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Phosphorylation of the EGF receptor from A431 epidermoid carcinoma cells by three distinct types of protein kinase C

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Three distinct types of protein kinase C obtained from rat brain cytosol phosphorylated the EGF receptor of A431 epidermoid carcinoma cells at different rates. This receptor was phosphorylated most rapidly by type III protein kinase C, but slowly by type I enzyme. Type II enzyme showed intermediate activity. Chromatographic analysis indicated that A431 cells possessed only one of the three types found in rat brain, which apparently corresponded to type III enzyme. This type of protein kinase C, that is encoded by the α-sequence or a closely related sequence, appeared to be expressed commonly in many tissues and organs. The result implies that type III enzyme may play roles in growth promotion.

Protein kinase C; EGF receptor; (A431 epidermoid carcinoma cell)

1. INTRODUCTION

The EGF receptor has been repeatedly shown to be phosphorylated by protein kinase C, resulting in the desensitization or down-regulation of this receptor function [1–6]. Recent analysis of cDNA clones has indicated that there are at least four subspecies of protein kinase C, namely α, βI, βII and γ, which have similar structures [7–10]. In addition, protein kinase C in rat brain can be separated into three fractions, type I, II and III, by hydroxyapatite column chromatography [11–13]. Expression of each of the rat brain cDNA clones in COS cells shows that α, βI and βII, and γ clones correspond to type III, II, and I protein kinase C, respectively [12,13]. The two subspecies having the βI- and βII-sequence are derived from alternative splicing from a single gene, and are indistinguishable chromatographically from each other [12]. The three types of protein kinase C show slightly different kinetic properties. To explore the functional difference of these protein kinase subspecies, the EGF receptor of A431 epidermoid carcinoma cells was employed as a test substrate for comparison of the three distinct types. A431 cells contain only the type III enzyme having probably the α-sequence or a closely related sequence.

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Abbreviations: EGF, epidermal growth factor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

2. MATERIALS AND METHODS

2.1. *Assay and purification of protein kinase C*

The enzyme was routinely assayed with calf thymus H1 histone as a substrate [14]. A mixture of the multiple forms of protein kinase C was purified from rat brain cytosol by DE-52 column chromatography, followed by a threonine-Sepharose column and a TSK phenyl-5PW column connected to a high-performance liquid chromatography (HPLC) system as described [14]. This apparently homogeneous preparation was resolved further into three fractions, type I, II and III, by chromatography on a hydroxyapatite col-

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umn connected to HPLC [12,13]. Each of the three fractions showed an apparently single band upon SDS-polyacrylamide gel electrophoresis, and the specific activities of these enzymes were practically identical. The three types of enzyme phosphorylated the same sites of H1 histone as judged by tryptic peptide analysis. Other procedures are indicated for each experiment.

2.2. *EGF receptor phosphorylation*

A431 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The membrane fraction of A431 cells was prepared as described by Cohen et al. [15], and stored in 10 mM Hepes buffer at −80°C. The membrane proteins were phosphorylated by protein kinase C as described by Cochet et al. [1]. The reaction mixture (60 μl) contained 20 mM Hepes buffer at pH 7.2, 10 mM $MgCl_2$, 0.5 mM Na_3VO_4, 2 μM [γ-^{32}P]ATP (8300 cpm/pmol), 60 μg/ml of phosphatidylserine, 6 μg/ml of diolein, 27 μg of A431 membrane protein, and each type of protein kinase C (0.25 units). One unit of protein kinase C was defined as that amount of enzyme which transferred 1 nmol P_i from [γ-^{32}P]ATP into H1 histone per min under the stated conditions. After incubation for 5 min at 30°C, the reaction was terminated by the addition of Laemmli sample buffer [16] or RIPA buffer [17]. The phosphorylated EGF receptor in RIPA buffer was precipitated by incubation with 10 μg/ml of an anti-EGF receptor monoclonal antibody (Amersham) for 1 h, and then with a sufficient volume of protein A-Sepharose (Pharmacia), for an additional 1 h at 4°C. After washing several times with RIPA buffer, Laemmli sample buffer was added to the suspension of protein A-Sepharose. The samples in Laemmli sample buffer were boiled for 2 min, and subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel. After staining with Coomassie blue, the gel was dried and exposed to an X-ray film with intensifying screen at −80°C. The absorbance of autoradiography was traced at 430 nm.

Autophosphorylation of the EGF receptor was carried out as described by Carpenter et al. [18]. The reaction mixture (50 μl), containing 27 μg A431 membrane protein, 20 mM Hepes buffer at pH 7.4, 1 mM $MnCl_2$, and 100 nM EGF, was preincubated for 10 min at 0°C. After the addition of 15 μM [γ-^{32}P]ATP (1300 cpm/pmol), the mixture was incubated for a further 10 min at 0°C. The reaction was then stopped by Laemmli sample buffer.

3. RESULTS AND DISCUSSION

The three types of protein kinase C were capable of phosphorylating several proteins associated with A431 cell membranes. In particular, EGF receptor proteins of 170 and 150 kDa as well as a

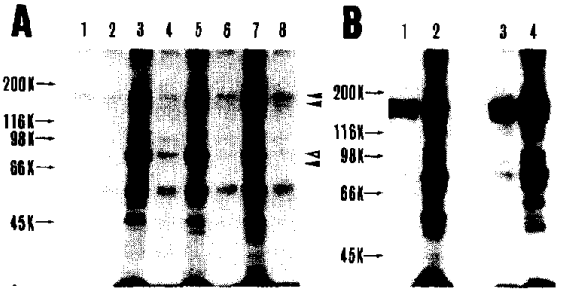

Fig.1. Protein phosphorylation in A431 cell membrane. A431 membrane proteins were phosphorylated by each type of rat brain protein kinase C or by EGF, and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography as described in section 2. Numbers with arrows indicate the molecular mass markers (K, kDa): myosin heavy chain (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (98 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa). (A) Protein phosphorylation in A431 cell membrane by three types of protein kinase C. Lanes: (1,2) A431 cell membrane alone; (3,4) plus type I protein kinase C; (5,6) plus type II protein kinase C; (7,8) plus type III protein kinase C; (1,3,5,7) with 1 mM EGTA and without lipid; (2,4,6,8) with 0.5 mM $CaCl_2$, 60 μg/ml phosphatidylserine, and 6 μg/ml diolein. The three closed arrowheads indicate 170 kDa and 150 kDa EGF receptor, and 70 kDa functionally unknown protein. The open arrowhead indicates protein kinase C. (B) Immunoprecipitation and autophosphorylation of the EGF receptor. A431 membrane was phosphorylated by type III protein kinase C and immunoprecipitated (lanes 1,2). In another set of experiments A431 membrane was incubated with or without EGF in the absence of exogenous protein kinase C (autophosphorylation) (lanes 3,4). Detailed conditions are given in section 2. Lanes: (1) EGF receptor immunoprecipitated from the sample shown in lane 2; (2) A431 membrane phosphorylated by type III protein kinase C; (3) A431 membrane incubated without EGF; (4) A431 membrane incubated with EGF.

protein of 70 kDa were phosphorylated most predominantly by type III enzyme as indicated by closed arrowheads in fig.1A. The function of the 70 kDa protein is unknown, but this protein phosphorylation has been described previously by Cochet et al. [1]. These proteins were phosphorylated by type I enzyme slowly, and by type II enzyme in an intermediate fashion. Conversely, autophosphorylation of the enzyme was most predominant for type I enzyme as shown by an open arrowhead. There was no indication that the different rates of autophosphorylation were due to the different amounts of preexisting phosphate that may have been attached to the enzyme molecules when initially purified. These results are shown more quantitatively in fig.2. All phosphorylation reactions were dependent on Ca^{2+}, diacylglycerol, and phosphatidylserine. It is unlikely that the three types of enzyme phosphorylated different sites of the EGF receptor, since brain protein kinase C was previously shown to phosphorylate only one threonine residue, Thr-654 [1,19,20].

The identity of the radioactive EGF receptor was determined from the experiments given in fig.1B. The two proteins were precipitated by an anti-EGF receptor monoclonal antibody (fig.1B, lanes 1,2). Autophosphorylation of EGF receptor molecules was greatly enhanced by the addition of EGF (fig.1B, lanes 3,4). It has been described by Cohen et al. [15] that the 150 kDa protein is a partially digested form of the 170 kDa intact EGF receptor.

In a separate set of experiments given in fig.3, it was shown that A431 cells possess only one type of protein kinase C that was eluted in the fraction similar to type III rat brain enzyme, when chromatographed on a hydroxyapatite column. Since the brain type III enzyme is known to be encoded by the α-sequence [13], A431 cell protein kinase C probably has the α-sequence or a closely related structure. This type of protein kinase C is found most commonly in many tissues and organs.

It has been reported that the EGF receptor of intact cells is phosphorylated by stimulation with tumor-promoting phorbol ester, which is a potent activator of protein kinase C [1–6]. EGF receptor phosphorylation by this enzyme was subsequently shown to be related to the down-regulation of the receptor [1–6]. In the present studies, it remains unproven whether the observed modification of the integral membrane proteins by exogenously added protein kinase C results directly in the functional alteration of the receptor. Nevertheless, type III enzyme, commonly present in many tissues including A431 cells, is most likely to be responsible for EGF receptor phosphorylation.

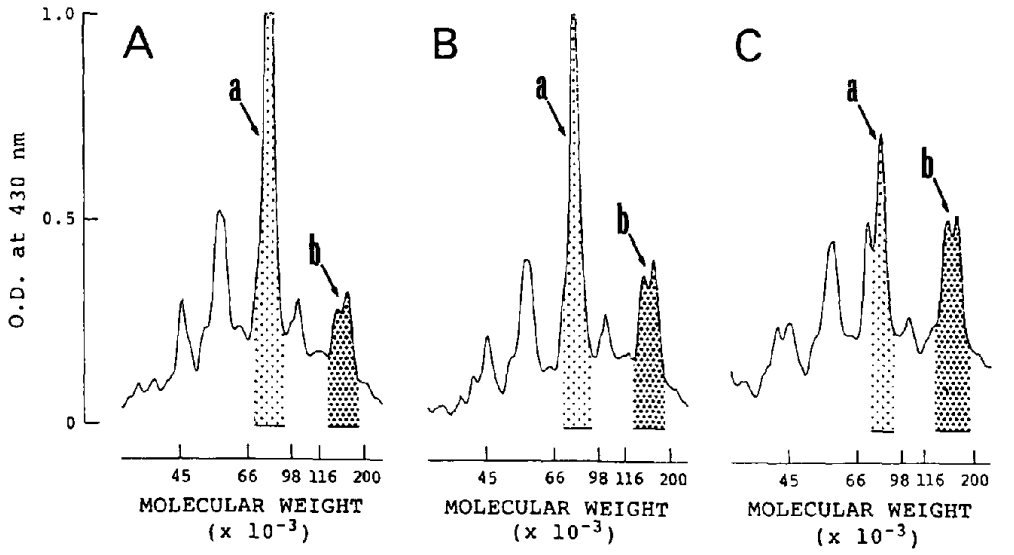

Fig.2. Densitometric tracing of autoradiograph shown in fig.1A. A, B and C; lanes 3, 5 and 7 in fig.1A, respectively. (a) Protein kinase C, (b) EGF receptors.

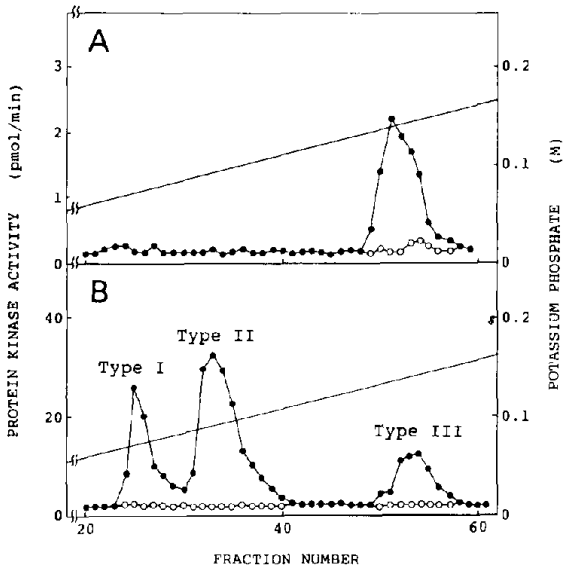

Fig.3. Hydroxyapatite column chromatography of protein kinase C. Protein kinase C from the cytosol of A431 cells (4×10^8) was purified by TSK DEAE-5PW column chromatography under conditions similar to those employed for the rat brain enzyme [14], and subjected to the hydroxyapatite column. Rat brain protein kinase C was purified as described in section 2. (A) A431 cell protein kinase C, (B) rat brain protein kinase C. (●——●) Protein kinase activity in the presence of 8 μg/ml phosphatidylserine, 0.8 μg/ml diolein, and 0.5 mM $CaCl_2$; (○——○) protein kinase activity in the presence of 1 mM EGTA instead of phosphatidylserine, diolein, and $CaCl_2$; (——) buffered potassium phosphate.

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