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Production and Characterization of Antibodies Against C-Terminal Peptide of Protein F1: A Novel Phosphorylation at Serine 209 of the Peptide by Protein Kinase C

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Protein F1 (GAP-43, B-50, neuromodulin, P-57), a neural tissue-specific phosphoprotein enriched in the growth cones of elongating neurites, is suggested to be involved in synaptic plasticity, neuronal development, and neurotransmitter release. In this study, a 21 amino acid polypeptide (AKPKES**AR*QDEGKEDPEADQE) that corresponds to the C-terminus sequence of protein F1 (from position 204–224) was synthesized and used to produce anti-protein F1 antibodies. Immunoblot analysis has demonstrated that the prepared antibodies recognized intact protein F1. Protein F1 and the synthesized F1 peptide were phosphorylated in vitro by PKC. Furthermore, phosphorylated protein F1 was immunoprecipitated by anti-F1 peptide antibodies demonstrating that these antibodies recognized both native, non-phosphorylated and phosphorylated protein. The anti-protein F1 antibodies also stained the plasma membranes of cell bodies and neurites of mouse neuronal cultures obtained from 14-day old spinal embryonic tissue. By contrast, no glial cells were stained. These data suggest that serine 209 at the C-terminus of protein F1 may be a substrate for PKC phosphorylation in vivo. In addition, antibodies raised against F1 peptide revealed protein F1 immunoreactivity that outlined all neurites of cultured mouse spinal neurons.

KEY WORDS: Protein F1; GAP-43; protein kinase C; antibodies, mouse spinal neurons.

INTRODUCTION

Protein F1 is a major membrane component of the neuronal growth cones in developing rat brains (1–3). The same protein has been identified according to the observed physiological effect as GAP-43 growth associated protein (4), B-50 phosphoprotein in synaptosomes (5), P-57, the calmodulin binding protein (6), and F1, or plasticity protein (7) by independent research groups. As protein F1 expression is higher in developing and regenerating neurons than in adult neurons, protein F1 may have an important role in axonal growth and regeneration (8–10). Unphosphorylated protein F1 binds calmodulin in the absence of calcium with the consequent regulation of calmodulin-dependent enzymes near the

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Abbreviations used: IgG, immunoglobulin G; KLH, keyhole limpet haemocyanin; OAG, L-α-1-oleoyl-2-acetoyl-sn-3-glycerol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PKC, protein kinase C; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

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neuronal membranes (6). Protein F1 also stimulates GTP binding to Go, providing intracellular regulation of Go protein that is enriched in growth cone membranes (11). In addition, phosphorylated protein F1 inhibits phosphatidyl inositol 4-phosphate kinase (12) which suggests a different signal transduction role of protein F1.

Protein F1 has an apparent Mr of 43,000 and pI of 4.3–4.6 (13,14). Although protein F1 is a very hydrophilic protein, it is attached to the cytosolic surface of the plasma membrane via palmitoylation of two cysteine residues at its amino terminus (15). According to the immunolocalization studies of protein F1 using electron microscopy, this protein exists mainly in the presynaptic terminals of adult brains (16). Nelson and Routtenberg (7) and Lovinger et al. (17) have reported that phosphorylation of protein F1 may be involved in the establishment of synaptic long term potentiation in rat hippocampus. This phosphorylation is catalyzed by a calcium/phospholipid dependent protein kinase C (PKC) (7,18,19).

Since protein F1 appears to play a central role in neuronal regeneration and plasticity, antibodies against this protein can be used as a tool to investigate the distribution and physiological function of protein F1 in mammalian neurons. Peptide conjugates provide a convenient way to produce antibodies against different domains of a protein. In this study, a 21 amino acid polypeptide was selected from the primary sequence of protein F1 that corresponds to its C-terminus. The selected peptide (AKPKES*AR*QDEGKEDPEADQE) has a possible phosphorylation site for PKC (SAR). The peptide was shown to be a good substrate for PKC and was used to produce anti-protein F1 polyclonal antibodies in rabbit. These antibodies showed good results on immunoblot analysis and in immunostaining of mouse spinal cord neurons in vitro.

EXPERIMENTAL PROCEDURES

Synthesis and Purification of F1 Peptide. Molecular cloning of F1 cDNA has revealed that protein F1 is a hydrophilic 226 amino acid protein encoded by 1.5 kb brain-specific mRNA. A 21 amino acid polypeptide (Mr 2300) was selected from the predicted primary structure of rat protein F1 (20), synthesized (Bio-Synthesis, Inc., Lewisville, TX), and confirmed by amino acid composition analysis. The peptide corresponds to the carboxy terminus of the protein from position 204–224.

The synthesized peptide was further purified by reversed-phase HPLC using a Waters C18 column eluted with 0–50% acetonitrile gradient in 0.1% trifluoroacetic acid (TFA)/H_2O at a flow rate of 1 ml/min and the purified peptide was stored at −20°C.

Production of Anti-Protein F1 Antibodies. Two milligrams of the synthetic F1 peptide and 18 mg of keyhole limpet haemocyanin (KLH, Calbiochem, San Diego, CA) were dissolved in 2 ml of 0.1 M phosphate buffer (pH 7.2). The peptide was conjugated to KLH by adding glutaraldehyde to a final concentration of 0.6% (v/v) (21). The mixture was incubated at room temperature for 3 hr then dialyzed against phosphate-buffered saline (PBS) overnight at 4°C against two changes of PBS (500 ml each). The dialyzed sample was made up to 5 ml with PBS, and 1.5 ml of the F1 peptide-KLH conjugate was emulsified with 1.5 ml Freund's complete adjuvant (Calbiochem) and injected into a rabbit. Preimmune serum was collected before immunization.

Three other preparations were made by emulsifying 0.75 ml of the immunogen with 0.75 ml Freund's incomplete adjuvant for each preparation. The animal was injected weekly with the preparation. The animal was bled after 4 weeks through ear veins. The animal was further boosted with 1.5 ml of F1 peptide-KLH conjugate and 30–40 ml of blood were collected by cardiac puncture a week after. The collected blood was decanted and centrifuged at 1,000 *g* for 20 min then the supernatant (serum) was stored at −20°C. To isolate the immunoglobulin G (IgG) fraction, the immune serum was dialyzed against 0.1 M potassium phosphate (pH 7.0) overnight at 4°C and purified by protein A affinity chromatography (Bio-Rad). The purified IgG was dialyzed overnight against PBS and stored at −20°C.

Purification of Protein F1. Twelve male Sprague-Dawley rats (Sasco Inc, Omaha, NE) were anesthetized using pentobarbital (Sigma, St. Louis, MO) at a concentration of 65 mg/kg body weight. Brains were then taken out and used as a source of protein F1. Purification was performed according to established procedures (22). Briefly, rat cortices (about 12 *g*) were homogenized in 60 ml of ice cold 0.32 M sucrose and 2 mM DTT using Teflon-Glass homogenizer (15 strokes). The homogenate was centrifuged in a Beckman J2-21 centrifuge at 1,000 *g* for 10 min and the supernatant was then centrifuged at 12,000 *g* for 20 min. The pellet was resuspended, centrifuged further at 17,500 *g* for 20 min. The supernatant was recovered as the source of PKC. The pellet from this same step (crude synaptosomal membranes) was resuspended in 10 ml of 1 mM magnesium acetate. Protein F1 was solubilized by pH extraction (23), precipitated with 40–80% ammonium sulfate, resuspended in 5 mM potassium phosphate and 1 mM EDTA (pH 7.5), and dialyzed overnight against the same buffer. The dialyzed sample was applied to hydroxylapatite column and the fractions containing protein F1 were pooled and further purified by phenyl sepharose column. All the procedures were carried out at 4°C.

Immunoblot Analysis of Protein F1. Fractions enriched in protein F1 (10 μg) were boiled for 3 min in sample buffer (0.125 M Tris-HCl, 2% sodium-dodecyl sulfate (SDS), 5% β-mercaptoethanol, 20% glycerol, and 0.0025% bromophenol blue). SDS-polyacrylamide gels (1 mm thick) were prepared according to the method of Laemmli (24). Proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was then either stained with Coomassie brilliant blue (R-250, Bio-Rad, Richmond, CA) or proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad) in 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% methanol for 2 hr at a constant current of 150 mA with a mini Trans-Blot cell (Bio-Rad). After transfer, the nitrocellulose membrane was blocked for 1 hr in 3% gelatin added to buffer A (20 mM Tris-HCl, pH 7.6; 0.5 M NaCl, and 0.05% Tween-20). The membrane was then washed 3 times with buffer A and incubated overnight at 4°C with anti-protein F1 antibodies at a dilution of 1:400 in buffer A containing 0.1% gelatin. The nitrocellulose membrane was then washed with buffer A followed by a 2 hr incubation with 1:1,000 dilution of sheep anti-rabbit IgG conjugated to horseradish peroxidase (Sigma) in buffer A and 0.1% gelatin. Immunoreactive bands were visualized by treatment with 4-chloro-1-naphthol (Bio-Rad) and 3% H_2O_2 in buffer A. Immunoblotting was performed according to manufacturer's protocol.

Purification of Protein Kinase C. Protein kinase C was purified according to the method of Murakami et al. (25). Briefly, whole brains of 12 Sprague-Dawley rats were homogenized in 6 volumes of ice cold 20 mM Tris-HCl buffer (pH 7.5) containing 0.3 M sucrose, 2 mM EDTA, 10 mM EGTA, and 2 mM DTT with Teflon-Glass homogenizer. The homogenate was centrifuged at 100,000 *g* for 1 hr (Beckman L5-50B ultracentrifuge). The supernatant was made 40 μM with respect to cAMP, to decrease contamination of cAMP-dependent protein kinase, and applied to DEAE-sepharose column (2.5 × 9 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM EGTA, 2 mM EDTA, 2 mM DTT, and 40 μM cAMP. The column was then washed with 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EGTA, 1 mM EDTA, and 2 mM DTT. The column was eluted with 600 ml of 0–0.3 M NaCl linear gradient in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EGTA, 1 mM EDTA, and 2 mM DTT at a flow rate of 1 ml/min. The active fractions (containing PKC) were pooled and concentrated down to 3 ml with an Amicon-Ultrafiltration cell (Amicon, Beverly, MA). The sample was applied to an Ultrogel AcA-44 column (2.5 × 112 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM EGTA, 0.5 mM EDTA, and 2 mM DTT. Elution was performed using the same buffer at a flow rate of 0.5 ml/min (all procedures were carried out at 4°C). Fractions with high PKC activity were pooled, concentrated, and stored in glycerol (1:1) at −80°C. Activity was maintained for at least 6 months following purification.

Protein Kinase C Assay. PKC activity was determined by subtracting the amount of $^{32}PO_4$ transferred from [γ-^{32}P] ATP (ICN Radioactive Chemicals, Irvine, CA) to histone type V-S (Sigma) in absence of phospholipid, from the amount of ^{32}P-incorporated in presence of the phospholipid (26). The reaction mixture (final volume 100 μl) included 50 μl of the eluted fractions containing PKC, 40 μg of histone, 5 mM Mg^{2+}, 0.75 mM Ca^{2+}, in the presence and absence of 25 μg phosphatidyl serine (Avanti Polar-Lipids, Inc., Pelham, AL) and 3 μg of L-α-1-oleoyl-2-acetoyl-sn-3-glycerol (OAG, Avanti Polar-Lipids, Inc, Pelham, AL) in 20 mM Tris, pH 7.6. The reaction was then initiated by adding 5 μl of [γ-^{32}P] ATP (final concentration 65 μM, specific activity 225 cpm/pmol). The reaction was allowed to proceed for 10 min at 30°C then terminated by the addition of 80 μl of 60% TCA. An aliquot (80μl) was spotted onto Whatman P-81 filter paper (Whatman, Hillsboro, OR) which was then washed in 30% acetic acid for 15 min, 15% acetic acid for 10 min (3 times), and acetone for 5 min. Filter paper was then dried and radioactivity retained determined by liquid scintillation counting (Beckman, LS 5000TD, Beckman Instruments Inc, Fullerton, CA). The typical protein kinase activity of the partially purified PKC was 170 pmol/min.

Immunoprecipitation of Protein F1. Immunoprecipitation of radiolabeled protein F1 was achieved by incubation in the presence of purified anti-protein F1 antibodies for 4 hr at 4°C. Prewashed standardized pansorbin (Calbiochem) (45 μl) was added, incubated for a further 1 hr at 4°C, and the conjugated antibodies sedimented by centrifugation (12,000 *g*) for 5 min at 4°C. The pellet was washed 3 times with NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, 0.05% NP-40) and finally resuspended in sample buffer. The mixture was boiled for 3 min then proteins were resolved by electrophoresis (10% gels, constant voltage of 200 V). The gel was then stained (Coomassie blue), dried, and exposed overnight to X-ray film (Kodak).

Cell Culture. Dissociated spinal tissue from 13–14-day mouse embryos was cultured according to the method of Ransom et al. (27) with some modifications. Cells were seeded on flamed, poly-L-lysine (10 mg/ml, Sigma) coated coverslips affixed to the bottom of the 60 mm culture dishes (Corning Inc, Corning, NY) with silicone rubber

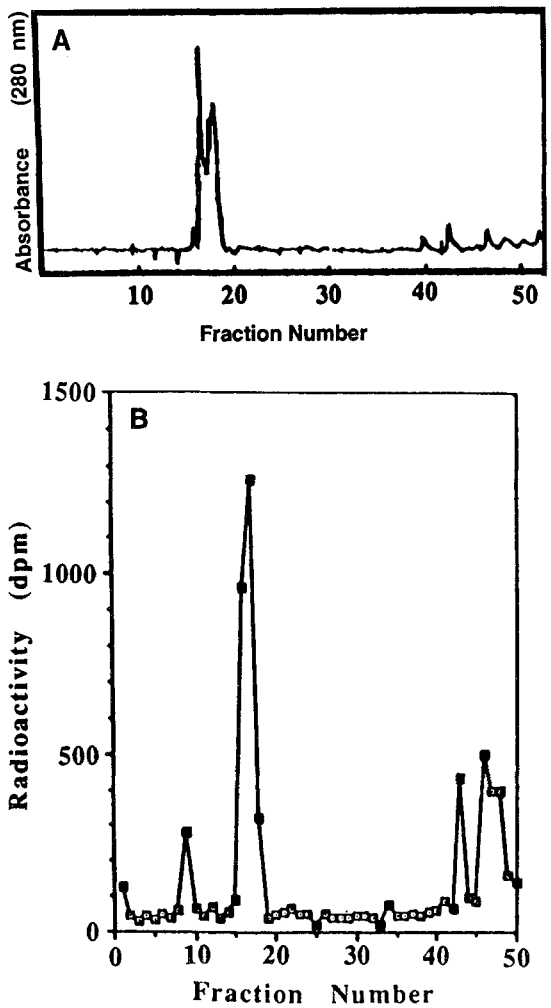


Fig. 1. Purification of unphosphorylated and phosphorylated F1 peptide by reversed-phase HPLC. The phosphorylation of F1 peptide was catalyzed by PKC, then the reaction mixture was applied to AG1X8 column (Bio-Rad) and the phosphopeptide was eluted with 15% acetic acid. The phosphopeptide generated above was purified by reversed-phase HPLC using a Waters C18 column eluted with 0–50% acetonitrile gradient (flow rate of 1 ml/min) as described in experimental procedures. (A) elution profile of phosphorylated F1 peptide. (B) Radioactivity of the fractions of HPLC elution profile of ^{32}P-phosphorylated F1 peptide.

(Dow Corning, Midland, MI). Spinal neurons were dissociated in papain (Boehringer Mannheim, Indianapolis, IN) for 15 min then seeded and maintained initially in minimal essential medium (MEM, GIBCO BRL, Gaithersburg, MD) containing 10% fetal bovine serum and 10% horse serum (HyClone Lab, Logan, Utah). Thereafter, cells were fed 2-times a week with MEM containing 10% horse serum. When the cultures were confluent, glial growth was inhibited by the addition of fluoro-deoxyuridine plus uridine (Sigma). The cells were grown at 37°C in an atmosphere of 90% air and 10% CO_2.

Immunocytochemical Stain. Spinal neuronal cultures (27) were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 20 min at room temperature followed by a rinse with PBS. Cultures were preincubated with goat serum (blocking agent) for 30 min, rinsed with PBS and

then reacted with a 1:1000 dilution of purified anti-protein F1 antibodies or with IgG from preimmune rabbit serum for 30 min at 37°C. Cultures were rinsed with PBS followed by incubation with biotinylated anti-rabbit IgG and stained with streptavidin conjugated to multiple alkaline phosphatase molecules and naphthol AS-MX phosphate (BioGenex Laboratories, CA) according to the manufacturer's procedures.

Protein Assay. Protein concentration of the samples was estimated by the Bradford method (Bio-Rad, Richmond, CA) using bovine serum albumin as standard.

RESULTS

Phosphorylation of Synthetic F1 Peptide by PKC (Figures 1–3). In order to test whether F1 peptide is a substrate for rat brain PKC, F1 peptide, purified by reversed-phase HPLC, was incubated with purified PKC in the presence of [γ-^{32}P] ATP. The reaction was allowed to proceed for 20 min at 30°C then the reaction mixture was applied to AG1X8 column (Bio-Rad), to remove extraneous [γ-^{32}P] ATP, and the peptide was eluted with 15% acetic acid. Reversed-phase HPLC analysis (C18 column using 0–50% acetonitrile gradient) of this mixture revealed a further peptide which eluted with a smaller retention time (Fig. 1A) relative to the native peptide. This is consistent with the introduction of a phosphate group in the peptide. Liquid scintillation spectrometric analysis of eluted fractions confirmed that this peptide with earlier elution time corresponded with the phosphorylated peptide.

The radioactive fractions (Fig. 1B) were pooled, concentrated by centrifugal vacuum concentrator (RC 10.10 Jouan Inc., Winchester, VA), and the phosphopeptide was analyzed by SDS-PAGE (20% gel). A single band was observed following Coomassie blue staining (Fig. 2A). By autoradiography (Fig. 2B), it was determined that all radioactivity was associated with this band which co-migrated with purified non-phosphorylated F1 peptide. These data suggest that F1 peptide is a substrate for protein kinase C.

In order to demonstrate that the phosphorylation of F1 peptide represented a specific effect, the concentration of F1 peptide was varied in the reaction mixture. As shown in Fig. 3, the phosphorylation of F1 peptide by PKC was linearly dependent on F1 peptide concentration. From this data, it was determined that PKC phosphorylated F1 peptide has a Km of approximately 0.25 μM which is consistent with the proposal that F1 peptide is a good substrate for PKC.

Immunoblotting of Protein F1 (Figure 4). The synthetic peptide was then used to generate polyclonal antibodies (see methods). These antibodies were shown initially to have strong reactivity with purified F1 peptide (data not shown). In order to test the ability of these antibodies to recognize intact protein F1, immunoblot analysis of protein F1 obtained from various stages of purification (ammonium sulfate precipitate, and fractions eluted from hydroxylapatite and phenyl sepharose columns) was performed. A single strong band (Mr 43,000) can be seen on immunoblotting of protein F1 eluted from phenyl sepharose column (Fig. 4) revealing that anti-F1 peptide antibodies can recognize the intact protein F1 from rat brain. Immunoblot analysis of crude

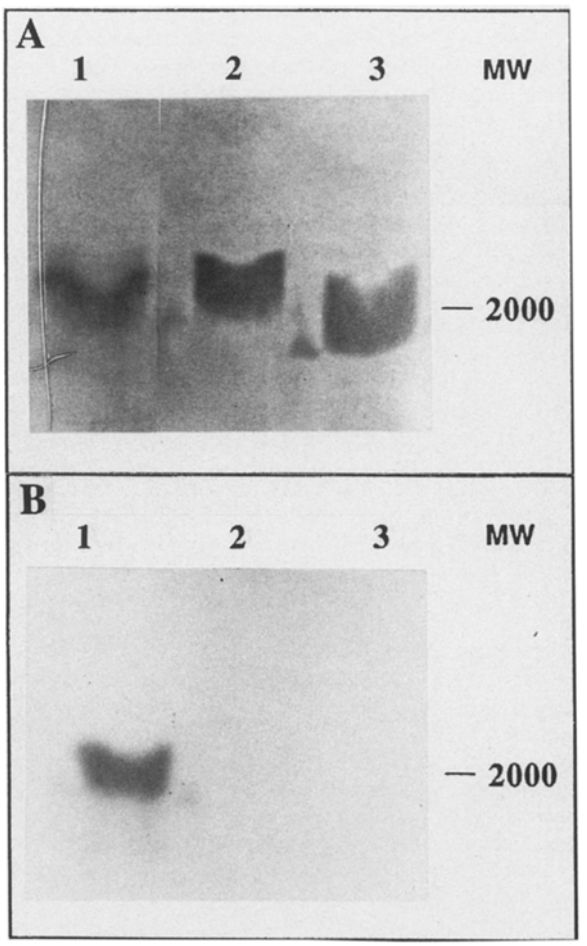


Fig. 2. SDS-PAGE analysis and autoradiogram of F1 peptide. F1 peptide, purified by reversed-phase HPLC, was used as substrate for PKC partially purified from rat brain. The reaction mixture was purified using AG1X8 (Bio-Rad). The phosphopeptide was further purified by reversed-phase HPLC using C18 column eluted with 0–50% acetonitrile gradient in 0.1% TFA/H_2O. Radioactive fractions were pooled, concentrated, and electrophoresed on 20% SDS-PAGE. The gel was dried and exposed to X-ray film (Kodak) at −80°C. Figure 2A represents a Coomassie blue stain of phosphorylated F1 peptide (1), unphosphorylated F1 peptide (2), and a molecular weight marker peptide (3) of Mr 2000. Figure 2B is an autoradiogram of the phosphorylated F1 peptide (1).

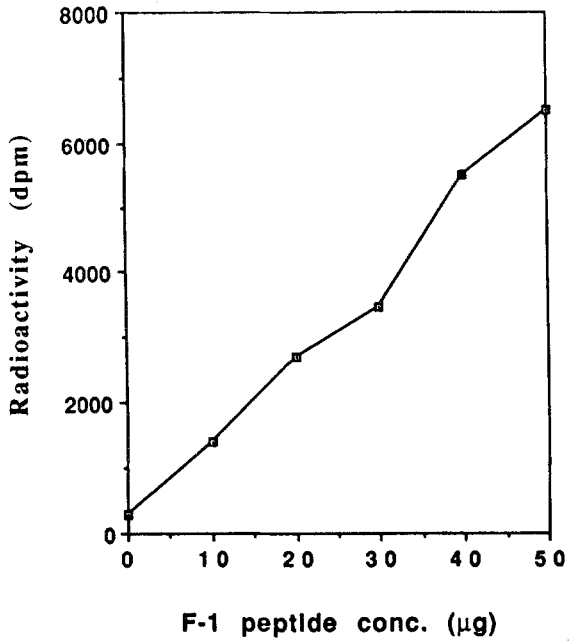


Fig. 3. Concentration-dependent phosphorylation of F1 peptide by PKC. The phosphorylation was carried out in a reaction mixture (final volume 100 μl) containing 0.75 mM Ca^{2+}, 5 mM Mg^{2+}, 25 μg phosphatidyl serine and 3 μg OAG in 20 mM Tris-HCl, pH 7.6, and 65 μM [γ-^{32}P] ATP, and different concentrations of F1 peptide (10–50 μg). The reaction was allowed to proceed for 10 min at 30°C, then stopped by adding 80 μl of 60% TCA. A fraction of the reaction mixture was spotted on P-81 filter. The filter was washed in acetic acid and acetone, dried and radioactivity was counted by liquid scintillation counter.

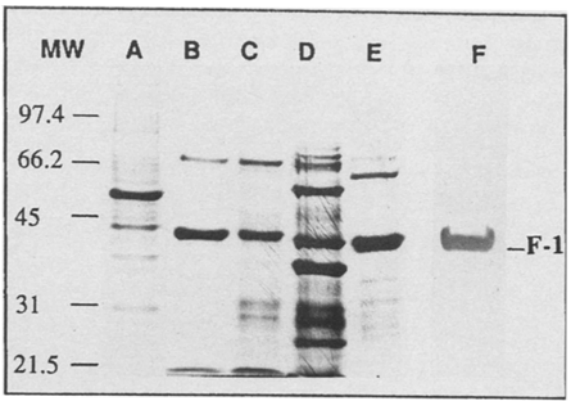


Fig. 4. SDS-PAGE and Western Blot Analysis of protein F1. Protein F1 purified at different steps was electrophoresed on 10% SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose and detected with anti-protein F1 polyclonal antibodies as described in experimental procedures. The molecular weight markers used are: phosphorylase B (97,400), Bovine serum albumin (66,200), Ovalbumin (45,000), Carbonic anhydrase (31,000), Soyabean inhibitor (21,500), and Lysozyme (14,400). Lanes (A), (B), (C), (D), and (E) are Coomassie blue stained SDS-PAGE of proteins in: 40–80% ammonium sulfate precipitate, 0-30 mM, 30-75 mM, and 75-400 mM potassium phosphate (pH 7.2) fractions eluted from hydroxylapatite column, and void volume eluted from phenyl sepharose column respectively. Lane (F) represents immunoblotting of protein F1 purified through phenyl sepharose column.

brain homogenate also revealed strong immunoreactivity to a protein of molecular weight similar to protein F1 (data not shown).

Immunoprecipitation of Protein F1 (Figure 5). The generated anti-F1 peptide antibodies were next used to immunoprecipitate protein F1 from partially purified preparation of brain homogenate that had been incubated in the presence of purified PKC and [γ-^{32}P] ATP. The mixture was then analyzed on SDS-PAGE (10% gels) and proteins were visualized with Coomassie blue staining. Following autoradiography of the dried gels, a protein of Mr 43,000 that corresponded to protein F1 was shown to be phosphorylated under these conditions (Fig. 5E). Another band was detected at Mr ~ 32,000 which is probably a proteolytic product of protein F1 (28). These results indicate that anti-protein F1 antibodies may recognize both the phosphorylated and unphosphorylated forms of protein F1.

Immunolocalization of Protein F1 (Figure 6). Since the protein F1 sequence is highly conserved in mammals, the antibodies raised against the C-terminus peptide of rat protein F1 could also be useful for the detection of protein F1 in growing cell cultures. To test this, 3-week old cultures of mouse spinal neurons (dissociated from 14-day old mouse embryos) were fixed then reacted with anti-protein F1 antibodies or preimmune rabbit IgG. Following staining, cultured mouse spinal neurons revealed protein F1 immunoreactivity in neurites and cell body membranes (Fig. 6B). Glial cells, however, are not stained. In contrast, preimmune rabbit IgG treated cells did not show any immunoreactivity (Fig. 6C).

DISCUSSION

In this study a peptide based on the primary sequence of protein F1 has been used to generate polyclonal antibodies against this protein. This peptide was selected because it shares sequence homology in human, bovine, rat, and mouse protein F1 (29). Furthermore, it contains a potential PKC phosphorylation site (serine 209) the function of which is not known. It is reasoned, therefore, that antibodies raised against this sequence may provide tools to answer the function of the phosphorylation of this site.

Data from this study indicated that serine 209 near the C-terminus of protein F1 can be phosphorylated by PKC in vitro in a concentration-dependent manner. The

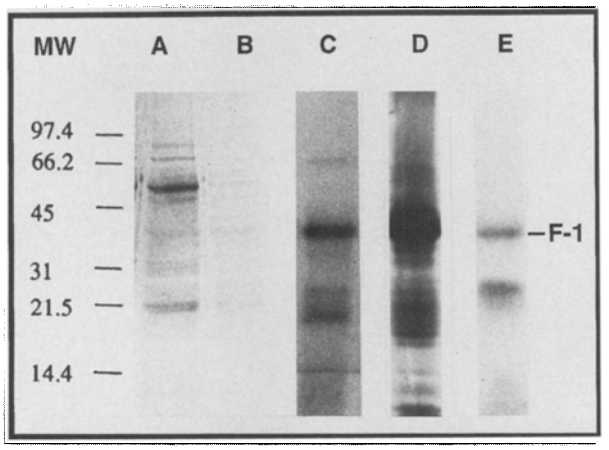


Fig. 5. Phosphorylation and immunoprecipitation of protein F1. Ammonium sulfate extract from rat brain was used as a substrate for PKC purified from the same tissue (as described in experimental procedures). Sample buffer was added to stop the reaction and the sample was purified on SDS-PAGE. The gel was then stained, dried and exposed to X-ray film. In a different experiment, anti-protein F1 antibodies were added at the end of the phosphorylation and the immune complex was separated using pansorbin and the proteins were analyzed by electrophoresis. The molecular weight markers used are: phosphorylase B (97,400), Bovine serum albumin (66,200), Ovalbumin (45,000), Carbonic anhydrase (31,000), Soyabean inhibitor (21,500), and Lysozyme (14,400). (A) and (B) are Coomassie staining of SDS-PAGE of ammonium sulfate precipitate and the F1 phosphorylation mixture respectively. (C) is an autoradiogram of F1 phosphorylation mixture. (D) is a Coomassie blue staining of SDS-PAGE analysis of protein F1 immunoprecipitated from ammonium sulfate precipitate (a strong band at Mr ~ 45,000 represents the heavy chain of the antibody) and (E) is an autoradiogram of the dried gel.

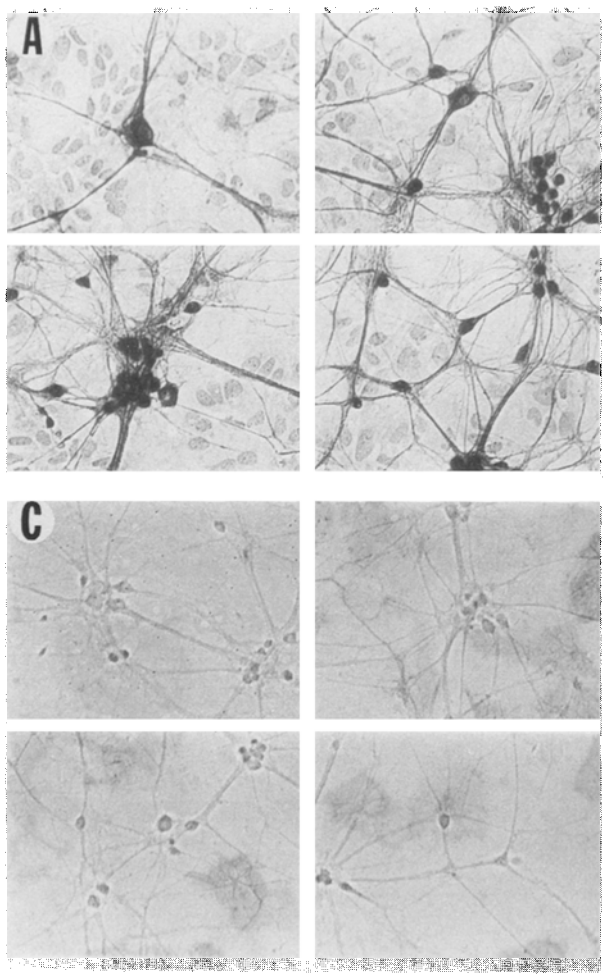
synthesized F1 peptide proved to be a good substrate of PKC (Km = 0.25 μM) and therefore raises the possibility that serine 209 on the intact protein may be a substrate in vivo. On the other hand, the PKC phosphorylation of serine 209 on the intact protein might not occur due to possible conformational differences between the synthetic peptide and the native protein F1. Hence, the physiological significance of the phosphorylation of serine 209 in vivo is yet to be determined. Several groups reported that PKC phosphorylation occurs exclusively at serine residues (22,29,30), whereas site-directed mutagenesis studies confirmed PKC phosphorylation at serine 41 (31). Although protein F1 can also be phosphorylated in vitro by casein kinase II at serines 192 and 193 (major sites) and threonines 88, 89, or 95 (minor sites) (32), no biological significance of casein kinase II catalyzed phosphorylation is known. Recently, Spencer et al. (33) reported that protein F1 is phosphorylated in cultured neurons and rat brains on three sites serine 41, serine 96, and threonine 172. These results suggest that protein F1 can be phosphorylated at different sites and this phosphorylation may play important functions. In vitro PKC phosphorylation of protein F1 inhibits calmodulin binding to the protein (34) and correlates with the persistence of long term potentiation (35). Inhibition of PKC phosphorylation also inhibited calcium-dependent neurotransmitter release from rat cortical synaptosomes (36).

The selected peptide has been shown in this study to be a potent immunogen for the production of anti-protein F1 antibodies. The antibodies showed strong immune reactivity to protein F1 on immunoblotting analysis. In addition, the antibodies were successfully used to immunoprecipitate phosphorylated protein F1 from a partially purified brain extract where two bands appeared on the autoradiogram, one detected at Mr ~ 43,000 (the expected molecular weight of protein F1). The second band was detected at Mr ~ 32,000 that is suspected to result from the action of a protease reported to cleave protein F1 at the N-terminal side of serine 41 (28).

Anti-protein F1 antibodies stained spinal neurons in cultures. Neuronal membranes and neurites exhibited strong reactivity to the antibodies while glial cells did not exhibit specific immunoreactivity (Fig. 6B). Previous immunolocalization studies with mature neuronal cultures reported protein F1 to exist mainly in growth cones, the growing tips of axons (2, 37). However, Burry et al. (38) reported that in one day old cultures of neonatal rat cerebellum, all membranes of cell bodies and neurites stained for protein F1 antibodies, but that cell body staining was greatly reduced by 10 days. In a different study, protein F1 immunoreactivity has been demonstrated in all neuronal plasma membranes of cultured hippocampal pyramidal neurons (39).

The antibodies raised against the protein F1 C-terminus peptide are currently being used to quantitate the expression of protein F1 in cultured mouse spinal neurons derived from embryonic spinal cord tissue. By using an enzyme-linked immunosorbent assay (40) it is hoped that protein F1 may be employed as a developmental marker via comparisons of protein F1 levels in vitro with those associated with different developmental stages in vivo.

The same antibody, or possibly antibodies produced against the functional domain of protein F1 (residues 41–51), that contains the major PKC phosphorylation site in vivo and calmodulin binding domain, might also be used to investigate the physiological roles supported by this protein. Specifically, it would be of great interest to determine changes in synaptic functions or effects on the spontaneous electrical activity of neuronal networks (41). Pilot experiments in this direction have shown that phospholipid-mediated delivery of anti-protein F1 antibodies (42) into cultured mouse spinal neurons causes excessive



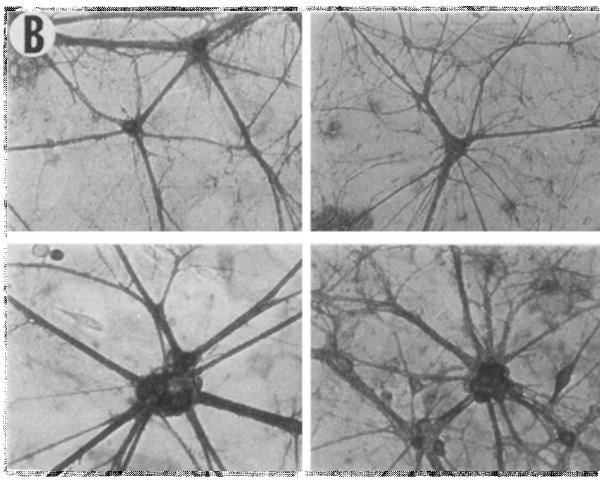


Fig. 6. Immunolocalization of protein F1 in mouse spinal cord neurons in culture. Spinal neuronal cultures, of the same tissue and age (3 weeks), were treated with anti-protein F1 antibodies or rabbit IgG. (A) Mouse spinal neurons fixed with Gregory reagent and stained with Loots modified Bodian stain (43). In this case both neurons and nuclei of glial cells stained. (B) Culture fixed with 4% paraformaldehyde in PBS and immunostained with anti-protein F1 antibodies (1:1000) using StrAviGen immunostaining kit (Biogenex). Neurons showed strong reactivity to the antibodies on cell membranes and neurites while glial cells did not stain. (C) Culture fixed with 4% paraformaldehyde in PBS and immunostained with rabbit IgG (the heavy staining evident in (B) is not present in this preparation). Light staining of some glial cells is visible in both panels (B) and (C) and may represent non-specific interactions. Each panel represents four different areas from the coverslip. All photographs were magnified at 200X.

neuronal death. Antibody transfer via liposomes or the use of antisense oligonucleotides to block protein F1 synthesis may be less damaging to neurons in cultures and are presently under consideration.

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REFERENCES

1. Skene, J. H. P., Jacobson, R. D., Snipes, G. J., McGuire, C. B., Norden, J. J., and Freeman, J. A. 1986. A protein induced during nerve growth (GAP-43) is a major component of growth-cone membranes. Science 233:783–786.
2. Meiri, K. F., Pfeninger, K. H., and Willard, M. B. 1986. Growth associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to pp-46, a major polypeptide of a subcellular fraction enriched in growth cones. Proc. Natl. Acad. Sci. USA. 83:3537–3541.
3. Skene, J. H. P. 1989. Axonal growth-associated proteins. Ann. Rev. Neurosci. 12:127–156.
4. Skene, J. H. P., and Willard, M. B. 1981. Changes in axonally transported proteins during axon regeneration in toad retinal ganglion cells. J. Cell Biol. 89:86–95.
5. Zwiers, H., Verhoef, J., Schotman, P., and Gispen, W. H. 1980. A new phosphorylation-inhibiting peptide (PIP) with behavioral activity from rat brain membranes. FEBS Lett. 112:168–172.
6. Andreasen, T. J., Luetje, C. W., Heideman, W., and Storm, D. R. 1983. Purification of a novel calmodulin binding protein from bovine cerebral cortex membranes. Biochem. 22:4615–4618.
7. Nelson, R. B., and Routtenberg, A. 1985. Characterization of protein F1 (47 kDa, 4.5 pl): A kinase C substrate directly related to neural plasticity. Exp. Neurol. 89:213–224.
8. Benowitz, L. I., and Lewis, E. R. 1983. Increased transport of 44,000 to 49,000-dalton acidic protein during regeneration of the

goldfish optic nerve: a two-dimensional gel analysis. J. Neurosci. 3:2153–2163.
9. Biffo, S., Verhaagen, J., Schrama, L. H., Schotman, P., Danho, W., and Margolis, F. L. 1990. B-50/GAP-43 expression correlates with process outgrowth in the embryonic mouse nervous system. Eur. J. Neurosci. 2:487–499.
10. Grafstein, B., Burmeister, D. W., McGuinness, C. M., Perry, G. W., and Sparrow, J. R. 1987. Role of fast axonal transport in regeneration of goldfish optic axons. Prog. Brain Res. 71:113–120.
11. Strittmatter, S. M., Valenzuela, D., Kennedy, T. E., Neer, E. J., and Fishman, M. C. 1990. Go is a major growth cone protein subject to regulation by GAP-43. Nature 344:836–841.
12. Van Dongen, C. J., Zwiers, H., De Graan, P. N. E., and Gispen, W. H. 1985. Modulation of the activity of purified phosphatidyl inositol 4-phosphate kinase by phosphorylated and dephosphorylated B-50 protein. Biochem. Biophys. Res. Commun. 128:1219–1227.
13. Masure, H. R., Alexander, K. A., Wakim, B. T., and Storm, D. R. 1986. Physiochemical and hydrodynamic characterization of P-57, a neurospecific calmodulin binding protein. Biochem. 25:7553–7560.
14. Zwiers, H., Ver Haagen, J., Van Dongen, C. J., De Graan, P. N. E., and Gispen, W. H. 1985. Resolution of rat brain synaptic phosphoprotein B-50 into multiple forms by two-dimensional electrophoresis: evidence for multisite phosphorylation. J. Neurochem. 44:1083–1090.
15. Zuber, M. X., Strittmatter, S. M., and Fishman, M. C. 1989. A membrane-targeting signal in the amino terminus of the neuronal protein GAP-43. Nature 341:345–348.
16. Gispen, W. H., Leunissen, J. L. M., Oestreicher, A. B., Verkleij, A. J., and Zwiers, H. 1985. Presynaptic localization of B-50 phosphoprotein: The (ACTH)-sensitive protein kinase substrate involved in rat brain polyphosphoinositide metabolism. Brain Res. 328:381–385.
17. Lovinger, D. M., Wong, K. L., Murakami, K., and Routtenberg, A. 1987. Protein kinase C inhibitors eliminate hippocampal long term potentiation. Brain Res. 436:177–183.
18. Aloyo, V. J., Zwiers, H., and Gispen, W. H. 1983. Phosphorylation of B-50 protein by calcium-activated, phospholipid-dependent protein kinase and B-50 protein kinase. J Neurochem. 41:469–473.
19. Akers, R. F., and Routtenberg, A. 1987. Calcium-promoted translocation of protein kinase C to synaptic membranes: Relation to the phosphorylation of an endogenous substrate (protein F1) involved in synaptic plasticity. J. Neurosci. 7:3976–3983.
20. Rosenthal, A., Chan, S. Y., Henzel, W., Haskell, C., Kuang, W. J., Chen, J. N., Wilcox, A., Ullrich, D. V., Goeddel, E., and Routtenberg, A. 1987. Primary structure and m-RNA localization of protein F-1, a growth-related protein kinase C substrate associated with synaptic plasticity. EMBO J. 6:3641–3646.
21. Goodall, G. J., Hempstead, J. L., and Morgan, J. I. 1983. Production and characterization of antibodies to thymosin β4. J. Immunol. 131:821–825.
22. Chan, S. Y., Murakami, K., and Routtenberg, A. 1986. Phosphoprotein F1: Purification and characterization of a brain kinase C substrate related to plasticity. J. Neurosci. 6:3618–3627.
23. Oestreicher, A. B., Van Dongen, C. J., Zwiers, H., and Gispen, W. H. 1983. Affinity-purified anti-B-50 protein antibody: Interference with the function of the phosphoprotein B-50 in synaptic plasma membranes. J. Neurochem. 41:331–340.
24. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.
25. Murakami, K., Chan, S. Y., and Routtenberg, A. 1986. Protein kinase C activity by cis-fatty acid in the absence of calcium and phospholipids. J. Biol. Chem. 261:15424–15429.
26. Farrar, W. L., Thomas, T. P., and Anderson, W. B. 1985. Altered cytosol/membrane enzyme redistribution on IL-3 activation of protein kinase C. Nature 315:235–237.
27. Ransom, B. R., Neale, E., Menkart, M., Bullock, R. N., and Nelson, P. G. 1977. Mouse spinal cord in culture. I. Morphological and intrinsic neuronal electrophysiological properties. J. Neurophysiol. 40:1132–1150.
28. McMaster, D., Zwiers, H., and Lederis, K. 1988. The growth associated neuronal phosphoprotein B-50: improved purification, partial primary structure and characterization and localization of proteolytic products. Brain Res. Bull. 21:265–276.
29. Benowitz, L. I., and Perrone-Bizzozero, N. I. 1991. The relationship of GAP-43 to the development and plasticity of synaptic connections. Ann. N. Y. Acad. Sci. 627:58–74.
30. Oestreicher, A. B., De Graan, P. N. E., Schrama, L. H., Lamme, V. A. F., Bloemen, R., Schotman, P., and Gispen, W. H. 1989. The protein kinase C phosphosite(s) in B-50(GAP-43) are confined to 15K phosphofragments produced by *Staphylococcus aureus* V8 protease. Neurochem Int. 14:361–372.
31. Apel, E. D., Byford, M. F., Au, D., Walsh, K. A., and Storm, D. R. 1990. Identification of the protein kinase C phosphorylation site in neuromodulin. Biochemistry 29:2330–2335.
32. Apel, E. D., Litchfield, D. W., Clark, R. H., Krebs, E. G., and Storm, D. R. 1991. Phosphorylation of neuromodulin (GAP-43) by casein kinase II. Identification of phosphorylation sites and regulation by calmodulin. J. Biol. Chem. 266:10544–10551.
33. Spencer, S. A., Schuh, S. M., Liu, W., and Willard, M. B. 1992. GAP-43, a protein associated with axon growth, is phosphorylated at three sites in cultured neurons and rat brain. J. Biol. Chem. 267:9059–9064.
34. Alexander, K. A., Cimler, B. M., Meier, K. E., and Storm, D. R. 1987. Regulation of calmodulin binding to P-57. J. Biol. Chem. 262:6108–6113.
35. Lovinger, D. M., Colley, P. A., Akers, R. F., Nelson, R. B., and Routtenberg, A. 1986. Direct relation of long-duration synaptic potentiation to phosphorylation of membrane protein F1: A substrate for membrane protein kinase C. Brain Res. 339:205–211.
36. Dekker, L. V., De Graan, P. N. E., Oestreicher, A. B., Versteeg, D. H. G., and Gispen, W. H. 1989. Inhibition of noradrenaline release by antibodies to B-50 (GAP-43). Nature 342:74–76.
37. Meiri, K. F., Willard, M., and Johnson, M. I. 1988. Distribution and phosphorylation of the growth associated protein GAP-43 in regenerating sympathetic neurons in culture. J. Neurosci. 8:2571–2581.
38. Burry, R. W., Lah, J. J., and Hayes, D. M. 1992. GAP-43 distribution is correlated with development of growth cones and presynaptic terminals. J. Neurocytolo. 21:413–425.
39. Van Lookeren Campagne, M., Dotti, C. G., Jap Tjoen San, E. R. A., Verkleij, A. J., Gispen, W. H., and Oestreicher, A. B. 1992. B-50/GAP43 localization in polarized hippocampal neurons in vitro: an ultrastructural quantitative study. Neurosci. 50:35–52.
40. Van der Neut, R., Oestreicher, A. B., Gispen, W. H., and Bär, P. R. 1990. The expression of B-50/GAP-43 during development of rat spinal neurons in culture is regulated by interneuronal contact. Neurosci. Lett. 105:36–41.
41. Gross, G. W., and Kowalski, J. M. 1991. Experimental and theoretical analysis of random nerve cell network dynamics. Pages 47–110, *in* Antognetti, P. and Milutinovic, V. (eds), Neural Networks: Concepts, Applications, and Implementations, Vol. IV, Prentice Hall, Englewood, N. J.
42. Shea, T. B., Perrone-Bizzozero, N. I., Beermann, M. L., and Benowitz, L. I. 1991. Phospholipid-mediated delivery of anti-GAP-43 antibodies into neuroblastoma cells prevents neuritogenesis. J. Neurosci. 11:1685–1690.
43. Loots, G. P., Loots, J. M., Brown, M. M., and Scheman, J. L. 1979. A rapid silver impregnation method for nervous tissue: a modified protargol-peroxide technic. Stain Technol. 54:97–100.