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# Purification of a phosphoprotein from rat brain closely related to the 80 kDa substrate of protein kinase C identified in Swiss 3T3 fibroblasts

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A phosphoprotein expressed in rat brain is closely related to the 80 kDa substrate of protein kinase C present in 3T3 cells. The protein kinase C substrates from both sources migrate identically on two-dimensional gel electrophoresis and give similar phosphopeptide fragments when digested with protease. Using a series of chromatographic steps, including DEAE-cellulose chromatography, Sephadex G150 gel filtration and reverse phase fast protein liquid chromatography, this phosphoprotein was purified 3800-fold from rat brain. The preparation appears homogenous by one- and two-dimensional gel electrophoresis, is an effective substrate of protein kinase C and contains a high proportion of the acidic amino acids glutamate and aspartate, and of alanine.

Growth control; Cellular signaling; Protein phosphorylation; Growth factor

## 1. INTRODUCTION

Protein kinase C, which is activated by diacylglycerols generated by receptor mediated phospholipid breakdown and serves as a major phorbol ester receptor [1], constitutes one of the signal transduction pathways leading to fibroblast proliferation [2]. An acidic protein of 80 kDa has been identified as a major and specific substrate for protein kinase C in intact, quiescent mouse 3T3 cells [3–10] and other cultured cells [3,6,9,11]. The phosphorylation of this protein is stimulated by phorbol esters [3], diacylglycerols [4], platelet-derived growth factor [3], bombesin [12,13] and vasopressin [14], all of which stimulate reinitiation of DNA synthesis in quiescent 3T3 cells [2].

Since activation of protein kinase C leading to a rapid increase in phosphorylation of the 80 kDa cellular protein may be a significant event in the initiation of mitogenesis, it is important to gain further understanding of the nature and role of this protein kinase C substrate. As an initial step, we decided to develop a procedure that would produce homogenous preparations of this protein. We found that a phosphoprotein closely related to Swiss 3T3 fibroblast 80 kDa protein is expressed in rat brain, making it a convenient source for large scale purification. A series of chromatographic steps were developed, resulting in a 3800-fold purification of this protein from rat brain, that is closely related to the 80 kDa protein identified in 3T3 cells.

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*Abbreviations:* FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; $PBt_2$, phorbol dibutyrate; PS, phosphatidyl serine

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Sephadex G150, phenyl-Sepharose, FPLC and liquid chromatography columns and FPLC apparatus were purchased from Pharmacia Biotechnology; [$\gamma$-$^{32}$P]ATP and $^{32}PO_4$ carrier-

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free were from the Radiochemical Centre (Amersham, England); DEAE-cellulose was from Whatman; $PBt_2$, histone IIIs, bombesin, PS and *Staphylococcus aureus* V8 protease were from Sigma (St Louis, MO); protein assay reagent from Pierce; and silver staining kits from Bio-Rad Laboratories (Richmond, CA). Rat brains were removed and snap-frozen immediately after sacrifice of rats and stored at −70°C until use. Protein kinase C was partially purified from rat brain by the method of Walsh et al. [15], and assayed using the phosphocellulose spot technique [16], as described [7].

### 2.2. *Methods*

#### 2.2.1. Cell culture procedures

Cell culture procedures [17,18], labelling of cells with $^{32}P_i$ [3], two-dimensional PAGE [7,19], and peptide mapping by limited proteolysis of phosphoproteins [7,20] were carried out as described previously. Slab gel SDS-PAGE was performed using 8% polyacrylamide gels with 3% stacking gels, by the method of Laemmli [21]. Protein was determined by the method of Bradford [22].

#### 2.2.2. Assay of the 80 kDa protein

Samples were adjusted to pH 7.5 or lyophilized if acetonitrile and trifluoroacetic acid were present, and made to 50 $\mu$l with 20 mM Tris-HCl, pH 7.5. The incubation mixture contained test proteins, protein kinase C (3 $\mu$l, spec. act. 50 nmol ATP/min per mg protein, using histone IIIs as substrate; 0.2 mg/ml), 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 ng/ml $PBt_2$, 100 $\mu$M $CaCl_2$, 100 $\mu$g/ml PS and 50 $\mu$M ATP (2 $\mu$Ci [$\gamma$-$^{32}$P]ATP) in a final volume of 65 $\mu$l. After incubation for 10 min at 37°C, the reaction was stopped by boiling and samples subjected to SDS-PAGE. Bands corresponding to 80 kDa on autoradiograms of dried gels were excised and quantified using a liquid scintillation counter (Beckman Instruments). A sample containing protein kinase C alone was routinely included.

#### 2.2.3. Procedures for the purification of the 80 kDa protein from rat brain

2.2.3.1. *Preparation of heat-stable extract* Thawed rat brains were homogenized in a Waring blendor in 5 vols homogenization buffer (20 mM Tris-HCl, 2 mM EDTA, 10 mM EGTA, 0.5% Triton X-100, pH 7.5) at 4°C. The homogenate was centrifuged at 10000 × *g* for 15 min at 4°C and the supernatant at 30000 × *g* for 90 min. The resultant supernatant (the rat brain extract – step 1 of table 1) was placed (in 200 ml aliquots) in a boiling waterbath, with continuous shaking, for 5 min. The boiled extract was placed on ice for 30 min and centrifuged at 30000 × *g* for 90 min. The final supernatant (the heat-stable extract) was stored at −20°C until used in subsequent steps, all of which were carried out at 4°C.

2.2.3.2. *DEAE-cellulose chromatography* A column of DEAE-cellulose (2.6 × 40 cm) was equilibrated with 20 mM Tris-HCl, pH 7.5. The heat stable extract of rat brain was applied and the column washed with 1.2 l of the equilibrating buffer. Bound proteins were eluted with a linear gradient from 0 to 1 M NaCl in 20 mM Tris-HCl, pH 7.5, at 0.8 ml/min and 90 fractions of 15 ml collected. After protein estimation and assay of 80 kDa protein activity, fractions were selected for the next stage. Combined fractions were dialysed against 3 changes of 4 l 20 mM Tris-HCl, 1 mM EDTA, pH 7.5, before being bound to minicolumns (1 ml) of DEAE-cellulose and eluted with 3 ml of 2 M NaCl in 20 mM HCl. Protein concentrated in this way was used for gel filtration.

2.2.3.3. *Sephadex G150 gel filtration* A column (70 × 2.5 cm) of Sephadex G150 was equilibrated with 20 mM HCl. Combined, concentrated fractions from the previous step were loaded onto the column and developed with the equilibrating solution at 0.25 ml/min. 2 ml fractions were collected. SDS-PAGE gels were silver stained, and protein and 80 kDa assays performed.

2.2.3.4. *Reverse phase chromatography on a pro RPC HR5/10 column* Fractions selected after the gel filtration step were dialysed overnight against 0.1% trifluoroacetic acid. The pro RPC column (attached to a Pharmacia FPLC system) was equilibrated with 0.1% trifluoroacetic acid and loaded with sample. Bound protein was eluted with a linear gradient of 0–20% acetonitrile in 0.1% trifluoroacetic acid over 40 min and isocratic elution with 20% acetonitrile in 0.1% trifluoroacetic acid, collecting 0.3 ml fractions at 0.3 ml/min. Fractions were monitored by silver staining of SDS-PAGE and by the standard 80 kDa assay, after lyophilization and resuspension.

#### 2.2.4. Amino acid analysis

After acid hydrolysis for 24 h [23] amino acids were separated and detected, as described [24].

## 3. RESULTS AND DISCUSSION

While phosphorylation of a 80 kDa cellular protein is seen within seconds of $PBt_2$ addition to quiescent cultures of Swiss 3T3 cells, the phosphorylation of other proteins, including one of 87 kDa, has also been reported after minutes of treatment [3]. Accordingly, addition of $PBt_2$ or bombesin for 5 min to quiescent cultures of Swiss 3T3 cells prelabelled with $^{32}P$ caused a marked increase in the phosphorylation of both 80 and 87 kDa proteins (fig.1). In extracts of rat brain fractionated by DEAE-cellulose chromatography, we also detected 80 kDa (eluting at 0.5 M NaCl) and 87 kDa (eluting at 0.2 M NaCl) substrates of protein kinase C. Thus, it was of importance to establish their relationship to those substrates detected in Swiss 3T3 cells. After boiling extracts from rat brain and Swiss 3T3 cells for 5 min, the 80 kDa substrate of protein kinase C remained in solution, in agreement with previous results [9]. Fig.2 compares the 80 kDa phosphoprotein produced in quiescent cultures of 3T3 cells by addition of $PBt_2$ with the 80 kDa phosphoprotein generated by the incubation of heat-stable proteins from rat

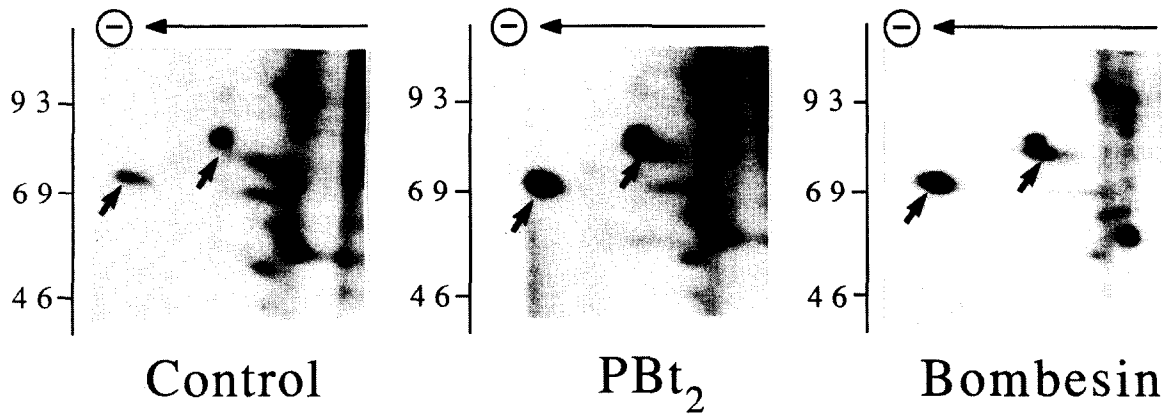


Fig.1. Stimulation of phosphorylation of 80 and 87 kDa proteins in Swiss 3T3 cells by $PBt_2$ and bombesin. Quiescent cultures were labelled with $^{32}P_i$ for 4 h. After incubation for 5 min with $PBt_2$ (200 nM), bombesin (6.2 nM) or an equivalent volume of solvent (control), phosphoproteins were resolved by two-dimensional PAGE. In the first dimension, separation was by isoelectric focusing from right (basic) to left (acidic). The arrows indicate the positions of 80 and 87 kDa phosphoproteins. Molecular mass standards ($\times 10^{-3}$) are shown on the left.

brain with protein kinase C, PS, $Ca^{2+}$, [$\gamma$-$^{32}$P]ATP and $PBt_2$. The 80 kDa phosphoproteins generated in vivo and in vitro migrated identically, and produced similar phosphopeptide fragments using *S. aureus* V8 protease (fig.2). These results indicate that rat brain expresses a protein kinase C substrate closely related to that identified in quiescent 3T3 cells. Therefore, we decided to use rat brain as a source for the purification of the 80 kDa protein.

The 80 kDa substrate of protein kinase C present in extracts of rat brain was purified through a series of steps, including DEAE-cellulose chromatography, Sephadex G150 gel filtration and

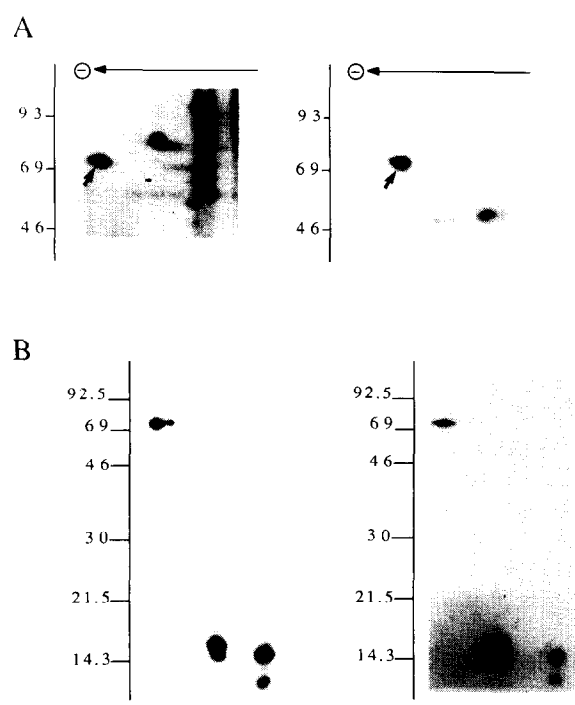


Fig.2. Comparison of phosphoproteins generated by protein kinase C in intact Swiss 3T3 cells (left-hand panels) and heat stable extracts of rat brain (right-hand panels). (A) Quiescent cultures of 3T3 cells were labelled for 4 h with $^{32}P_i$ before incubation for 5 min with 200 nM $PBt_2$. Heat-stable proteins from rat brain (20 $\mu$g) were incubated for 10 min with protein kinase C, PS, $Ca^{2+}$, $PBt_2$ and [$\gamma$-$^{32}$P]ATP. In both cases, phosphoproteins were resolved by two-dimensional PAGE, as described in the legend to fig.1. Arrows indicate the position of the 80 kDa protein. (B) Peptide mapping of 80 kDa phosphoproteins generated in quiescent Swiss 3T3 cells in response to 200 nM $PBt_2$ and in the heat-stable extract of rat brain when incubated with protein kinase C and activators. Spots corresponding to the 80 kDa protein on two-dimensional PAGE (fig.2A) were excised and re-run through a 15% polyacrylamide gel in the presence of 0 $\mu$g (lane 1), 0.05 $\mu$g (lane 2) or 1 $\mu$g (lane 3) of *S. aureus* V8 protease.

reverse phase FPLC. Typical profiles of the 80 kDa protein and protein elution from these columns is illustrated in fig.3. A summary of the purification of the 80 kDa protein from 500 g rat brain is depicted in table 1. A 3800-fold purification of the 80 kDa protein was achieved and protein purified to apparent homogeneity, as judged by SDS-PAGE.

Two-dimensional SDS-PAGE of the final preparation is compared with crude extract from rat brain in fig.4. Silver staining shows that while multiple proteins are detected in the crude extract (left-hand panel), a single 80 kDa protein is seen in the purified sample (middle panel) which co-migrates with the 80 kDa substrate of protein kinase C (right-hand panel). Peptide mapping of the final preparation gave an identical pattern of phosphopeptide fragments as the 80 kDa protein from Swiss 3T3 cells (results not shown). Amino acid analysis of the 80 kDa protein gave the results shown in table 2. The protein has a unique composition, with a high proportion of acidic amino acids (Glu, Asp) and of alanine.

While this work was in its final stages, two studies reported the purification of 87 kDa substrates of protein kinase C from bovine [25] and rat [26] brain. The protein from bovine brain differed from the heat stable substrate found in rat brain (reported here) in its behaviour in SDS-PAGE [25], immunological cross-reactivity [9], elution from anion exchange columns [25] and amino acid composition (28.6% alanine was reported) [25]. Although the 87 kDa substrate purified from rat brain [26] could be related to the 80 kDa phosphoprotein purified in the present study, no comparison with the 80 kDa substrate found in Swiss 3T3 fibroblasts was performed. Hence, the present results describe a series of chromatographic steps leading to the purification to apparent homogeneity of a rat brain 80 kDa phosphoprotein which is closely related to the substrate of protein kinase C in Swiss 3T3 cells. This acquires a special significance in view of the fact that in quiescent cultures of these cells, the 80 kDa protein is phosphorylated within seconds of addition of tumour promoters, mitogenic neuropeptides and growth promoting factors [3,12] and is also dephosphorylated rapidly upon their removal [14,24].

Activation of protein kinase C rapidly elicits a

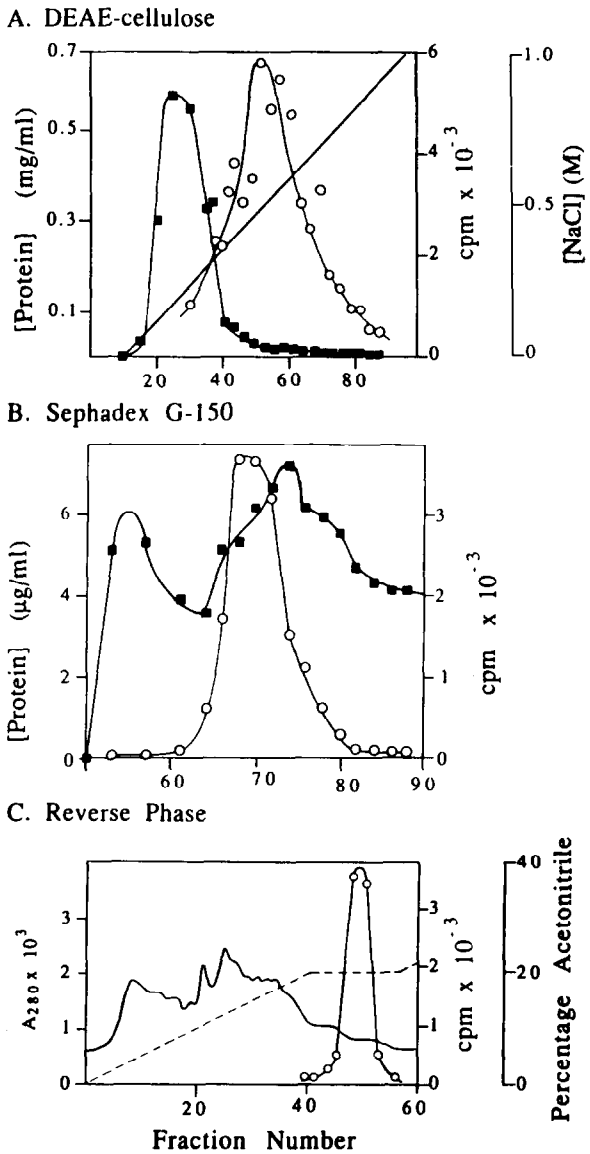


Fig.3. Steps in the purification of the 80 kDa protein. (A) Chromatography of heat-stable proteins from rat brain on a column of DEAE-cellulose (step 3). (■) Protein concentration (mg/ml); (○) $^{32}P_i$ incorporation into the 80 kDa protein when aliquots from individual fractions were incubated with protein kinase C, PS, $PBt_2$, $Ca^{2+}$ and [$\gamma$-$^{32}$P]ATP, before separation of phosphoproteins by SDS-PAGE; (——) concentration of NaCl. (B) Gel filtration of combined concentrated fractions from step 3 on a column of Sephadex G150 (step 4). Symbols as in A . (C) Chromatography of combined, dialyzed fractions from the gel filtration step on a pro RPC HR5/10 column. Elution of the 80 kDa protein was achieved with a gradient of 0–20% acetonitrile in 0.1% trifluoroacetic acid, followed by isocratic elution at 20% acetonitrile. (——) Optical density ($A_{280}$); (– – –) concentration of acetonitrile; (○) incorporation of $^{32}P_i$ into the 80 kDa protein. All details are described in section 2.

Table 1

The purification of the 80 kDa protein from rat brain

| Stage | [Protein] (mg/ml) | Volume (ml) | Total protein (mg) | Specific activity ($10^4$ cpm/mg) | Total activity | Fold purification | Yield (%) |
|---|---|---|---|---|---|---|---|
| Step 1 | | | | | | | |
| Rat brain extract | 13.6 ± 3[a] (17) | 880 | 11968 | 0.8 ± 0.1 (10) | 91.5 | 1 | 100 |
| Step 2 | | | | | | | |
| Boiled extract | 1.07 ± 0.14 (17) | 800 | 856 | 4.4 ± 0.5 (15) | 37.6 | 5.75 | 41 |
| Step 3 | | | | | | | |
| DEAE-cellulose | 0.017 ± 0.001 (12) | 420 ± 42 (12) | 7.02 ± 0.86 (12) | 178 ± 18 (15) | 12.5 | 233.0 | 13.7 |
| Step 5 | | | | | | | |
| Sephadex G150 | 0.035 ± 0.007 (10) | 14.6 ± 2.2 (10) | 0.512 | 777 ± 231 ( 9) | 3.98 | 1017 | 4.3 |
| Step 6 | | | | | | | |
| Reverse phase pro RPC HR5/10 | 0.051 ± 0.09 ( 3) | 2.01 ± 0.4 ( 3) | 0.082 ± 0.04 ( 3) | 2964 ± 800 ( 3) | 2.43 | 3877 | 2.66 |

[a] Standard error of the mean, number of determinations in brackets. All procedures described in section 2

variety of responses in quiescent 3T3 cells, including stimulation of $Na^+$ uptake, $Na^+/K^+$ pump [28,29], cytoplasmic alkalinization [29,30], enhancement of cyclic AMP accumulation [31], inhibition of phosphoinositide breakdown [32] and $Ca^{2+}$ mobilization [33], and increased expression of the photo-oncogene c-fos [34]. As these responses occur shortly after the increase in phosphorylation of the 80 kDa protein detected in 3T3 cells, the possibility that this phosphoprotein could participate in the elicitation or modulation of these early molecular events warrants further experimental work. The availability of the highly purified 80 kDa protein should assist in the elucidation of the physiological significance of this protein in the initiation of cell proliferation.

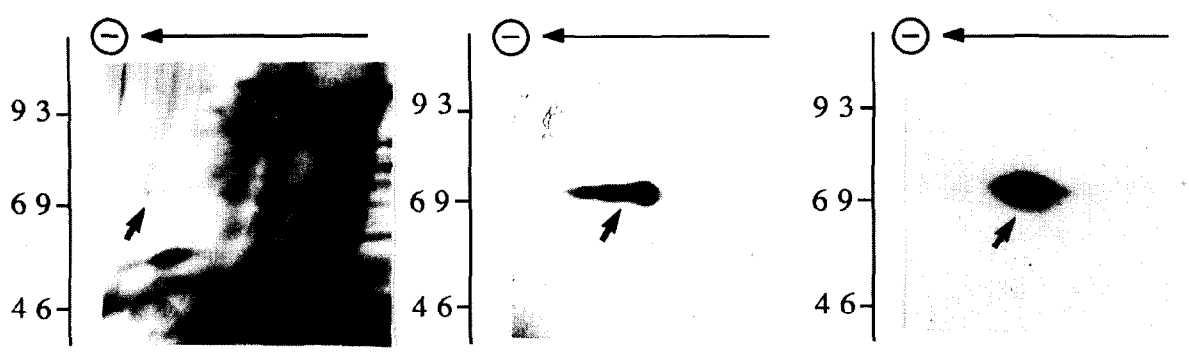


Fig.4. Analysis of crude extract of rat brain and the purified 80 kDa protein by two-dimensional PAGE. (Left and middle panels) Silver staining of 50 μg crude extract (left) or 0.2 μg purified sample (middle) after two-dimensional PAGE. (Right-hand panel) 0.2 μg purified 80 kDa was incubated for 10 min with protein kinase C, activators and [$\gamma$-$^{32}$P]ATP. Two-dimensional PAGE was performed and gels autoradiographed. After this time of incubation, 1.8 mol of phosphate per mol of the purified 80 kDa protein was incorporated.

Table 2

Amino acid composition of the 80 kDa protein

| Amino acid | Mol% |
|---|---|
| Asp | 7.2 |
| Thr | 4.5 |
| Ser | 7.7 |
| Glu | 17.2 |
| Pro | 8.6 |
| Gly | 9.7 |
| Ala | 17.8 |
| Cys | N.D. |
| Val | 5.4 |
| Met | 1.3 |
| Ile | 2.9 |
| Tyr | 0.9 |
| Phe | 2.2 |
| His | 1.0 |
| Lys | 7.0 |
| Arg | 2.2 |

Results are the mean of 2 independent determinations

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