The EMBO Journal vol.6 no.12 pp.3641–3646, 1987

Primary structure and mRNA localization of protein F1, a growth-related protein kinase C substrate associated with synaptic plasticity

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Communicated by F.Grosveld

Protein F1 is a neuron-specific, synaptic-enriched, membrane-bound substrate of protein kinase C (PKC) whose phosphorylation is related to synaptic plasticity in the adult. The sequence of 26 N-terminal amino acids was determined from purified rat protein F1. A 78-mer synthetic oligonucleotide designed from the partial N-terminal sequence enabled identification of protein F1 cDNA clones in a rat brain library. F1 protein is a 226 amino acid protein encoded by a 1.5 kb brain-specific, developmentally-regulated mRNA. Transcripts for protein F1 can be detected at birth, and their level declines after maturation. A full-length cDNA clone was transcribed and translated *in vitro*. Translation products could be immunoprecipitated with anti-F1 antibodies. *In situ* hybridization analysis revealed protein F1 transcripts in hippocampal pyramidal cells, but not in granule cells. In cerebellum, granule cells contained protein F1 mRNA, while Purkinje cells did not. Co-localization of protein F1 with protein kinase C-II [PKC-II (β)], rather than PKC-I (γ) suggests that PKC-II may phosphorylate protein F1.

Key words: protein kinase C/protein F1/axonal elongation/synaptic plasticity

Introduction

Protein F1, a substrate for protein kinase C (PKC, Nelson and Routtenberg, 1985; Chan *et al.*, 1986), has been linked to adult synaptic plasticity (Routtenberg, 1986; Akers *et al.*, 1986; Lovinger *et al.*, 1985, 1986). This protein is probably identical to (i) the GAP43 nerve growth- and regeneration-associated protein (Snipes *et al.*, 1987; Benowitz *et al.*, 1987); (ii) the pp46-growth cone protein (Nelson *et al.* 1985) and (iii) B-50 protein (Gispen *et al.*, 1986) and therefore belongs to a small family of membrane-bound proteins whose induction, synthesis and rapid axonal transport to the nerve terminal is related to successful axon elongation (Skene and Willard, 1981; Skene *et al.*, 1986; Benowitz and Lewis, 1983). The level of these proteins increases 10–100-fold during normal development of the central nervous system (Skene *et al.*, 1986; Jacobson *et al.*, 1986), during regeneration of the pyramidal tract (Kalil and Skene, 1986) and optic nerve (Skene and Willard, 1981) and during neurite outgrowth of primary cultured cells (Perrone-Bizzozero *et al.*, 1986). Their decline coincides with cessation of axon elongation and with a failure to regenerate after injury (Skene and Willard, 1981; Kalil and Skene, 1986).

Phosphorylation of protein F1 by PKC is increased after hippocampal long-term potentiation. Because of its link to axonal growth, protein F1 may regulate synaptic plasticity by modulating presynaptic terminal axon growth (Routtenberg, 1985). To investigate the role of protein F1 in neural growth and synaptic plasticity, and to examine its mechanism of action, we characterized cDNA clones for protein F1 and analyzed the tissue- and developmentally-regulated expression of the corresponding mRNA.

Results

Isolation of rat protein F1 cDNA clones

A single most-probable (Lathe, 1985) 78-mer oligonucleotide was synthesized based on the N-terminus amino acid sequence of protein F1 (Met-Arg-Thr-Lys-Gln-Val-Glu-Lys-Asn-Asp-Glu-Asp-Gln-Lys-Ile-Glu-Gln-Asp-Gly-Val-Lys-Pro-Gln-Asp-Lys). It was used as a probe for screening a λgt10-based cDNA library made using adult Sprague–Dawley rat brain mRNA. Screening of 1.6×10^6 plaques yielded six positives, four of which were subcloned into M13 vectors and sequenced. The cDNA clones were found to be overlapping and together spanned 1.5 kb. Two potential polyadenylation signal sequences can be found, one between nucleotides 1385–1404, containing three overlapping AATAAA sequences, and another between nucleotides 1463–1470, which was actually used in the cDNA examined (Figure 1). A single open reading frame contained in the cDNA clones between nucleotides 216–894 can encode a 226 amino acid 23.6 kd protein. The predicted protein F1 contains two possible in-frame translation initiation sites, one around positions 215–217 (ACCATGC) and another at positions 227–229 (TGTATGA). The first translation initiation site shows better agreement with the consensus sequences usually surrounding an initiator methionine codon (Kozak, 1986). Surprisingly N-terminal amino acid sequencing indicates the second methionine as the N-terminal amino acid (Figure 1), suggesting that either the first translation initiation site is not used or that the first four amino acids are removed following synthesis to yield the mature protein. The predicted protein F1 is highly hydrophilic (Figure 2) and does not contain any potential N-glycosylation sites. The protein contains three repeats of the peptide Glu-Lys-Lys (Figure 1) that might serve as potential processing sites.

Immunoprecipitation of the protein F1

In order to establish the identity of the cloned cDNA with the protein F1, a cDNA fragment containing the entire coding region was cloned into an SP6-derived vector. F1 mRNA was transcribed *in vitro* using SP6 RNA polymerase, and the resulting transcripts were translated in a rabbit reticulocyte lysate. The translation products were immunoprecipitated using anti-F1 antiserum and electrophoresed on a 12% polyacrylamide SDS gel (Figure 3). The immunoprecipitated protein migrates anomalously as an ~40 kd protein, in agreement with previous studies (Benowitz *et al.*, 1987), despite its low mol. wt of 23.6 kd.

Expression of protein F1 mRNA in rat tissues

In order to determine tissue specificity and developmental regulation of the protein F1, we have analyzed the level of F1 mRNA

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   1 CTGATCGCTGTAGACCTTACAGTTGCTGCTAACTGCCCTGGTGTGTGTGAGGGAGAGAGAGGGAGAGAGAGGGAGAGGGAGGGAGGGAGGGAGGGAGAGCGCGCTAGCGCGAGAGAGCGA
                                                                                                                         *
 121 GTGAGCAAGCGAGCAGAAAAGAGGTGGAGAGGGGGGGAATAAGGAAGAGAGGAGGAAAGGAGAGAAGGCAGGAAGAAGGCAGGGGAAGATACCACCATGCTGTGCTGTATGAGAAGAACC
   1                                                                                                 MetLeuCysCysMetArgArgThr

 241 AAACAGGTTGAAAAGAATGATGAGGACCAAAAGATTGAACAAGATGGTGTCAAACCGGAGGATAAGGCTCATAAGGCTGCAACCAAAATTCAGGCTAGCTTCCGTGGACACATAACAAGG
   9 LysGlnValGluLysAsnAspGluAspGlnLysIleGluGlnAspGlyValLysProGluAspLysAlaHisLysAlaAlaThrLysIleGlnAlaSerPheArgGlyHisIleThrArg

 361 AAAAAGCTCAAAGACGAGAAGAAGGGTGATGCACCAGCTGCTGAGGCCGAGGCCAAGGAGAAGGATGATGCTCCCGTTGCTGATGGTGTGGAGAAGAAGGAGGGAGATGGCTCTGCTACT
  49 LysLysLeuLysAspGluLysLysGlyAspAlaProAlaAlaGluAlaGluAlaLysGluLysAspAspAlaProValAlaAspGlyValGluLysLysGluGlyAspGlySerAlaThr

 481 ACCGATGCAGCCCCAGCCACCAGCCCTAAGGCTGAGGAGCCCAGCAAGGCAGGAGATGCACCTTCTGAGGAGAAGAAGGGCGAAGGGGATGCGGCCCCTTCAGAGGAAAAGGCCGGCTCA
  89 ThrAspAlaAlaProAlaThrSerProLysAlaGluGluProSerLysAlaGlyAspAlaProSerGluGluLysLysGlyGluGlyAspAlaAlaProSerGluGluLysAlaGlySer

 601 GCTGAGACAGAAAGTGCTGCTAAAGCTACCACTGATAACTCGCCGTCCTCCAAGGCCGAAGATGGCCCAGCCAAGGAGGAGCCTAAACAAGCCGATGTGCCTGCTGCTGTCACTGATGCT
 129 AlaGluThrGluSerAlaAlaLysAlaThrThrAspAsnSerProSerSerLysAlaGluAspGlyProAlaLysGluGluProLysGlnAlaAspValProAlaAlaValThrAspAla

 721 GCTGCCACCACCCCTGCTGCAGAGGATGCTGCCAAGGCAGCGCAGCCTCCAACGGAGACTGCAGAAAGCAGCCAAGCTGAGGAGGAGAAAGAAGCTGTAGATGAAGCCAAACCTAAGGAA
 169 AlaAlaThrThrProAlaAlaGluAspAlaAlaLysAlaAlaGlnProProThrGluThrAlaGluSerSerGlnAlaGluGluGluLysGluAlaValAspGluAlaLysProLysGlu

 841 AGTGCCCGACAGGATGAGGGTAAAGAAGACCCCGAGGCTGACCAAGAACATGCCTGAACTTTAAGAAACGGCTTTCCACGTTGCCCCCACCTGAACCCTGTCTCTCCTGCCCTTTCTCAG
 209 SerAlaArgGlnAspGluGlyLysGluAspProGluAlaAspGlnGluHisAlaOP*

 961 ATCCACTCTGAAGTTTCCTCTCCTGTCCTGCTCACGTGTGTGAGCCTGTCCTCTCCTACCTATGAGCCCTCTCTCTCTGTGTGGCAAACATTTAAAAAAAAAAAAGCAGGAAAGATCCCA

1081 AGTCCAACAGTGTGGCTTAAACATTTGTTTCTTGGTGTTGTTATGGCGAGTTTTTGGTAATGATGATGCAGTCATCTTGGGAAATTCTTGCACTGTACCCCGGTTTTTTGATCTGGTGCG

1201 TGTGGCCCTGTGGGAGTCCACTTTCCTCTCTATTTCTCTCTGTTCCAAGTGTGTGTGTGCAATGTTCCGTTCTGAGGAGTCCAAAATATTAAGTGAATTCAAAAAACCATTTCTGTTTCC

1321 TCATTTTCAATGTGATGGAATGAACAAAAAGGTTAAAAAAATTTAAAAAAAAAAACCGGTTTGTTTTAAAAATAAATAAATAAAGCAAATGTGCCAATTAGCGTAACTTAAGGCTGTGAG

1441 GCTCCTTTTTCAATCTGAATATTAATAAATCATGAGAGTAATCAAAA polyA
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Fig. 1. cDNA sequence and predicted primary structure of the protein F1. The nucleotide sequence was derived from two full length and two partial cDNA clones. Potential polyadenylation sites are underlined. The predicted amino acid sequence is shown below the DNA. N-Terminal methionine as predicted from N-terminal protein sequence is marked by an asterisk. The first in-frame methionine and amino acid predicting the putative mature N-terminus are shown in bold lettering. Repeated amino acid sequence Glu-Lys-Lys is shaded.

Fig. 2. Hydropathy plot of the protein F1.

in 21-day-old rat brain, spleen, kidney, liver and heart tissues, and in developing rat brain. Northern blot analysis reveals the existence of 1.5 kb RNA in the brain but not in the other tissues (Figure 4A). Protein F1 mRNA could be detected in newborn rat brain. Its level increased during the first week and declined during maturation (Figure 4B). Protein F1 mRNA could be detected in adult hippocampus, amygdala, cortex and midbrain (Figure 4C).

Fig. 3. Immunoprecipitation of protein F1 translated *in vitro*. Uncapped or capped mRNA transcribed *in vitro* from an F1 cDNA template using SP6 RNA polymerase were translated in rabbit reticulocyte lysate. Translation products were immunoprecipitated and analyzed on a 12% SDS polyacrylamide gel (see Materials and methods). Anti-sense RNA derived from *in vitro* transcription of F1 cDNA template in the wrong orientation, and viral RNA, were used as negative controls.

Localization of F1 mRNA in rat brain by in situ hybridization

The expression of protein F1 mRNA in rodent brain is selective for certain brain regions (Figure 5) and certain cell types (Figure 6). High levels of protein F1 transcripts can be found in olfactory structure (mitral and periglomerular cells of the olfactory bulb, as well as the olfactory tubercle and cortex) (Figure 6). Expression is also detected throughout the neocortex, thalamus, substantia nigra, preoptic area, inferior colliculus, scattered cells of the caudate nucleus, amygdala, brain stem and in selected hippocampal zones (see below). Other structures demonstrating detectable protein F1 mRNA are indicated in Figure 5.

Protein F1 mRNA is specifically detected with a high signal-to-noise ratio. Even in brain regions which show uniform labeling, like the neocortex, there are cells which clearly do not contain F1 mRNA (Figure 6B). Protein F1 mRNA shows selective expression in certain cell types. In the hippocampus pyramidal cells express protein F1 mRNA while granule cells do not (Figure 6C); in contrast, in the cerebellum, granule cells express protein F1 mRNA but Purkinje cells do not (Figure 6E). Entorhinal cortex, a major source of afferents projecting to the hippocampus, contains protein F1 transcripts in both layer II and

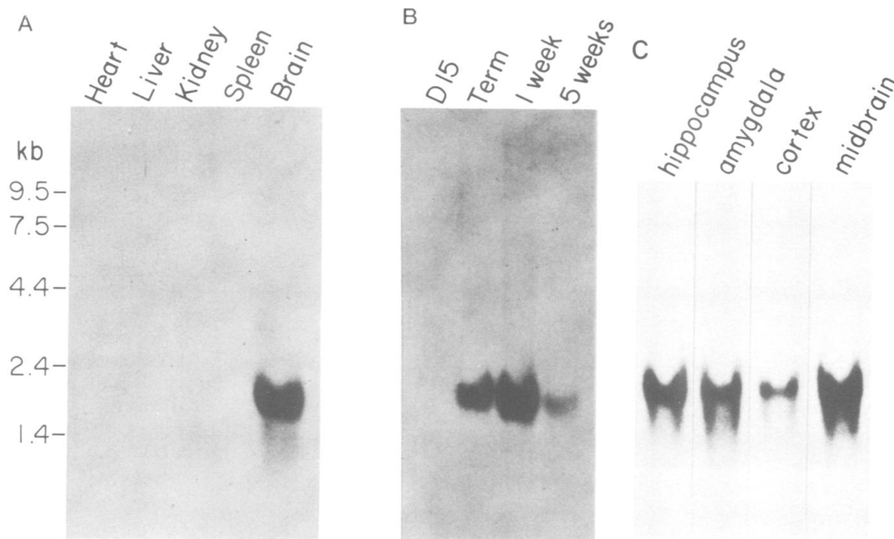

Fig. 4. Northern hybridization analysis of protein F1 mRNA from rat tissue. (**A**) 20 μg of poly(A) RNA from 21-day-old rat heart, liver, kidney, spleen and brain were separated on agarose formaldehyde and hybridized to ^{32}P-labeled protein F1-cDNA. (RNA size markers were obtained from BRL.) (**B**) 5 μg of poly(A) RNA from 15-day-old embryo (D15), newborn (term), 1-week-old and 5-week-old rat brain were analyzed as in (a). (**C**) 10 μg of poly(A) RNA from 21-day-old rat hippocampus, amygdala, cortex and midbrain (other brain parts were not analyzed).

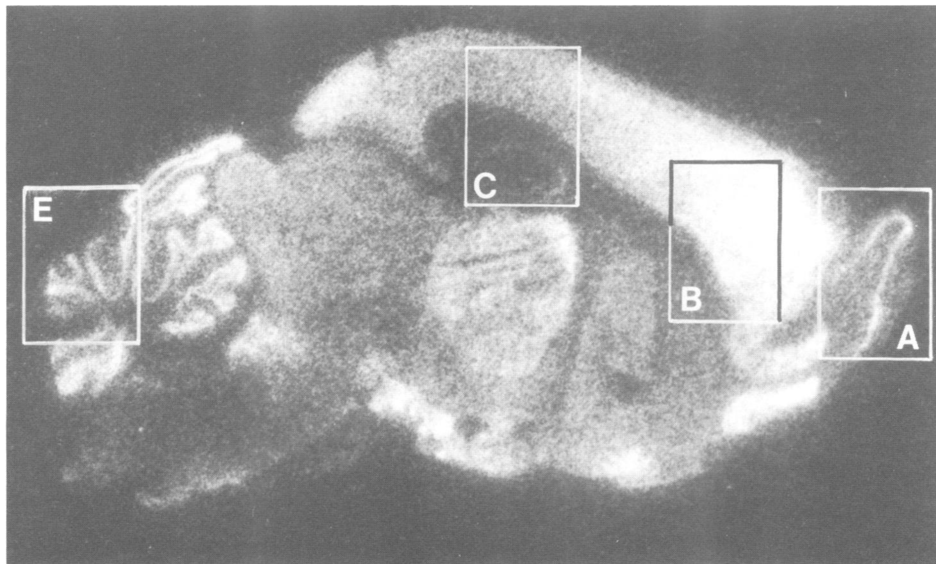

Fig. 5. Localizaton of F1 mRNA-containing cells in rat brain by *in situ* hybridizaton to ^{32}P-labeled riboprobe. After hybridization the sections were exposed to Kodak X-ray film for 48 h at room temperature and then developed. The boxed regions indicating the olfactory bulb (**A**), neocortex (**B**), hippocampus (**C**), and cerebellum (**E**) correspond to the localization studies with ^{35}S-labeled probes as shown in Figure 6.

layer III cells (Figure 6D). The specificity of the hybridization is confirmed by parallel hybridization of serial brain sections to the F1 sense riboprobe; in no case was labeling seen with this probe (Figure 6F).

Discussion

We have isolated cDNA clones encoding the rat protein F1 using as a probe a synthetic oligonucleotide, deduced from N-terminus amino acid sequencing. The cDNA clones obtained encode a 226 amino acid, 23.6 kd hydrophilic protein. The sequence contains no potential transmembrane binding domain or N-linked glycosylation sites. The availability of the complete nucleotide and amino acid sequence of protein F1 revealed its identity with the GAP-43 protein (Karns *et al.*, 1987) and will enable the study of its relation with the pp46 and B-50 proteins. The identity of protein F1 with GAP-43, a nerve growth and regeneration associated protein, strongly indicates that both neuronal outgrowth and synaptic plasticity are mediated through the same molecular mechanism.

Protein F1 is associated with synaptosomal membranes (Chan *et al.*, 1986). However from the hydropathy plot (Figure 2), it is a very hydrophilic molecule, as was previously indicated by elution from phenyl-Sepharose column (Chan *et al.*, 1986). Immunocytochemical evidence showed that protein F1, as GAP-43, may be attached only to the inner plasma membrane (Meiri *et al.*, 1986). The hydrophobic region (residues 161–175), that showed the only peak of hydrophobicity, may be the attaching point to the membrane.

The synthesis of protein F1 occurs in several cell types within

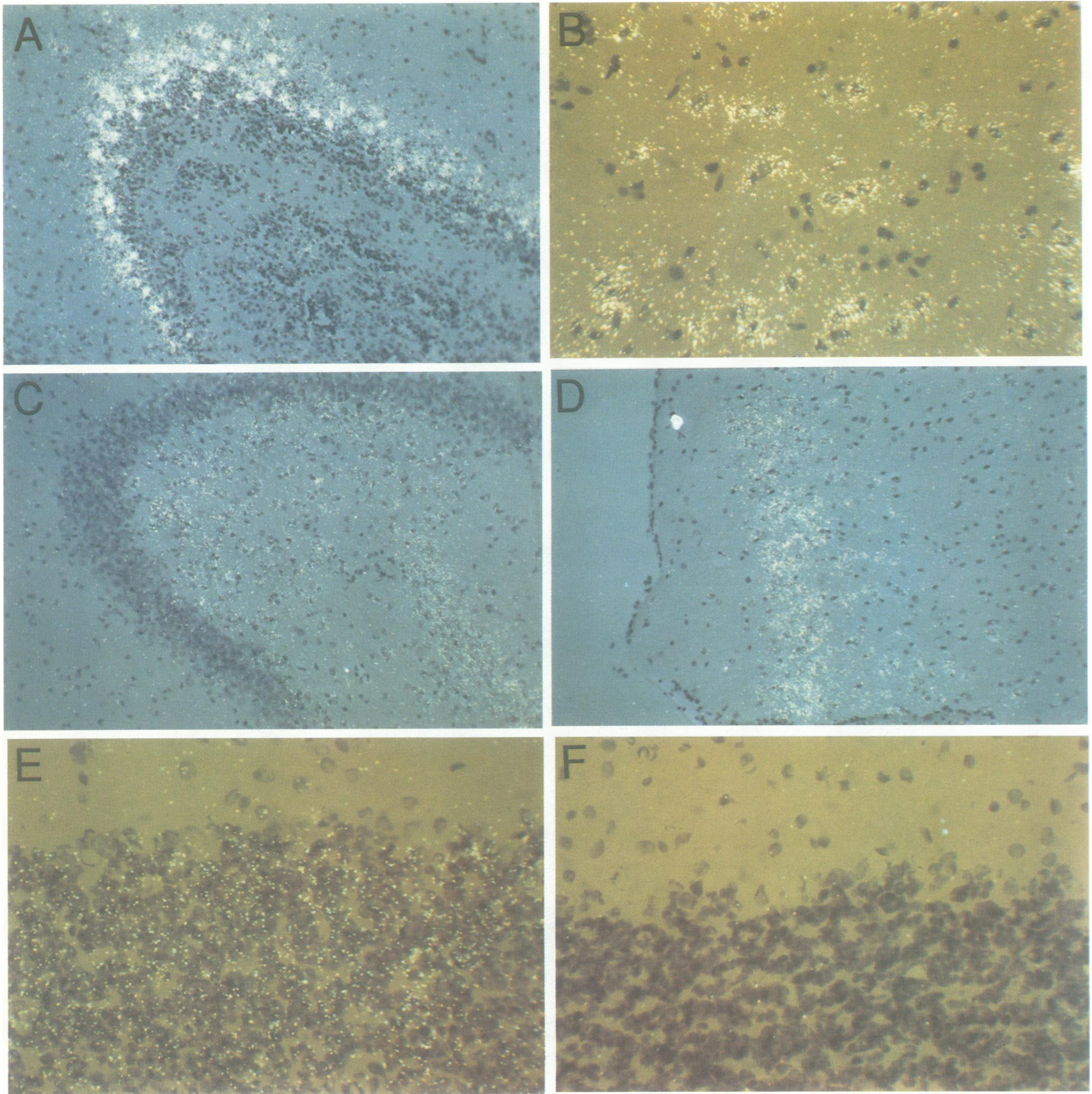

Fig. 6. Localization of F1 mRNA in rat brain by *in situ* hybridization. 10 μm frozen sections were hybridized to ^{35}S-labeled riboprobe as described in Materials and methods and exposed for 1 week. Cells containing F1 mRNA were found in the olfactory bulb (**A**), the neocortex (**B**) and the pyramidal cells of the CA3/CA4 region of the hippocampus, but not in the dentate gyrus (**C**), the entorhinal cortex (**D**) or the granule cells of the cerebellum (**E**). For comparison, a section of the cerebellum immediately adjacent to that shown in E was hybridized with a ^{35}S-labeled F1 sense riboprobe and shows the specificity of the hybridization reaction (**F**).

the hippocampal formation. Protein F1 transcripts were observed in all pyramidal cell subdivisions of the hippocampal gyrus (CA1−CA4) and in the layer 2 and 3 cells of the entorhinal cortex. In contrast, dentate gyrus granule cells did not show a hybridization signal for protein F1 mRNA. Consistent with recent immunocytochemical evidence (Snipes *et al.*, 1987; Chan *et al.*, 1987), the present evidence indicates that, at synapses made by entorhinal cortex afferents (perforant path) onto granule cell dendrites, protein F1 is present in the presynaptic terminal but not in the postsynaptic target.

Recently, localization of three sub-types of PKC in rat brain has been reported (Brandt *et al.*, 1987). Since protein F1 is a PKC substrate (Chan *et al.*, 1986), the co-localization of F1 with PKC is of interest. Comparison of PKC distribution with F1 suggests that PKC-II (β) sub-type co-localizes with protein F1, which PKC-I (γ) does not. In the hippocampus, PKC-II (β) and pro-

tein F1 are both present in pyramidal cells, and both are absent in granule cells. Taken together with co-expression of PKC-II and protein F1 in cerebellum, we suggest that protein F1 is phosphorylated by PKC-II. PKC-II (β) (Brandt *et al.*, 1987) and protein F1 (Gispen *et al.*, 1986) are probably localized presynaptically, consistent with the proposed role of protein F1 in presynaptic terminal plasticity of long-term potentiation (Routtenberg, 1986).

Since protein F1 is made in hippocampal pyramidal cells, but is not observed immunocytochemically in their dendrites (Chan *et al.*, 1987), protein F1 has a process preference for export via axons rather than dendrites. This is consistent with its role, as GAP-43 and pp46, in developmental and regenerative axonal growth.

If the selective localization of protein F1 signals the capacity for plasticity as was proposed (Nelson *et al.*, 1987), then the synaptic terminal targets of those cells which make protein F1 are likely zones of synaptic plasticity. The present results indicate that some of the synapses that are likely to be plastic in the adult are: (i) in hippocampus: perforant path; Schaffer collateral; hippocampal–septal; hippocampal–subicular; subicular–entorhinal; but not mossy fiber synapses; (ii) in cerebellum: parallel fiber, but not Purkinje cell output synapses.

In the hypothalamus, protein F1 message is present, but no PKC sub-type has been described. This suggests, since endogenous phosphorylation of protein F1 occurs in the hypothalamus (Gonzalez-Mariscal and Routtenberg, unpublished), that a hypothalamic PKC localized to this brain region may phosphorylate protein F1. Recent localization of the gonadotropin releasing hormone (GnRH) receptor in the central nervous system indicated its existence in regions CA1–CA4 of the hippocampus, in the entorhinal cortex, and in the basal hypothalamus (Haour *et al.*, 1987), all of which contain F1-producing cells. Since GnRH might mediate its effects through PKC, it is tempting to speculate that GnRH might mediate physiological responses through activation of PKC and subsequent F1 protein phosphorylation.

In the olfactory bulb and tubercle, and related olfactory cortex, an intense hybridization signal is observed. In this region there is considerable turnover of synaptic connections (Grafe, 1983) requiring axonal growth and synaptic plasticity. Additionally the capacity for olfactory-dominated learning in the rat is well documented (Brunjes and Frazier, 1986). The presence of protein F1 in this region may thus serve a synaptic remodeling role and a role in the plasticity of learning.

Long-term potentiation has been observed in several structures apart from the hippocampus (Racine *et al.*, 1983). The duration and time course of this enhancement varies. It will be of interest to determine whether a quantitative relationship exists between the durability of enhanced synaptic connection and extent of protein F1 production.

With the availability of cDNA clones of both protein F1 and of PKC (Parker *et al.*, 1986) and the capacity to translate these proteins selectively, the synaptic/physiological functions of protein F1 and PKC and their interrelationships can be studied.

Materials and methods

Materials

Restriction endonucleases and DNA polymerase I Klenow fragment were purchased from Boehringer Mannheim Biochemicals; T4 polynucleotide kinase was purchased from NEN, SP6 RNA polymerase, SP6-derived vectors and rabbit reticulocyte extract from Promega Biotech; oligo-dT cellulose and protein A-Sepharose from Pharmacia, $\gamma[^{32}P]$ATP, $\alpha[^{32}P]$dATP, $\alpha[^{32}P]$dCTP, $\alpha[^{35}S]$CTP and $[^{35}S]$methionine from Amersham; nitrocellulose was from Schleicher and Schuell.

Purification of protein F1

Protein F1 was purified from adult rat (Sprague–Dawley, male) cortex according to published procedures (Chan *et al.*, 1986), except that the final phenyl-Sepharose column was eluted with 2 M KCl instead of 1.5 M. The resulting fraction (eluted at void volume and the first two fractions of 2 M KCl eluate) approached homogeneity in a silver-stained sodium dodecyl sulfate (SDS) gel. This fraction was dialyzed in 5 mM Tris (pH 7.5) and lyophilized. A small portion (5%) of the purified fraction was phosphorylated in the presence of $\gamma[^{32}P]$ATP and purified PKC for 1 h at 30°C as described (Chan *et al.*, 1986). The ^{32}P-labeled phospho-F1 was mixed with the purified fraction and electrophoresed on a 10% polyacrylamide gel. After electrophoresis and autoradiography, the protein F1 band was cut from the gel and the protein eluted by incubation with 500 μl H_2O overnight at 37°C.

Amino acid sequence analysis

Automated Edman degradation was performed with a 470A Applied Biosystems gas phase sequencer equipped with an on-line 120A phenylthiohydantoin amino acid analyzer.

Screening of the λgt10 cDNA library

Adult Sprague–Dawley rat brain RNA was used to make a λgt10-based cDNA library. *Escherichia coli* strain c600 HFL were infected with the recombinant phage and replica plated on nitrocellulose filters ($3-5 \times 10^4$ plaques per filter). Filters were baked at 80°C for 2 h, prehybridized for 3–4 h and then transferred to hybridization solution containing 40% formamide, 5× SSC, 50 mM sodium phosphate (pH 6.8), 0.1% SDS, 0.1% sodium pyrophosphate, 10% dextran sulfate, 5× Denhardt's, 50 μg/ml salmon sperm DNA, at 42°C. The synthetic DNA probe (ATG-CGG-CGG-ACC-AAG-CAG-GTG-GAG-AAG-AAT-GAT-GAG-GAC-CAG-AAG-ATT-GAG-CAG-GAT-GGC-GTG-AAG-CCC-CAG-GAC-AAG) was labeled with $\gamma[^{32}P]$ using T4 polynucleotide kinase and added to the hybridization solution. Filters were hybridized for 12–16 h before washing in 0.2× SSC, 0.1% SDS at 42°C.

DNA sequencing

DNA sequencing was done according to the standard dideoxy chain termination method following subcloning into M13 derivatives (Smith, 1980). At least two independent cDNA clones were sequenced for each region of the cDNA. cDNA were cloned into the M13 mp18 vector and sequenced for both orientations. Specific primers were used to extend the sequences to cover the complete cDNA.

Northern blot analysis

Polyadenylated RNA from different tissues were electrophoresed into formaldehyde 1.2% agarose gels and transferred onto nitrocellulose. The nitrocellulose filters were hybridized with ^{32}P-labeled protein F1 cDNA in 50% formamide, 5× SSC, 0.1% SDS, 0.1% sodium pyrophosphate, 50 mM sodium phosphate (pH 6.8), 5× Denhardt's solution, 10% dextran sulfate at 42°C for 12–15 h. Filters were washed twice for 30 min in 0.1× SSC, 0.1% SDS at 60°C.

In vitro transcription

Protein F1 cDNA was cloned into an SP6-derived vector and transcribed according to manufacturer's (Promega Biotech) instructions using SP6 RNA polymerase.

Production of antiserum and purification of polyclonal antibody

Calf protein F1 was purified as described (Chan *et al.*, 1986). Two hundred micrograms of purified protein, mixed with RIBI adjuvant (RIBI Immunochemicals), was injected s.c. into rabbits at 3-week intervals. Three weeks after the third injection, serum was extracted after bleeding the rabbits. Each batch of antiserum was tested by immunoprecipitation for titer and specificity against calf and rat F1 before use.

In vitro translation and immunoprecipitation

In vitro transcribed mRNA was translated in rabbit reticulocyte lysate in the presence of $[^{35}S]$methionine according to manufacturer's instructions. The resulting translation products were diluted 1× with wash buffer [0.15 mM NaCl, 0.04 mM Tris (pH 8.6), 2 mM EDTA, 1% NP40, 1 mg/ml bovine serum albumin); 10 μl of anti-F1 polyclonal antibodies were added, and the samples were incubated at 4°C. Twenty-five microliters of protein A-agarose beads were added to the samples after 16 h and the samples were incubated at 22°C for 1–2 h with gentle agitation. The protein A-agarose beads were then washed five times with wash buffer. The washed beads were incubated with 100 μl of 2-fold-concentrated electrophoresis sample buffer [0.125 M Tris–HCl (pH 6.8), 5% glycerol, 5% SDS, 10% β-mercaptoethanol, 0.1 mg/ml bromophenol blue] at 100°C for 5 min. After removal of the beads by centrifugation, the samples were analyzed by electrophoresis in denaturing 12% polyacrylamide gels. Prestained molecular weight markers were purchased from Bethesda Research Laboratories (BRL). The gels were fixed with 30% methanol, 10% trichloroacetic acid, dried and exposed to Kodak XAR-2 film at −70°C.

In situ hybridization

In situ hybridization was performed as described (Wilcox *et al.*, 1986a,b), except as follows. Animals were killed and the brains fixed by immersion for 2 h in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The tissue was immersed in 15% sucrose−PBS overnight and frozen in Tissue Tek embedding medium compound. Cryostat sections (10 μm) were thaw-mounted on polylysine-coated slides and stored at −70°C until use. Prior to hybridization, the sections were thawed, fixed for 10 min in 4% paraformaldehyde, washed in 0.5× SSC and treated with 1 μg/ml proteinase K for 10 min. The sections were then hybridized to a ^{35}S-labeled SP6 riboprobe generated from the rat F1 cDNA fragment using [^{35}S]UTP (Amersham, 1000 Ci/mmol) as described (Melton *et al.*, 1984). The tissue was hybridized overnight at 55°C in hybridization buffer containing 50% formamide, 0.3 M NaCl, 20 mM Tris (pH 8.0), 5 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, and 10 mM dithiothreitol. After hybridization, the slides were washed twice for 10 min in 2× SSC, treated with ribonuclease A (20 μg/ml) for 30 min, and subsequently washed with 0.1× SSC at 55°C for 3 h. All washes after hybridization contained 10 mM β-mercaptoethanol and 1 mM EDTA. After two final 10 min washes in 0.5× SSC without β-mercaptoethanol, the sections were dehydrated and dipped in Kodak NTB2 nuclear emulsion and exposed for 1 week at 4°C. After development, the sections were counterstained with hematoxylin.

Acknowledgements

This work was supported, in part, by grants MH25281 from NIMH and AFOSR87-0042 from the Air Force Office of Scientific Research to A.Routtenberg. The authors would like to thank A.Mason for the cDNA library and for technical assistance, M.Matteucci and M.Vasser for synthetic oligonucleotides, and T.Bringman for assistance with the immunoprecipitation.

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Received on August 4, 1987; revised on August 28, 1987