification was provided by the use of a creatine affinity column to which the radioactive p40 of BALB/MK-2 was absorbed to and eluted with 0.6 - 1.0 M NaCl (Fig. 5).

Phosphorylation of CKB by PKC. Using a cell-free phosphorylation system, we demonstrated that CKB was a substrate for PKC. For this, PKC and CKB were purified from mouse brain as described in the Materials and Methods. When incubated with PKC in the presence of [γ-^{32}P]ATP and activators of PKC [i.e. Ca^{2+}, phosphatidylserine and TPA] CKB was found to be phosphorylated along with autophosphorylation of PKC itself (Fig. 6A). However, CKB was not phosphorylated in vitro by cAMP-dependent kinase (Fig. 6B) or casein kinase II (Fig. 6C) in spite of the presence of putative phosphorylation sites for these kinases.

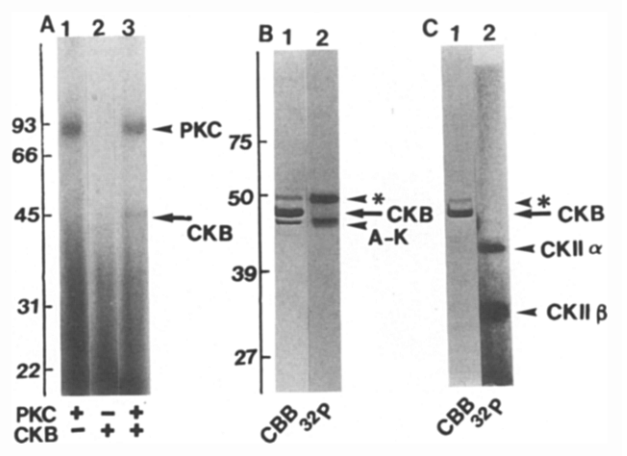

Fig. 6. Phosphorylation in vitro of CKB by PKC **(A)**, cAMP-dependent protein kinase (A-K) **(B)** and casein kinase II (CKII) **(C)**. **A**, Lane 1, PKC alone; lane 2, CKB alone; lane 3, CKB plus PKC. **B** and **C**, lane 1, CBB staining; lane 2, autoradiography; *, a contaminated protein which was phosphorylated by cAMP-dependent kinase.

DISCUSSION

Although a number of proteins phosphorylated by PKC have been detected by gel electrophoresis, only a few of them have been identified. Moreover little if anything is known about the biological significance of phosphorylation of the proteins identified. These include glycogen synthetase (9), ribosomal S6 kinase (10), adenylate cyclase (11) and inositol triphosphate 5'-phosphomonoesterase (12). Here we add CKB to this list.

One of the most prominent cellular substrates for PKC is the "80KDa" protein. This protein is considered to be a possible early target in the pathway of PKC-mediated signalling. Recently Stumpo et al. (13) reported molecular cloning and characterization of the 80KDa protein.

We found that p40 and p34 were phosphorylated by PKC in mouse skin in vivo (3) and BALB/MK-2 keratinocytes, suggesting that these proteins are keratinocyte specific substrates for PKC. We consider that p40 and p34 are involved in tumor promotion, because they are phosphorylated specifically by tumor promoters and because they are present in the target tissue for tumor promotion. In the present study, we identify p40 as CKB based on the amino acid sequences, immunoprecipitation, absorption to a creatine-affinity column and phosphorylation in vitro by PKC. Recently, Quest et al (14) reported that chicken CKB contained a phosphorylated derivative showing a shift of pI on 2-dimensional gel, although a protein kinase(s) responsible for this phosphorylation was not identified. p34 was found to be a novel protein by computor aided homology search. The nature of this protein will be elucidated by cDNA cloning using its amino acid sequences.

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