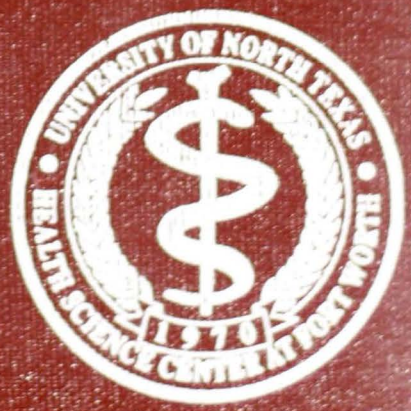
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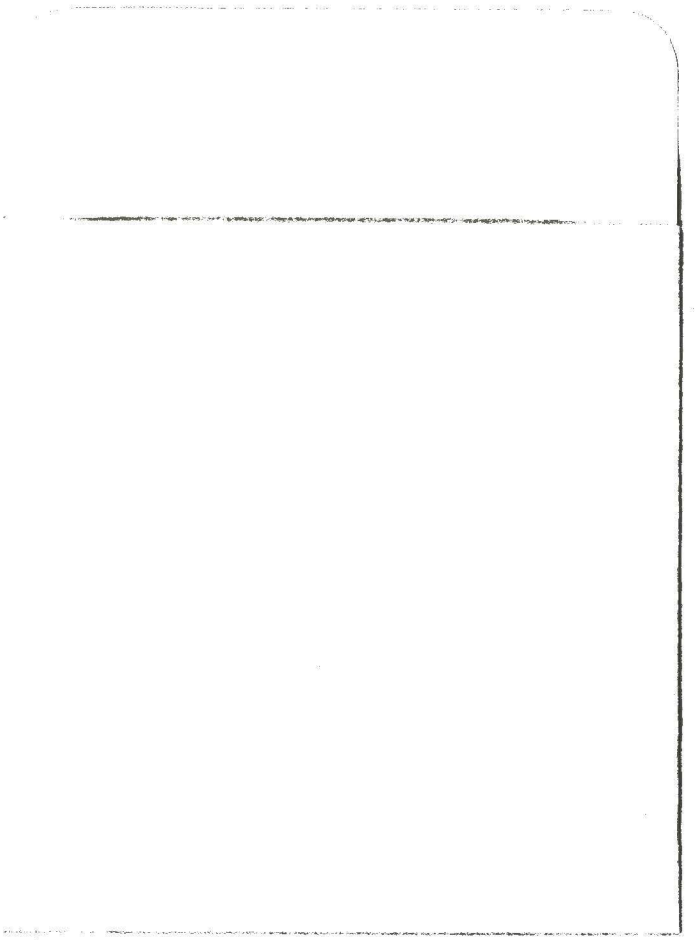

El-Badawy, Hassan M. E. Azzazy, Studies of Protein F1 (GAP-43) Expression and Function in Spinal Neuronal Cultures, Doctor of Philosophy (Biochemistry and Molecular Biology), August 1994, 167 pp., 32 illustrations, References, 194 titles.

Protein F1 (GAP-43, B-50, neuromodulin) is a membrane-bound phosphoprotein that has been studied mainly in neurons and is implicated in synaptic plasticity, axonal growth and regeneration, and neurotransmitter release. In this study, a 21 amino acid polypeptide that corresponds to the C-terminus sequence of protein F1 and contains a potential PKC phosphorylation sequence (SXR) was synthesized. The synthetic peptide was phosphorylated by rat PKC in a concentration-dependent manner suggesting that this site in the intact protein may be phosphorylated by PKC *in vivo*. Polyclonal antibodies against the peptide were produced in a rabbit and used to: (i) recognize native non-phosphorylated protein F1 purified from rat brain, (ii) immunoprecipitate phosphorylated protein F1, and (iii) stain the cell bodies and neurites of cultured neurons. Electron microscopic studies revealed intracellular protein F1 immunoreactivity but no specific subcellular association of the gold label could be demonstrated. The antibodies were also used to compare protein F1 levels during the development of spinal neurons in culture and *in vivo*. The highest levels of protein F1 were detected, by ELISA, at 2 days in culture. These results are in accordance with previous reports that correlate high expression of protein F1 to neurite outgrowth. *In vivo*, however, protein F1 reached maximal level at one day after parturition. Two approaches were utilized to investigate the potential physiological functions of protein F1 in spinal neural networks. First, interaction of positively charged, rhodamine-labeled liposomes with spinal neurons was characterized by fluorescence microscopy and electrophysiological recording. Uniform, non-toxic, and preferential interaction of

liposomes with spinal neurons over glia was established. These liposomes were used to deliver anti-protein F1 antibodies into spinal neurons but did not affect neurite formation by these cells. Second, antisense oligodeoxynucleotides internalized into spinal neurons in order to interfere with protein F1 expression had no effect on the development of these cells in culture. Data from this study suggest that Ser-210 at the C-terminus of protein F1 may be a substrate for PKC phosphorylation *in vivo*. Antibodies raised against F1 peptide revealed protein F1 immunoreactivity that outlined cell bodies and neurites of cultured spinal neurons. Positively charged liposomes were characterized as a potential delivery system for macromolecules into spinal neurons. Protein F1 levels were shown to be developmentally regulated in mouse spinal neurons in culture and *in vivo*. Finally, the use of antisense oligodeoxynucleotides against protein F1 mRNA revealed that protein F1 may not be essential for neurite outgrowth of mouse spinal neurons in culture.

STUDIES OF PROTEIN F1 (GAP-43) EXPRESSION AND FUNCTION IN SPINAL NEURONAL CULTURES

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STUDIES OF PROTEIN F1 (GAP-43) EXPRESSION AND FUNCTION IN SPINAL NEURONAL CULTURES

DISSERTATION

Presented to the Graduate Council of the
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For the Degree of

DOCTOR OF PHILOSOPHY

By

Hassan M. E. Azzazy El-Badawy
Fort Worth, Texas

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AD	Alzheimer disease
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
cAMP	3', 5'-Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary cells
CKII	Casein kinase II
CNS	Central Nervous System
COS	African green monkey kidney cell line
DAG	Diacylglycerol
DEAE	Diethylaminoethyl
DNase I	Deoxyribonuclease I
DOPE	Dioleoylphosphatidylethanolamine
DRG	Dorsal root ganglion
DTT	Dithiothreitol
EDTA	Ethylene diamine-tetra-acetic acid
EGTA	Ethylene glycol-bis-(β-amino-ethyl ether) N, N'-tetra acetic acid
ELISA	Enzyme-linked immunoadsorbent assay
FCS	Fetal calf serum
H_2O_2	Hydrogen peroxide
HEPES	N-(2-hydroxyethyl) piperazine-N'-2-ethane sulfonic acid
HPLC	High performance liquid chromatography

HRP	Horse radish peroxidase
IgG	Immunoglobulin-G
IL-1β	Interleukin 1-β
IP3	Inositol 1,4,5-trisphosphate
KCl	Potassium chloride
kDa	Kilodalton
KPi	Potassium phosphate buffer
LM	Light microscope
LMB	Loot's modified bodian
LPC	L-α-Lysophosphatidylcholine
LTP	Long-term potentiation
MEM	Minimal essential medium
Mr	Relative molecular weight
NaCl	Sodium chloride
NFT	Neurofibrillary tangles
NGF	Nerve growth factor
NOS	Nitric oxide synthetase
NP-40	Nonidet-40
OAG	L-α-1-oleoyl-2-acetoyl-sn-3-glycerol
ODN	Oligodeoxynucleotides
PAGE	Polyacrylamide gel electrophoresis
PB	Phosphate buffer
PBS	Phosphate-buffered saline
PC12	Pheochromocytoma cells
PDL	Poly-D-lysine
pI	Isoelectric point

CHAPTER I

INTRODUCTION

Protein F1 was first discovered by Zwiers et al. (1976) as a synaptic protein whose phosphorylation was inhibited by an ACTH-derived peptide. Inhibition of protein F1 phosphorylation abolished learning task in experimental animals. The same protein was later identified according to the observed physiological effect as GAP-43 growth-associated protein (Skene and Willard, 1981), B-50 phosphoprotein in synaptosomes (Zwiers et al., 1980), P-57, the calmodulin binding protein (Andreasen et al., 1983), and F1, or plasticity protein (Nelson and Routtenberg, 1985). Other discoveries of the same protein have been reported by Rodnight (1982), Benowitz and Lewis (1983), and Katz et al. (1985) who named it γ 5, GAP-48, and neuromodulin, respectively. Using biochemical and immunological approaches all these proteins were shown to be the same (for a review see: Benowitz and Routtenberg, 1987). Moreover, sequencing studies have shown high sequence homology among these forms as described below.

The name protein F1 was chosen as a title for this study because of the following: (i) protein F1 was first reported as a synaptic protein that inhibited learning in animals upon inhibition of its phosphorylation by ACTH-derived peptide, (ii) it has been correlated with long-term potentiation (LTP), and (iii) because the main scope and system used in this study is neural networks whose dynamics underlie behavior.

Tissue Specificity of Protein F1

Protein F1 has been localized exclusively to the cytoplasmic surface of the plasma membrane of neurons (McGuire et al., 1988; Gorgels et al., 1989). However, more recent reports using immunohistochemistry or *in situ* hybridization of protein F1 mRNA, have also found protein F1 in astroglia, oligodendroglia, nonmyelin-forming Schwann cells of the peripheral nervous system, and satellite cells (Vitkovic et al., 1988; Vitkovic and Mersel, 1989; Da Cunha et al., 1990; Deloulme et al., 1990; Woolf et al., 1990; Da Cunha et al., 1991; Curtis et al., 1992; Plantinga et al., 1993).

Protein F1 and its mRNA were also shown to be transiently expressed in 2 populations of non-neuronal cells in developing chick limbs (for a review see: Stocker et al., 1992). In addition, the same authors reported: (i) high levels of protein F1 mRNA and protein in the chick interdigital mesenchyme that undergoes apoptosis or programmed cell death, and (ii) protein F1 immunoreactivity in cells that are also immunoreactive to meromycin, a muscle-specific marker. Protein F1 was, therefore, suggested to play a role in muscle development and possibly programmed cell death (Stocker et al., 1992).

Localization of Protein F1 and Its Role in Development of CNS

Nervous system development is characterized by a specific series of morphological changes together with a differential expression of genes. During the first phase of neuronal development, immature neurons (generated by mitotic division of stem cells) undergo a complex process of morphological and functional maturation. Neurons then migrate to their specific positions where neurite formation is initiated. Finally, synapses are formed and neurons that fail to form functional connections die (Cowan, 1979).

Protein F1, a membrane-bound phosphoprotein, has been proposed as a molecular marker to define the commitment of neuronal precursors to neurite formation (Biffo et al., 1990). Protein F1 is highly expressed in the developing nervous system (Jacobson et al.,

1986; Kalil and Skene, 1986; Karns et al., 1987; Moya et al., 1988; Fitzgerald et al., 1991). However, except for certain brain regions, protein F1 levels are low in the adult central nervous system (CNS, Neve et al., 1987; de la Monte et al., 1989). Electron microscopic immunolocalization studies have shown that protein F1 is located at the cytoplasmic surface of the neuronal plasma membranes (Van Hooff et al., 1988; Campagne et al., 1990; Gorgels et al., 1989). This protein has been shown to exist mainly in the presynaptic terminals of adult rat brains (Gispen et al., 1985). Protein F1 is enriched in neuronal growth cones where it is thought to affect growth cone motility and elongation of neurites (Benowitz and Routtenberg, 1987; Skene, 1989) through interaction with Go protein (a GTP-binding protein enriched in the growth cones, Strittmatter et al., 1990), regulation of calcium levels and signaling (Skene, 1990), and phosphorylation by protein kinase C (PKC, Akers and Routtenberg, 1985; Aloyo et al., 1983).

Conservation of Protein F1 Structure

High homology of protein F1 sequence between human and several animal species (including mouse, rat, cow) has been revealed by several cloning studies (Cimler et al., 1987; Karns et al., 1987; Rosenthal et al., 1987; Kosik et al., 1988; Ng et al., 1988; LaBate and Skene, 1989). Despite the high degree of homology observed in mammalian protein F1 molecules (Fig. 1), the fish and chick proteins are different in sequence (Apel and Storm, 1992). The protein F1 N-terminus domain (first 57 amino acids) that contains the growth cone targeting domain, membrane attachment sites, G-protein regulating domain, major PKC phosphorylation site, and the calmodulin binding domain, is particularly conserved through vertebrate evolution (for a review see: Benowitz and Perrone-Bizzozero, 1991) which further documents the significance of this region in protein F1 functions. On the other hand, the C-terminus of protein F1, with a highly negative extended rod, is much less conserved. It contains a conserved portion that has

been proposed to be a cytoskeleton binding domain and includes casein kinase II (CKII) *in vitro* phosphorylation sites (Masure et al., 1986; LaBate and Skene, 1989).

Biochemical Features of Protein F1

Protein F1 (pI 4.3-4.6) is an very acidic protein (Zwiers et al., 1985; Masure et al., 1986). Although it is associated with plasma membranes, this protein is very hydrophilic as evident from its elution in the void volume of a hydrophobic phenyl Sepharose column (Chan et al., 1986). Earlier estimations of protein F1 apparent molecular mass ranged between 43-67 kDa on sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) depending on the concentration of the polyacrylamide gel used and the species tested. The actual molecular mass of the protein, estimated from sequence analysis data from different species (Basi et al., 1987; Cimler et al., 1987; Karns et al., 1987; Nielander et al. 1987), ranged between 23.7-25 kDa.

Protein F1 has a distinctive amino acid composition. It has a single aromatic amino acid (Phe-42), that is adjacent to Ser-41, the major PKC phosphorylation site, situated within the calmodulin binding domain. Protein F1 has a high percentage (40%) of charged residues and about 25% proline and alanine residues (Wakim et al., 1987; Masure et al., 1986). The N-terminus of protein F1 has a net positive charge contributed by many basic residues in this part of the protein. The rest of the protein contains a very high percentage of acidic residues that are responsible for its pI of 4.3 (Apel and Storm, 1992).

Circular dichroism spectroscopic analysis of protein F1 indicated that the protein secondary structure is 1% alpha-helix, 21% beta sheet, and 78% random coil (Masure et al., 1986). Sequence analysis of protein F1 together with its reported hydrodynamic properties demonstrated that protein F1 is an elongated protein with little secondary structure (Masure et al., 1986; LaBate and Skene, 1989).

Protein F1 is not a glycoprotein. This was shown by its inability to bind a number of lectins, such as wheat germ agglutinin, and the absence of hexosamine after acid hydrolysis of the protein and subsequent gas chromatographic analysis (Masure et al., 1986). In addition, protein F1 amino acid sequence does not contain N-glycosylation sites.

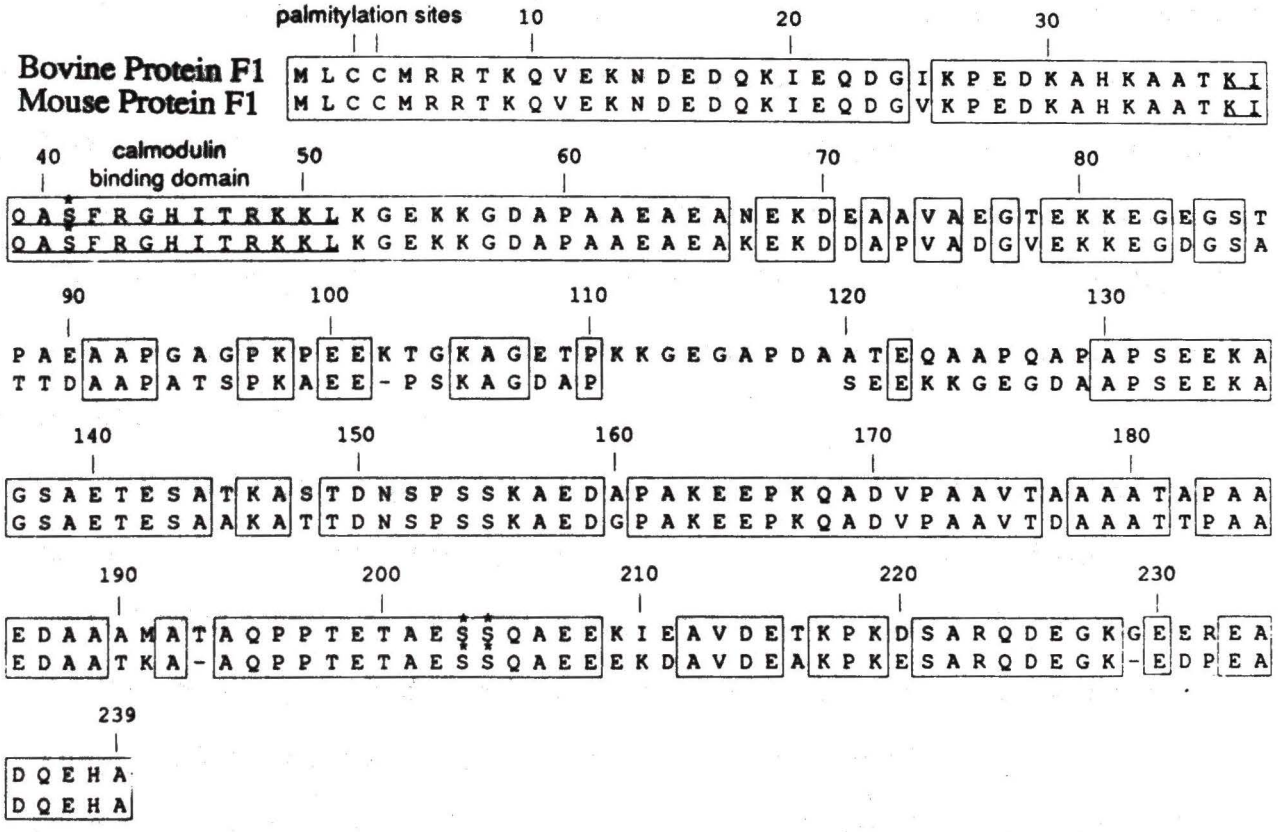


Fig. 1. Sequence and functional domains of bovine and mouse protein F1 (Apel and Storm, 1992). Sites of membrane attachment (Cys-3, Cys-4) are noted, calmodulin binding domain is underlined, and PKC phosphorylation site at Ser-41 and CKII phosphorylation sites at Ser-192 and Ser-193 are marked with asterisks.

Functional Domains of Protein F1

1. Membrane Binding Domain

The protein F1 primary sequence (Fig. 1) does not contain any hydrophobic sequences that are consistent with membrane spanning domains (Apel and Storm, 1992). Membrane attachment of protein F1 is believed to occur via palmitoylation of 2 cysteine residues (Cys-3 and Cys-4) near the N-terminus of the protein (Skene and Virag, 1989). In cultures of PC12 cells or rat cerebral cortex cells, radiolabeled palmitic acid was shown to be incorporated into protein F1 whereas myristic acid was not incorporated (Skene and Virag, 1989). In addition, palmitic acid incorporation was hydroxylamine-sensitive which suggests formation of thioester bonds between palmitic acid and sulfhydryl group of cysteine residues (Apel and Storm, 1992). Furthermore, mutated protein F1 (that lacks Cys 3 and Cys 4) did not attach to the membranes when expressed in COS-7, PC12, or CHO cell lines (Zuber et al., 1989a; Liu et al., 1991).

Several reports suggest that palmitoylation of protein F1 is a dynamic process that can play an important role in the regulation of the protein function. First, in isolated growth cones, labeled palmitic acid was incorporated in protein F1 which indicates that enzymes and other cofactors required for palmitoylation are present in the growth cones (Skene and Virag, 1989). Second, Cimler et al. (1985) detected both cytosolic and membrane pools of protein F1 extracted from bovine brain. Third, palmitoylation of protein F1 purified from rat brains, reversibly abolishes its activation of Go protein (Sudo et al., 1992).

2. Calmodulin Binding Domain

In comparison to other calmodulin regulators that selectively bind calcium-rich calmodulin, protein F1 binds calmodulin with high affinity at low calcium concentration (Andreasen et al., 1983; Cimler et al., 1985). In this sense protein F1, with its high affinity for calcium-poor calmodulin, could affect calmodulin-mediated target activation during the

early stage of calcium influx into neurons, and unlike other regulators, it would not compete for the calcium-rich form of calmodulin under conditions of increased intracellular calcium concentrations (McIlroy et al., 1991). Binding of protein F1 to calmodulin was shown to be inhibited: i) under high calcium concentration conditions (Alexander et al., 1987), and ii) by PKC-activated phosphorylation of protein F1 at Ser-41 (located within calmodulin binding domain of protein F1) (Chapman et al., 1991).

Recently, Slemmon and Martzen (1994) demonstrated that protein F1 can modulate the activity of nitric oxide synthase (NOS), an enzyme that requires calcium and calmodulin for its activity (Bredt and Snyder, 1990). Protein F1 decreased NOS activity in a concentration-dependent manner and under physiological calcium conditions. Phosphorylation of protein F1 by PKC abolished its ability to inhibit NOS, and calcineurin (phosphatase 2b) regained its inhibitory action (Slemmon and Martzen, 1994).

3. Phosphorylation of Protein F1

Protein F1 is a major substrate for calcium-activated and phospholipid-dependent protein kinase C (Aloyo et al., 1983, Nelson and Routtenberg, 1985; Alexander et al., 1987). *In vitro*, protein F1 has been shown to be phosphorylated at a single site: Ser-41 (Alexander et al., 1988; Coggins and Zwiers, 1989; Apel et al., 1990; Chapman et al., 1991). Because the protein F1 calmodulin binding domain (amino acids 39-56) overlaps Ser-41, phosphorylation at this site reduces the calmodulin binding affinity of protein F1 (Alexander et al., 1987; Chapman et al., 1991). Spencer et al. (1992) demonstrated that protein F1 in cultured superior cervical ganglion cells is phosphorylated at 3 sites (Ser-41, Ser-96, and Thr-172). Of these sites, only Ser-41 phosphorylation is induced by phorbol esters which supports the previous reports that Ser-41 is the major *in vivo* protein kinase C substrate (Spencer et al., 1992).

The physiological significance of protein F1 phosphorylation by protein kinase C has been demonstrated on several occasions. First, Pisano et al. (1988) reported that protein F1 purified from adult rat brain cortex is phosphorylated *in vitro* by CKII on serine residues. Phosphoamino acid analysis of protein F1 phosphorylated by CKII revealed major CKII phosphorylation sites at Ser-192 and Ser-193 and minor phosphorylation sites at Thr-88, Thr-89, or Thr-95 (Apel et al., 1991). Calmodulin, which binds to the N-terminus of protein F1, inhibited CKII phosphorylation of protein F1 and consequently seems to regulate protein F1 phosphorylation by CKII. This inhibition may indicate that the calmodulin binding domain and the CKII phosphorylation sites at the C-terminus of protein F1 are located side by side as induced by the minimal secondary and tertiary structures displayed by protein F1 (Apel et al., 1991). No physiological significance of CKII phosphorylation of protein F1 is known, as phosphoamino acid analysis of protein F1 phosphorylated in living neonatal rat brains and living cultured neurons, indicates no phosphorylation on any of the *in vitro* CKII phosphorylation sites (Spencer et al., 1992). That finding was important because CKII is activated in response to various growth-inducing factors such as epidermal growth factor and insulin (Sommercorn et al., 1987; Ackerman and Osheroff, 1989). In addition, CKII is implicated in neurite growth (Diaz-Nido et al., 1988).

One other kinase that phosphorylates protein F1 at its calmodulin binding domain is phosphorylase kinase [E.C. 2.7.1.38 (Paudel et al., 1993)], a calcium-dependent enzyme (Brostrom et al., 1971). This enzyme, which is phosphorylated by cAMP-dependent kinase (Walsh et al., 1968), regulates the activation cascade of glycogenolysis and phosphorylates glycogen phosphorylase b to produce its active form, phosphorylase a (Pickett-Gies and Walsh, 1986). Protein F1 is a better substrate of PKC than of phosphorylase kinase. Approximately 30% of phosphorylase kinase activity is associated with synaptic membranes where protein F1 is localized (Paudel et al., 1993). Protein F1 is

not phosphorylated by either calmodulin-dependent protein kinases I and II (Baudier et al., 1989) or by cAMP-dependent protein kinase (Chan et al., 1986).

4. Cytoskeletal Binding Domain

Protein F1 has been reported to interact with the membrane cytoskeletal fraction (Meiri and Gordon-Weeks, 1990; Moss et al., 1990). Also, protein F1 has been thought to contain a cytoskeleton binding domain near its C-terminus. This domain contains CKII phosphorylation sites that may regulate protein F1 interaction with cytoskeleton components such as actin (LaBate and Skene, 1989; Apel et al., 1991). Both observations indicate that protein F1 may play a role in regulating cytoskeletal dynamics (Apel and Storm, 1992).

Multiple Roles of Protein F1 in Signal Transduction

1. Calmodulin Binding and Calcium Signaling

Protein F1 has been suggested to bind and concentrate calmodulin at specific regions in neurons (Andreasen et al., 1983; Alexander et al., 1987). Because protein F1 is known to be a fast-axonally transported protein, it may also transport associated calmodulin from the soma to the tips of growing axons facilitating its rapid distribution (Spencer and Willard, 1992). Increases in calcium and phosphorylation of protein F1 by PKC would result in local release of calmodulin (Fig. 2) and subsequent activation of calmodulin-regulated proteins that are important in neurotransmitter release, synaptic plasticity, and axonal growth and regeneration. Concentration of calmodulin would remain high until dephosphorylation of protein F1 (Alexander et al., 1987; Liu and Storm, 1990; Skene, 1990). Protein F1 has a relatively low affinity for calmodulin (Kd=3 μM). Protein F1 concentrations in growth cones and specific synaptic terminals however were estimated to

be 100 μM (Cimler et al., 1985; Alexander et al., 1988; Skene, 1990). Furthermore, protein F1 is highly enriched in growth cones, probably in excess of other calmodulin-

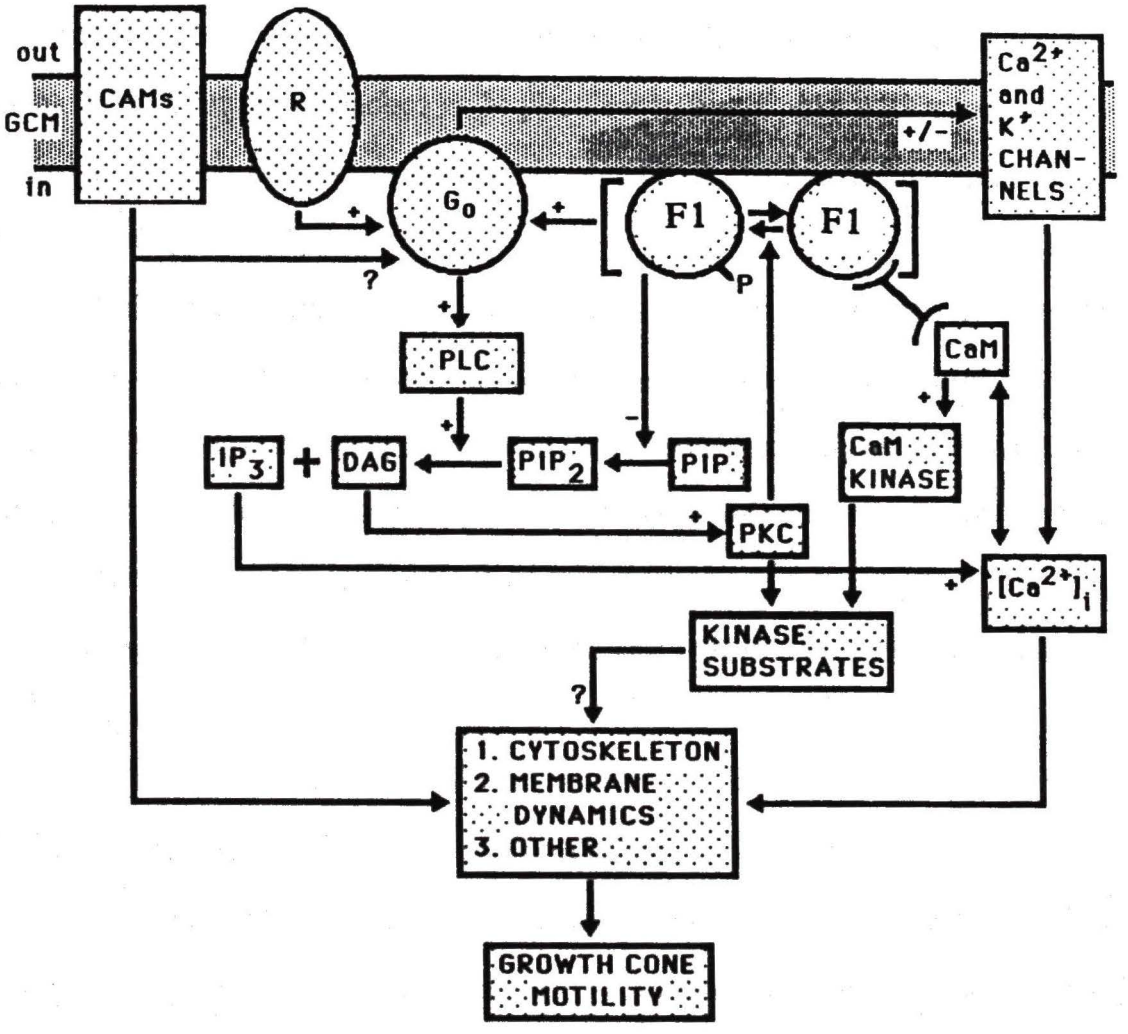


Fig. 2. Possible roles of protein F1 in signal transduction (Strittmatter et al., 1991). Dephospho-protein F1 binds to free calmodulin and releases calmodulin upon phosphorylation of by PKC thus modulating the concentrations of intracellular free calcium (Alexander et al., 1988). Phospho-protein F1 also inhibits the formation of phospholipase C substrate, PIP_2 (Van Hooff et al., 1986). In addition, protein F1 regulates Go protein, a major GTP-binding protein in the growth cone membranes (Strittmatter et al., 1990). Therefore, protein F1 through its interaction with several signal transducing pathways may modulate the motility and functions of growth cones (Strittmatter et al., 1991).

binding proteins (Meiri et al, 1986; Skene et al., 1986). These observations suggest that protein F1 may be able to sequester a large amount of calmodulin at the growth cones.

Several dynamic rearrangements of the cytoskeleton underlie axonal growth and regeneration (Bray and Hollenbeck, 1988; Smith, 1988). A number of such processes are calmodulin-dependent. Attached to the plasma membrane, protein F1 is located between the cytoskeleton and plasma membranes in a suitable position to regulate rearrangement processes (Apel and Storm, 1992).

Calmodulin release from protein F1, upon a depolarization-induced increase in intracellular free calcium, can activate calcium calmodulin-dependent protein kinase II. This kinase phosphorylates synapsin I, a synaptic vesicle protein, in such a process that is suggested to enhance synaptic vesicle fusion to plasma membranes and neurotransmitter release (Benfenati et al., 1989). Protein F1 may also regulate the activity of calmodulin-sensitive adenylate cyclase that has been correlated with synaptic plasticity (Hawkins et al., 1983; Levin et al., 1992).

2. Effect on Phosphoinositide Metabolism

This second messenger system starts with receptor-mediated hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP2), initially produced by phosphorylation of phosphatidyl inositol 4-phosphate (PIP). This hydrolysis leads to the release of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (for a review see: Berridge, 1987). DAG activates protein kinase C. On the other hand, IP3 causes release of calcium from intracellular stores. PKC-phosphorylated protein F1 has been reported to inhibit the activity of PIP kinase (Fig. 2) (Van Dongen et al., 1985). In addition, antibodies against protein F1 inhibited its phosphorylation and stimulated PIP2 production in synaptosomes (Oestreicher et al., 1983).

According to the previous data, protein F1 was proposed as a feedback inhibitor of both PKC activation and calcium mobilization (Jolles et al., 1980; Oestreicher et al., 1983; Van Dongen et al., 1985; Van Hooff et al., 1988). PIP was reported to be hydrolyzed under specific conditions to produce DAG and inositol biphosphate that can not mobilize calcium (Berridge, 1987). Under such conditions, phosphorylated protein F1 would act as a modulator to uncouple PKC activation from calcium mobilization (Skene 1989). Whether or not protein F1-induced inhibition of PIP2 production and consequent termination of IP3-stimulated calcium mobilization occur also *in vivo*, is unknown.

3. Interaction between Protein F1 and Go Protein

Several pieces of evidence document the role of protein F1 as a modulator of Go-protein function in the growth cones (for review see: Strittmatter, 1992). First, both protein F1 and Go protein are highly enriched in the membrane fractions of the growth cones (Meiri et al., 1986; Skene et al., 1986; Strittmatter et al., 1990). Second, both protein F1 and a pertussis toxin-sensitive G protein are involved in similar functions including growth cone motility, (Mattson et al., 1988; Rodrigues et al., 1990; Vartanian et al., 1991; Suidan et al., 1992; Strittmatter et al., 1992), neurotransmitter release (Haydon et al., 1991), and long-term potentiation, a model of memory (Goh and Pennefather, 1989). Third, GTP-binding to purified Go protein is stimulated by protein F1 (Strittmatter et al., 1990). Fourth, the G protein-activating domains of receptors and of protein F1 have similar sequences (Strittmatter et al., 1990; Okamato et al., 1990; Sudo et al., 1992; Okamato et al., 1991). Finally, palmitoylation of protein F1 was shown to modulate the interaction of protein F1 and Go (Fig. 3) (Strittmatter et al., 1992). The N-terminal 25 amino acid residues of protein F1 contain the G protein-interacting domain (Strittmatter et al., 1990). Microinjection of protein F1 (0.2 μM) into *Xenopus laevis* oocytes increased the oocyte response to a G-protein-coupled receptor agonist (serotonin, 5-hydroxytryptamine) by up

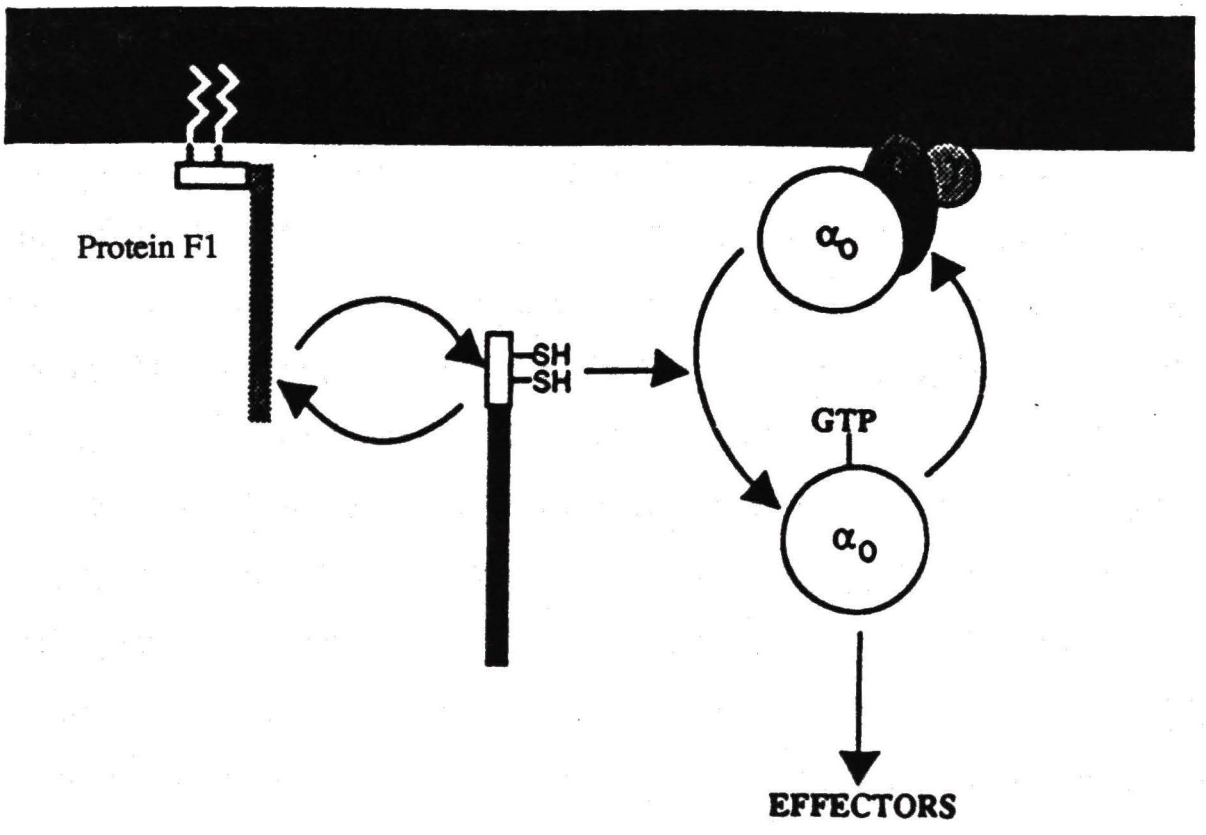


Fig. 3. Interaction of protein F1 with Go protein (Sudo et al., 1992). Palmitoylation of protein F1 is a dynamic process that occurs in the growth cones. Cytoplasmic protein F1 is believed to enhance GTP binding to the α-subunit of Go protein and consequently affect second messenger systems (Strittmatter et al., 1991).

to 100-fold (Strittmatter et al., 1993). Therefore, protein F1 may act as an intracellular effector that enhances the transduction of G-protein-coupled receptors (Fig. 3).

Proposed Physiological Functions of Protein F1

1. Axonal Growth and Regeneration

The complex series of events that regulate neurite outgrowth, axonal elongation, and ultimately the formation of synapses are collectively termed synaptogenesis.

Mechanisms that regulate these events, which result in the formation of a highly organized pattern of neuronal circuitry, are poorly understood.

Protein F1 is abundant in the brain during neurite outgrowth and elongation, then decreases after formation of mature synapses (Jacobson et al., 1986; Basi et al., 1987; Karns et al., 1987; Burry et al., 1991). Following establishment of neuronal polarity, protein F1 is conveyed by fast axonal transport from the soma to the growth cones where it is incorporated into the plasma membrane (Goslin and Banker, 1990; Goslin et al., 1990; Burry et al., 1991; Campagne et al., 1992 a, b).

Several studies have been performed to elucidate the role of protein F1 in the cell by changing protein F1 levels and subsequent evaluation of morphological changes. Upon transient expression of protein F1 in NIH-3T3, CHO, COS, L6, or PC12 cells, the growth of filopodial-like structures was increased (Zuber et al., 1989b; Yanker et al., 1990; Widmer and Caroni, 1993). But transfection of COS-7 cells with a mutated protein F1 (Ala-3 Ala-4) construct, a membrane-attachment negative mutant, produced no change in the cell morphology (Widmer and Caroni, 1993). Furthermore, inhibition of protein F1 translation in PC12 cells with an antisense oligonucleotide with a complementary sequence to protein F1 mRNA 5'-end has been reported to interfere with both NGF-induced increase in protein F1 expression and with neurite outgrowth (Jap Tjoen San et al., 1992). In addition, intracellular delivery of anti-protein F1 antibodies into mouse NB2a/d1 cells and human neuroblastoma lines (Sh-SY-5Y and LAN5) reduced neurite outgrowth (Shea et al., 1991). Recently, Aigner and Caroni (1993) reported that they depleted cultured dorsal root ganglion (DRG), obtained from chick lumbar embryonic day-16, of protein F1 using three different antisense oligonucleotides. This depletion affected neurite outgrowth in a manner that was dependent on the substratum. In the same study, inhibition of protein F1 expression prevented growth cone and neurite formation when neurons were seeded on

poly-L-ornithine. By contrast, protein F1-depleted DRG plated on laminin extended neurites that were longer, thinner, and with smaller growth cones than control neurons.

By contrast, overexpression of protein F1 in mouse neuroblastoma cells (Neuro-2a), that differentiate to a neuronal phenotype upon treatment with retinoic acid, did not induce neurite extension (Morton and Buss, 1992). Additional evidence by Baetge and Hammang (1991), indicated that a protein F1-deficient PC12 cell line was able to extend neurites in response to NGF or bFGF. Because these cells were less adherent than PC12 cells that express protein F1, these authors speculated that protein F1 might have a role in the process of growth-cone-substrate adhesion processes with the consequent hypothesis that previously reported overexpression of protein F1 in PC12 or COS cells might have increased the number of the filopodial extensions observed by making them more adherent and less transitory. Finally, Kumagai-Tohda et al. (1993) observed that neurite formation in NG108-15 cells transfected with protein F1 cDNA, was higher than controls only after treatment with dibutyryl cAMP. Previous data indicated that protein F1 might not be essential for neurite outgrowth.

2. Synaptic Plasticity in Adult Brains

Nelson and Routtenberg (1985) reported a correlation between protein F1 phosphorylation and synaptic long-term potentiation (LTP). LTP has been proposed as a model to study the cellular mechanisms underlying learning (Teyler and DiScenna, 1987). LTP is produced following a train of high frequency stimulation at certain synapses in the hippocampus (Bliss and Lomo, 1973). LTP induction involves activation of N-methyl-D-aspartate receptors with subsequent stimulation of several calcium-dependent enzymes (for a review see: Bliss and Lynch 1988). Activation of protein kinase C, a calcium-phospholipid-dependent kinase, was correlated to neurotransmitter release and learning (Malenka et al., 1986; Olds et al., 1989). PKC activation by phorbol esters was reported to

enhance LTP in intact animals (Routtenberg et al., 1986; Linden et al., 1987). In hippocampus, LTP was shown to involve PKC translocation to membranes (Akers et al., 1986; Akers and Routtenberg). PKC inhibitors (mellitin, polymyxin B, H-7) prevented persistence of LTP in intact hippocampus (Lovinger et al., 1987). These studies, however, did not determine whether PKC-induced changes in PKC occurred in pre- or postsynaptic compartments.

Protein F1 was shown to be exclusively localized at presynaptic membranes in adult brains (Gispen et al., 1985; Goslin et al., 1988; Coggins and Zwiers, 1991). Phosphorylation of protein F1 by protein kinase C has been correlated with neurotransmitter release in hippocampal slices and brain synaptosomes (Dekker et al., 1989 a, b). Phorbol ester-induced PKC activation increased protein F1 phosphorylation and induced LTP-like potentiation (De Graan et al., 1988). Furthermore, Gianotti et al., (1992) reported increase in protein F1 phosphorylation after LTP induction in slices of rat hippocampus.

3. Neurotransmitter Release

Exocytosis of neurotransmitters from presynaptic membranes is stimulated by depolarization of presynaptic terminals and subsequent calcium influx. Calcium entry into synaptosomes changes the phosphorylation states of several proteins through activation of different calcium-dependent protein kinases and phosphatases, of which calcium-phospholipid-dependent protein kinase C phosphorylates protein F1 (Aloyo et al., 1983). Protein F1 has been suggested to play a role in the molecular mechanism of neurotransmitter release because: i) it is mainly localized at the presynaptic membranes (Gispen et al., 1985; Campagne et al., 1990), ii) it is correlated with several signal transducing mechanisms (for a review see Skene, 1989), and iii) of the strong correlation

between protein F1 phosphorylation by PKC and neurotransmitter release (De Graan and Gispen, 1993).

The involvement of protein F1 in neurotransmitter release has been studied by introducing monoclonal antibodies to protein F1 domains in synaptosomes made permeable with the bacterial toxin Streptolysin-O, that does not affect the exocytosis process (Dekker et al., 1989; Dekker et al., 1991; Hens et al., 1993a). Introduction of a monoclonal antibody that recognizes the calmodulin binding domain of protein F1 (amino acids 39-51) containing the only PKC phosphorylation site Ser-41 inhibited calcium-induced release of endogenous noradrenaline and the neuropeptide cholecystokinin-8 (Hens et al., 1993a, b). In contrast, monoclonal antibodies against the C-terminal domain of protein F1 (amino acids 132-213) did not have any major effect on the previous process, which in turn excluded non-specific steric hindrance as a cause of release inhibition. Another important characteristic of the protein F1 N-terminus is its ability to bind calmodulin under low calcium concentrations (Alexander et al., 1987). At the plasma membranes, protein F1 may act as a local calmodulin store (Liu and Storm, 1990). Calmodulin inhibitors (Robinson, 1991; Trifaro et al., 1992) and anti-calmodulin antibodies (Momayezi et al., 1987; Ahnert-Hilger et al., 1989) were reported to inhibit exocytosis. Calmodulin might increase neurotransmitter release through stimulation of synapsin I phosphorylation by activation of calcium-calmodulin protein kinase II (Nichols et al., 1990). This evidence also supports the role of the protein F1 N-terminus in neurotransmitter release from large dense-cored vesicles.

Protein F1 in Neuropathology: Clinical Relevance

In schizophrenia, synaptic connections of associative cortical areas of the brain are thought to have disturbed organization. Protein F1 levels, that are correlated to development of neural networks, were found to be higher in associative visual cortex of

schizophrenic brains as compared to controls (Sower et al., 1994). No significant changes were found in the primary visual cortex. However, since schizophrenic individuals are mainly known to have auditory hallucination, it would be interesting to determine whether or not protein F1 displays abnormal levels in the auditory cortex of schizophrenic brains.

In Alzheimer's disease (AD), a neurodegenerative disorder characterized by memory loss and cognitive impairment, protein F1 mRNA levels were found to be 6-fold lower compared to those of controls (Coleman et al., 1992). This reduction was demonstrated in AD brains with high neurofibrillary tangles (NFT) density. Furthermore, phosphorylation of protein F1 was shown to be reduced in the membrane compartment compared to that in the cytosol of AD brains with high NFT density (Martzen et al., 1993). This reduction might be related to mechanisms that underlie memory loss of AD or it may be secondary or even unrelated to AD.

Spinal Neural Networks

Many different embryonic central nervous system tissues can be dissociated and grown in cultures, however, not all tissues are robust in culture or can be easily isolated to produce constant cell ratios. In this study, whole murine embryonic spinal cords obtained at 13-14 day gestation were dissociated and seeded coverslips coatd with different adhesion substrata. This produced cocultures of neurons and glia (1:10). These cultures form monolayer networks where all neuronal cell bodies are in one plane and are clearly visible with phase contrast or fluorescence microscopy. Spinal monolayers survive for over 6 months in culture and mature ones (3-4 weeks) show coordinated patterns of electrical activity (Gross and Kowalski, 1991).

In this study, the main goal was to investigate the potential roles of protein F1 in the development and electrical activity of murine spinal neural networks *in vitro*. No purification of neurons was attempted as it has been shown in different studies that glia and

neurons regulate each other and pure cultures of neurons (or of glia) will behave completely differently than if co-cultured together. Furthermore, cocultures are more representative of the microenvironment of the original tissue.

In comparison to the routine recording techniques that depend on invasive single cell recording using the patch clamp technique with the consequent cell loss, recording techniques used in this study provide extracellular, multi-site (up to 64 different locations within the network), non-invasive, and mechanically stable techniques that record from active system (Gross et al., 1985). In addition, data obtained from neural networks seeded on multimicroelectrode plates (MMEPs) can be recorded on both analog and digital format for off-line analysis (for a review see: Gross, 1994).

Liposomes as Tools for Drug Delivery

A major problem in studying the physiological functions of protein F1 is the inaccessibility of this protein to experimental manipulations since it is located on the cytoplasmic surface of the neuronal plasma membranes. The small size of axonal terminals, where protein F1 is enriched, precludes microinjection. In addition, this technique that can not be applied to a population of neurons to study their network dynamics. For some time, liposomes (phospholipid vesicles) have been considered attractive vehicles for the delivery of impermeable bioactive molecules, including drugs and DNA, to cells (Papahadjopoulos et al., 1974; Fraley et al., 1980). However, early experiments used negatively charged liposomes that were transferred by endocytotic mechanisms. Under such conditions, liposomes are transported to the secondary lysosomes, resulting in the destruction of the bioactive molecules delivered (Straubinger et al., 1983). In order to achieve fusion, more recent *in vitro* experiments have used polyethylene glycol and membrane active molecules (for reviews see: Hong et al., 1991; Hui and Boni, 1991). Fusogenic virosomes, which are viral protein-containing liposomes, have also been used for delivering macromolecules to

cells (Kato et al., 1991; Bagai and Sarkar, 1993). Cationic liposomes were recently introduced as vehicles for both *in vitro* and *in vivo* gene delivery (Zhu et al., 1993; Felgner et al., 1994). Although, it is generally believed that the latter experiments involve fusion of liposomes with cell membranes, this result has not yet been clearly demonstrated (Debs et al., 1992). Hence, despite substantial efforts directed at the chemistry of liposome formation and structure, an efficient, reliable transfer of bioactive molecules to cells has not been achieved.

In this study, we demonstrate transfer of rhodamine-labeled phospholipids to mouse spinal cord neurons in cell culture. We show with microscopic observations and multichannel recording of spontaneous activity before, during, and after liposome exposure, that the transfer of rhodamine-labeled phospholipids can occur without cell death and without major effects on the spontaneous activity of the neuronal networks. Finally, the anti-protein F1 antibodies were encapsulated in positively charged liposomes and delivered into cultured neurons.

Specific Aims and Significance of this Study

Despite the extensive amount of information available on protein F1, most of the results have been observed in isolated systems or in neuroblastoma cell lines. In this research, several approaches have been applied to shed more light on the molecular functions and expression of protein F1 (GAP-43). This project had the following specific aims:

1. PKC phosphorylation of protein F1 has been reported to be crucial for several physiological functions of the protein, including its possible role in LTP, effect on phosphoinositide metabolism, and calmodulin binding. In addition, protein F1 sequence has several serine residues that have not been investigated. Therefore, we investigated

whether or not Ser-210 at the C-terminus of the protein can act as a substrate for PKC purified from rat brain. This region is a less investigated domain of the protein and is thought to be involved in the interactions of protein F1 with the cytoskeletal proteins and is also located within a potential PKC phosphorylation motif (SXR).

2. Since protein F1 may play a role in neuronal regeneration as well as in plasticity, antibodies against this protein can be used as a tool to investigate the distribution and confirm the 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*ARQDEGKE-DPEADQE) has a possible phosphorylation site for PKC (SAR). In addition, this peptide has almost identical sequence in several mammalian species. It was of interest to produce antibodies against this domain for the following reasons: i) the C-terminus domain has not yet been well defined as the N-terminus domain, ii) the C-terminus domain has been thought to be involved in interaction with other cytoskeletal proteins, iii) the antibodies produced can be used to study the physiological function(s), if any, of the phosphorylation of Ser-210, and finally, iv) production of antibodies against other parts of the protein in the N-terminus (as its calmodulin binding domain) might not be very specific because other proteins such as neurogranin have been reported to have consensus amino acid sequences with protein F1 that corresponds to the calmodulin binding domain and PKC phosphorylation site (Baudier et al., 1991).

3. In order to study the relationship between the localization and function of protein F1 in mammalian neurons, anti-protein F1 antibodies were used to localize protein F1 in cultured spinal neurons at both the light and electron microscope levels. Primary cultures of mouse

spinal neurons were used as a system to investigate expression and functions of protein F1 as they have the following advantages: i) these cells are primary neurons and not a neuroblastoma cell line, ii) spinal neurons are robust and have been shown to grow in culture and remain electrically active for up to 10 months, iii) this system represents cocultures of neurons and glial cells which is similar to the microenvironment of neurons in the original tissue, iv) spinal cultures contain a single layer of neurons growing atop of a glial carpet. The morphology and the activity of these dissociated neurons can be monitored efficiently using microscopical and electrophysiological techniques.

4. As protein F1 has been correlated to growth cone motility and neurite outgrowth of growing neurons, an enzyme-linked immunoadsorbent assay (ELISA) was used to determine whether or not protein F1 levels are regulated during the development of spinal neurons *in vitro*. In parallel, the same assay was used to observe protein F1 levels in spinal tissues in order to compare spinal neuronal development in culture versus *in vivo*.

5. Because protein F1 may be involved in several signal transducing mechanisms at the synaptic terminals and has been reported to influence neurotransmitter release and enhance LTP; investigation of the potential role of protein F1 in the gross spontaneous activity of neural networks was attempted using anti-protein F1 antibodies. A major problem in this study was the presence of protein F1 on the cytoplasmic face of plasma membranes requiring the transport of the antibodies across the plasma membranes. Direct application of antibodies, cell permeabilization, and finally the use of specific positively charged liposomes were attempted to introduce anti-protein F1 antibodies into neurons of neural networks followed by monitoring of their electrical activity.

6. Whether or not protein F1 is essential for neurite outgrowth is unknown. Furthermore, the exact role of this protein in neurite outgrowth has been a matter of controversy among investigators. In this research, the role of protein F1 in the outgrowth and extension of neural processes was further studied using both thio-modified and unmodified antisense oligodeoxynucleotides (ODN, 18-mer), with a complementary sequence to protein F1 mRNA 5' end that overlaps the start codon in order to interfere with processing and/or translation of protein F1 mRNA (Fig. 4) in cultures of mammalian neurons.

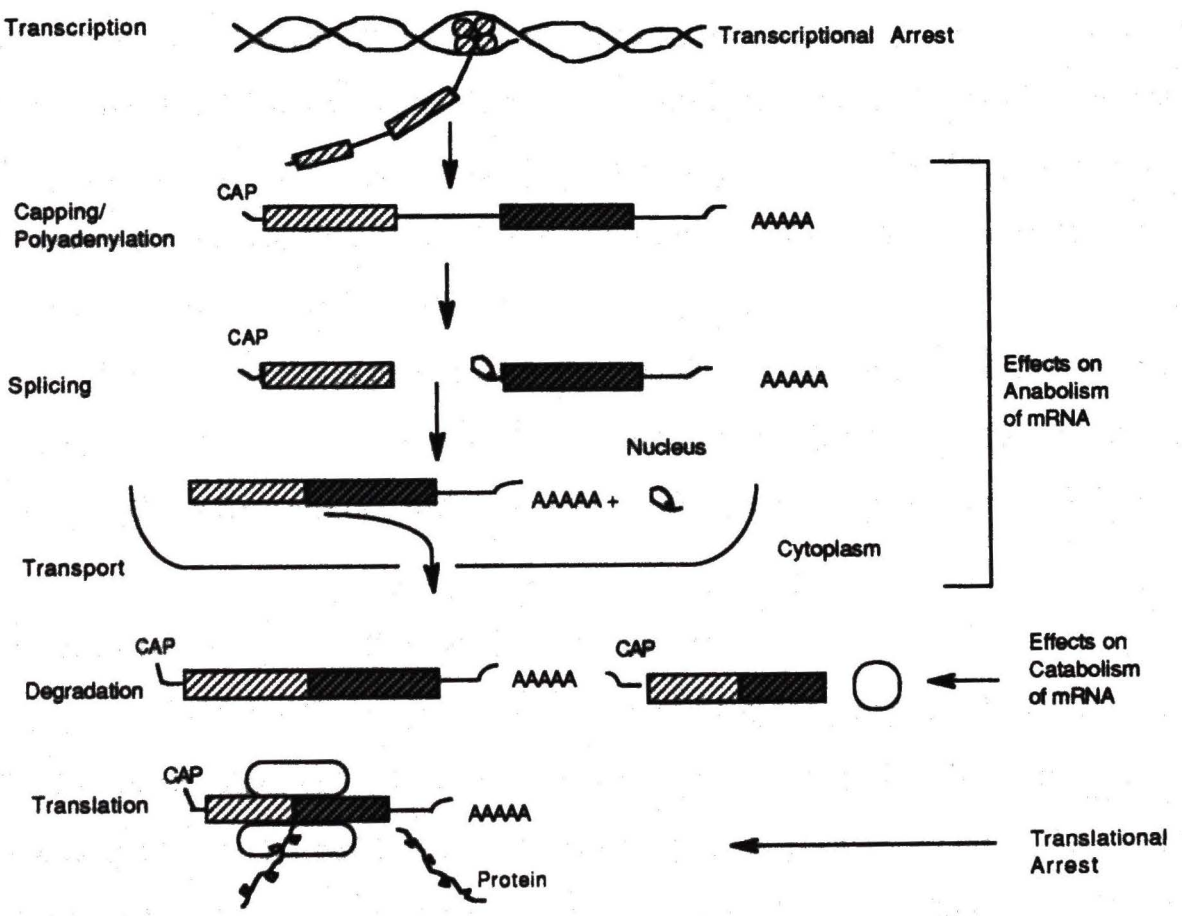


Fig. 4. Possible effects of ODN on mRNA processing (Crooke, 1993). Antisense oligodeoxynucleotides may interfere with mRNA anabolism, degradation, and/or translation.

CHAPTER II

MATERIALS and METHODS

Synthesis and Purification of F1 Peptide

Molecular cloning of F1 cDNA has revealed that mouse protein F1 is a hydrophilic 227 amino acid protein encoded by 1.5-kb nervous tissue-specific mRNA. A 21 amino acid polypeptide (Mr 2300) was selected from the predicted primary structure of mouse protein F1 (Cimler et al., 1987), 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 205-225.

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.

Purification of Protein Kinase C from Rat Brain

Protein kinase C was purified according to the method of Murakami et al. (1986). 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 Potter-Elvehjem tissue grinder with Teflon pestle (Wheaton, Milliville, NJ). The homogenate was centrifuged at 100,000 x 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 a DEAE-Sepharose column (2.5 x 9 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.5)

containing 5 mM EGTA, 2 mM EDTA, 2 mM DTT, 10% glycerol, and 40 μM cAMP. The column was then washed with 20 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol, 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 the wash buffer at a flow rate of 1 ml/min. The active fractions were pooled and concentrated to 3 ml with an Amicon-Ultrafiltration cell (Amicon, Beverly, MA). The sample was applied to an Ultrogel AcA-44 column (2.5 x 112 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol, 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, St. Louis, MO) in absence of phospholipid, from the amount incorporated in presence of the phospholipid (Farrar et al., 1985). The reaction mixture in a final volume of 100 μl contained: 50 μl of the sample, 40 μg of histone, 5 mM Mg^{2+}, 0.75 mM Ca^{2+}, in the presence and absence of 25 μg phosphatidylserine (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-HCl, pH 7.6. The reaction was then initiated by adding 5 μl of [γ-^{32}P] ATP (final concentration 65 μM, specific activity 225 dpm/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 (80 μl) was spotted onto Whatman P-81 filter paper (Whatman, Hillsboro, OR) which was then washed 3 times in 30% acetic acid for 15 min, 15% acetic acid for 10 min, and acetone for 5 min. The filter paper was then dried and radioactivity

retained determined by liquid scintillation counting (Beckman, LS 5000TD, Beckman Instruments Inc, Fullerton, CA). Typical protein kinase activity of the partially purified PKC was 170 pmol/min/μg protein.

Phosphorylation of F1 Peptide

F1 peptide (20 μg) purified on RP-HPLC, was used as a substrate for PKC partially purified from rat brain. The reaction mixture included 50 μl of the F1 peptide solution in water, 5 mM Mg^{2+}, 0.75 mM Ca^{2+}, 25 μg phosphatidylserine, 3 μg OAG in 20 mM Tris-HCl, pH 7.6. The reaction was then initiated by adding 5 μl of [γ-^{32}P] ATP (final concentration 65 μM, specific activity 225 dpm/pmol). The reaction proceeded for 20 min at 30°C after which the reaction mixture was either applied to AG1X8 column (Bio-Rad, Richmond, CA) and the peptide eluted with 15% acetic acid for analysis on HPLC and SDS-PAGE, or stopped by the addition of 60% TCA and phosphate incorporation assayed as described above.

Gel Electrophoresis and Autoradiogram of F1 Peptide

Unphosphorylated F1 peptide (20 μg) or the phosphorylated peptide were boiled for 3 min in sample buffer (0.125 M Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 20% glycerol, and 0.0025% bromophenol blue). SDS-polyacrylamide gels (1mm thick) were prepared according to the method of Laemmli (1970). F1 peptide was analyzed by 20% SDS-PAGE. The gel was then stained with 0.1% Coomassie brilliant blue (R-250, Bio-Rad) for 30 min, destained, dried, and exposed overnight to X-ray film (Kodak) at -80°C.

Production of Anti-Protein F1 Antibodies

Two milligrams (2 mg) 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 sodium phosphate buffer (pH 7.2). The peptide was conjugated to KLH using glutaraldehyde at a final concentration of 0.6% (v/v) (Goodall et al., 1983). The mixture was incubated at room temperature for 3 hr then dialyzed against phosphate-buffered saline (PBS) overnight at 4°C with 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 via ear veins 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 and the serum was analyzed by dot-blot for anti-F1 peptide reactivity. A strong antibody activity was detected at that time. 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 one week later. The collected blood was centrifuged at 1,000 x g for 20 min and the supernatant (serum) was stored at -20°C. To isolate the immunoglobulin G (IgG) fraction, 4 ml of immune serum were 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) 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 (Chan et al., 1986). 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 x g for 10 min and the supernatant was then centrifuged at 12,000 x g for 20 min. The pellet was washed by resuspension in lysis buffer (containing 10 mM EGTA, 2 mM DTT, pH 7.5) and centrifugation (17,500 x g, 20 min, 4°C). The supernatant was recovered as a 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 (Oestreicher et al., 1983). The resuspended synaptosomal membranes were adjusted to pH 11.5 by adding 1 N NaOH then centrifuged at 130,000 x g in a Beckman L5-L75 ultracentrifuge for 30 min. The supernatant was recovered and adjusted to pH 5.5 with 1 M Na-acetate-acetic acid, pH 5.0, and centrifuged at 58,000 x g for 20 min. Solubilized protein F1 in the supernatant was precipitated with 40-80% ammonium sulfate, resuspended in 5 mM potassium phosphate (KPi) and 1 mM EDTA (pH 7.5), and dialyzed overnight against the same buffer. The dialyzed sample was applied to a hydroxylapatite column (1.5 x 4.5 cm) eluted with 30, 75, and 400 mM KPi, pH 7.5, at a flow rate of 0.2 ml/min. Fractions containing protein F1 (30-75 mM KPi) were pooled and further purified on a phenyl Sepharose column (1 x 2.5 cm) and eluted with a step gradient of 1.5 M KCl, 1 M KCl, and 0.0 M KCl all in 20 mM Tris, pH 7.5 (10 ml of each). Protein F1 in fractions was detected by SDS-PAGE analysis and Western blotting. 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 and resolved by 10% SDS-PAGE. The gel was then either stained with Coomassie brilliant

blue (R-250, Bio-Rad) or proteins were electrophoretically transferred to nitrocellulose membranes (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 (10 μg/ml) 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.

Phosphorylation of Protein F1

Ammonium sulfate precipitate (0.1 mg) obtained after homogenization of whole rat brains and pH extraction, was phosphorylated by PKC (5 μg) purified from the same tissue. The reaction mixture included 5 mM Mg^{2+}, 0.75 mM Ca^{2+}, 25 μg phosphatidyl-serine, 3 μg OAG, and [γ-^{32}P] ATP (final concentration 65 μM), in 20 mM Tris-HCl, pH 7.6, and was allowed to proceed for 30 min at 30°C. At the end of the reaction, sample buffer was added and the reaction mixture was analyzed on 10% SDS-PAGE. The gel was stained, dried, and exposed to X-ray film overnight at -80°C.

Immunoprecipitation of Protein F1

Immunoprecipitation of radiolabeled protein F1, from the phosphorylation mixture prepared as described above, was achieved by incubation in the presence of anti-protein F1 antibodies (100 μg) 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 x 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 SDS-PAGE sample buffer. The mixture was boiled for 3 min then the 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).

Protein Assay

Protein concentration of the samples was estimated by the method of Bradford (1976) using bovine serum albumin (BSA, Sigma) as a standard. For monitoring protein concentrations in column chromatography fractions, absorbance at 280 nm was used. For ELISA assay, protein concentration was determined using bicinochoninic acid method (BCA, Pierce, Rockford, IL) and absorbance at 562 nm with BSA (Sigma) as a standard.

Sources and Preparation of Spinal Embryonic Tissues

In this study, ICR mice (Harlan Sprague Dawley Inc., Idianapolis, IN), maintained in the animal care facility at the University of North Texas, were used. Animals were fed rodent chow (Harlan Teklad, Madison, WI) and regular water and maintained under 12 hr light/dark cycles at 24°C.

Pregnant female mice at 13-14 days of gestation were sacrificed by rapid cervical dislocation. The abdominal cavities were opened and the uteri were isolated and washed several times in D1SGH (Puck's salt solution containing 0.75% sucrose and 0.3% glucose, pH 7.3). The embryos were then removed from the isolated uteri, washed in D1SGH, and the spinal cords surgically removed. Then spinal cords were freed from meningies, and pooled and used as a source for culturing spinal neurons.

Cell Culture of Spinal Neurons

Dissociated spinal tissue from 13-14-day mouse embryos was cultured according to the method of Ransom et al. (1977) with some modifications that included the use of papain (3 U/ml, Boehringer Mannheim Corp., Indianapolis, IN) and deoxyribonuclease I (DNase I, 40 μg/ml, Boehringer Mannheim) to enhance tissue dissociation. Cells (mixed population of neurons and glia, 1:10) were seeded on flamed, poly-D-lysine (PDL, 1.5 $\mu g/cm^2$, Sigma Immunochemicals, St. Louis, MO) and laminin (1 $\mu g/cm^2$, Sigma) coated coverslips affixed to the bottom of the 60 mm culture dishes (Corning Inc, Corning, NY) with silicone sealant (Dow Corning, Midland, MI) or on MMEPs containing 64 thin film electrodes in 1 mm^2 area (Gross et al., 1985). Initially, spinal cords prepared as described above were chopped with a scalpel and treated with papain in D1SGH for 20 min at 37°C. Papain action was terminated by adding minimal essential medium (MEM, GIBCO BRL, Gaithersburg, MD) containing 10% fetal bovine serum and 10% horse serum (HyClone Lab, Logan, Utah) (MEM10/10). The tissue was then centrifuged at 800 rpm for 4 min using clinical Dynac II centrifuge (Clay Adams/Becton Dickinson) and the pellet was resuspended in MEM10/10 containing DNase I. The tissue was then dissociated by trituration and seeded (500,000 cells/coverslip) and maintained initially in MEM10/10. Thereafter, cells were fed 2-times a week with MEM containing 10% horse serum (MEM10). When the glial carpet was confluent, further growth was inhibited by the addition of 5.2×10^{-5}M fluoro-deoxyuridine plus 1.2×10^{-4} M uridine (Sigma). The cells were grown at 37°C in an atmosphere of 90% air and 10% CO_2. These conditions allowed neurons to survive and remain electrically active for up to 312 days. For transmission electron microscopy (TEM) the dissociated cells were seeded on Lux-Permanox culture dishes.

For coating, coverslips were sonicated first with detergent for 30 min then rinsed in water, sonicated in 1 N HCl for 30 min, then washed with deionized water extensively. They were finally coated with PDL (1.5 $\mu g/cm^2$).

All cultures were provided to this investigation by the culture staff of the Center for Network Neuroscience, UNT.

Immunocytochemical Stain

Spinal neuronal cultures of 3-4 weeks (Ransom et al., 1977) 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 10% normal goat serum in PBS (blocking agent) for 30 min, rinsed with PBS and then incubated with 4 μg/ml 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 goat anti-rabbit IgG and stained with streptavidin conjugated to multiple alkaline phosphatase molecules and naphthol AS-MX phosphate according to the manufacturer's procedures (BioGenex Laboratories, San Ramon, CA).

Ultrastructural Localization of Protein F1 in Cultured Spinal Neurons

1. Cryoultramicrotomy

In this protocol, cells (4 weeks) were scraped using a cell scrapper or detached from the petri dish by adding papain (3 U/ml) and incubation at 37°C for 30 min. The cells were then centrifuged and the supernatant was discarded. The pellet was fixed in periodate-lysine-paraformaldehyde fixative (0.01 M sodium periodate, 0.075 M lysine-HCl, 2% paraformaldehyde, 0.037 M KPi, pH 7.3) for 30 min. The pellet was centrifuged, treated with 1% BSA in the same buffer as the fixative, postfixed for 2 hr in the previous fixative, and centrifuged. The pellet was infiltrated for 1 hr with 2.1 M sucrose in the same buffer as

fixative, centrifuged, frozen in liquid nitrogen, and sectioned using a diamond knife on a Sorvall MT-6000 ultramicrotome. All centrifugations were done at 1000 x g (DynaCII centrifuge) for 5 min at room temperature. Sections were transferred onto Formvar-coated nickel grids. For labeling, sections were rinsed in 0.02 M glycine to quench aldehydes, blocked in 5% BSA, and incubated with various concentrations of primary and secondary antibodies. Sections were contrast stained in 2% methyl cellulose then in 2% uranyl acetate, air dried, and examined with a JEOL JEM-100CXII transmission electron microscope (TEM) at 80 kilovolts.

2. Pre-embedding Labeling Procedure for TEM

Mature neurons (4 weeks) were fixed in 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M KPi, pH 7.4, for 30 min at room temperature. Cells were either permeabilized with 0.2% Triton X-100 or directly blocked in 5% goat serum, 1% BSA, 0.5% gelatin (bloom number 60, Sigma) in PB. Sections were then incubated with anti-protein F1 antibodies or rabbit IgG (4 μg/ml and 40 μg/ml), then incubated with goat anti-rabbit IgG conjugated to 10 nm gold particles (Sigma Immunochemicals). Tissues were washed, postfixed in 2.5% glutaraldehyde for 15 min, and treated with 1% osmium tetroxide for 60 min. Cultures were double stained with uranyl acetate and lead citrate, dehydrated, mounted, sectioned, and examined under TEM. Sectioning and photography were performed by Dr. L. M. Higgins (Texas Women University).

3. Post-embedding Labeling Procedure for TEM

The following procedure was modified from the method of Ribeiro-Da-Silva et al., (1993) and applied in the following order:

i) Fixation:

Cultured spinal neurons were briefly rinsed in cold 0.1 M KPi, pH 7.4, then fixed in 2% glutaraldehyde, 1% paraformaldehyde in 0.1 M KPi, pH 7.4, for 30 min at 4°C.

ii) Postfixation, Dehydration, and Embedding:

Fixed cultures were rinsed twice in 0.1 M KPi then treated with 1% osmium tetroxide for 60 min at 4°C. Cultures were then washed in 0.1 M KPi 3 x 5 min, in water (2 x 5 min), and then dehydrated in ethanol (series 30, 50, 70, 90, and 100%). After two more changes in 100% ethanol, tissues were then treated with propylene oxide, embedded in Epon-araldite and cured at 55°C until completely hardened (36 hours). Epon-araldite was prepared as a mixture containing 25% poly-Bed 812, 35% araldite resin 502, and 40% dodecenylsuccinic anhydride. DMP-30 [2,4,6- tri(dimethylaminomethyl) phenol, 0.3%] was then added to the mixture as a catalyst. All chemicals were purchased from Polysciences, Warrington, PA.

iii) Sectioning:

Squares of Epon-araldite (0.5 x 0.5 mm^2), containing neurons selected with a light microscope, were cut out under a dissecting microscope, mounted on a stub and sectioned using a diamond knife on a Sorvall MT-6000 ultramicrotome to produce sections of 80-100 nm thick. Sections were collected on 300 mesh nickel grids (Electron Microscope Sciences, Fort Washington, PA).

iv) Etching:

The sections on the grids were etched by floating them on drops of saturated sodium-*m*-periodate (Sigma) in deionized water for 30 min at room temperature before washing with deionized water for 5 min. Sections were incubated in 1% sodium borohydride (Sigma) solution for 5 min at room temperature then washed in water for 5 min.

v) Permeabilization:

A brief permeabilization of the sections was performed by treatment with BSA-Tris buffer (15 mM Tris-HCl, pH 8.0, 0.1% BSA, 0.5 M NaCl, 0.05% Tween 20) containing 1% Triton X-100 for 2 min.

vi) Blocking Non-specific Binding:

Sections were incubated with 10% normal goat serum in BSA-Tris buffer for 60 min at room temperature.

vii) Primary Antibody Incubation:

Anti-protein F1 antibodies were diluted to 80 μg/ml in BSA-Tris buffer with 1% normal goat serum and incubated with "blocked" sections overnight at 4°C. Following incubation, sections were washed 3 x 5 min in BSA-Tris buffer.

viii) Secondary Antibody Incubation:

Goat anti-rabbit IgG conjugated to 10 nm gold particles (Sigma) was diluted 1:40 in BSA-Tris buffer and applied to the sections for 60 min at room temperature after which sections were washed (3 x 5 min) in BSA-Tris buffer then (2 x 5 min) in water.

ix) Contrast Staining:

Sections were stained in uranyl acetate (saturated solution in 50% ethanol) for 8 min and washed in water for 5 min. They were then stained in 0.1% lead citrate solution (prepared in CO_2-free water) and washed in 0.2 M NaOH followed by water. Sections were examined in a TEM and photographed.

Controls:

Two different controls were employed in this study: i) sample control in which the primary antibodies were either omitted or replaced with pre-immune serum, and ii) background control that compares gold particles located in neuronal tissue and in cell-free areas.

ELISA Assay of Protein F1 Levels in Spinal Tissues

1. Preparation of the Antigen enriched in Protein F1:

Mouse spinal tissues collected at different time points were homogenized in 2.5 ml of ice-cold buffer [50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 2% Triton X-100, 0.01% PMSF, 1 μg/ml leupeptin, 0.1 μg/ml pepstatin A, 1 μg/ml TPCK, Sigma] with 12 up and down strokes of a Teflon-glass homogenizer (1 ml capacity) (Chang et al., 1991). The homogenized tissue was centrifuged at 17,000 x g for 15 min at 4°C and the supernatant was recovered, aliquoted, and stored at -80°C. Protein assay was performed using BCA method.

2. ELISA Assay:

Protein F1 enriched fractions [10 μg/well, diluted in 250 μl of 0.1 M phosphate buffer, pH 7.4] were added to Maxisorp 96-well plate (Nunc Inc, Naperville, IL) and incubated at 37°C for 3 hr. The wells were then washed 3 times in PB then blocked with 10% normal goat serum (Sigma) in PB for 1 hr at room temperature. Wells were then reacted with anti-protein F1 antibodies or preimmune rabbit IgG (4 μg/ml of PB containing 10% normal goat serum) for 1 hr at room temperature (each well received 200 μl of the antibody solution). Wells were then washed 3 times with PB then treated with goat anti-rabbit IgG-HRP conjugate (Bio-Rad) diluted 1:500 in 0.1 M PB, for 1 hr at room temperature (100 μl/well). Wells were washed 3 x 5 min in 0.1 M PB and 2 x 5 min in deionized water. Substrate solution (9 ml of 2,2'-azino-di-[3-ethyl-bezthiazoline-6-sulfonic acid], and 1 ml hydrogen peroxide solution, Bio-Rad) was then added for 15 min at room temperature (100 μl/well). Color development was stopped by adding 2% oxalic acid and color intensity was measured at 415 nm with 2 seconds plate shake at single reference wavelength using a Bio-Rad plate reader.

Controls:

Controls included: i) the omission of the antigen, ii) the omission of primary antibody, iii) the omission of the second antibody, and vi) using rabbit IgG (Sigma) as primary antibody.

ELISA Assay of Protein F1 Levels in Spinal Neuronal Cultures

1. Cell Cultures:

Primary mouse neurons and glial cells isolated from embryonic spinal cords were plated on PDL (1.5 $\mu g/cm^2$) coated 96-well microtitre plates (Nunc) at a density of 50,000 cells/well (experiment I) or 100,000 cells/well (experiment II). The cells were cultured in a humidified atmosphere containing 10% CO_2 at 37°C.

2. ELISA Assay:

At every time point (experiment I: days 1, 2, 3, 4; and experiment II: days 1, 7, 21, 35), cultures were fixed in 4% paraformaldehyde in PBS, pH 7.4, and stored at 4°C. When a complete set of plates was ready for further processing, cells were rinsed in PBS, permeabilized, blocked for non-specific binding, and assayed for protein F1 content as described above.

In a different set of experiments, spinal neurons were treated with NGF (12.5 ng/ml, Sigma), EGF (50 ng/ml, Sigma), or IL-1 β (10 ng/ml, Chemicon Intl, Temecula, CA) and incubated for 1, 3, 7, and 14 days in order to modulate protein F1 levels.

Direct Application of Antibodies to Cultured Neurons

Anti-protein F1 antibodies produced in rabbit against the C-terminus peptide of protein F1 conjugated to KLH were applied to three, randomly-selected, spinal neuronal cultures at 400 μg/ml in PBS 1, 3, and 5 days after seeding (neurons derived from embryonic tissues). In this particular experiment, 3 control cultures were treated with PBS.

In a different experiment, spinal neurons were treated with anti-protein F1 antibodies, rabbit IgG, or mouse IgG (all at 400 μg/ml in PBS).

Cell Permeabilization

Neuronal cells were permeabilized utilizing the method of Normura et al. (1986) as modified by Shea and Beermann (1991). Spinal neurons (3-4 weeks in culture) in 35-mm^2 petri dishes were rinsed with serum free MEM then treated with 1 ml of prewarmed 1.2 M glycerol (Sigma) in PBS, pH 7.4. After incubation on ice for 10 min, L-α-lysophosphatidylcholine (LPC; 40 μg/ml, Sigma) was added and the cells incubated for a further 8 minutes. One milliliter (1 ml) of anti-protein F1 antibodies diluted in MEM (200 μg/ml) or media alone was added and cultures were incubated at 37°C for 10 min to reverse the permeabilization and then received 1 ml of MEM containing 10% normal horse serum. Cultures were incubated further for 4 hr (the likely time course of antibody degradation) after which cells were fixed. The intracellular delivery of the antibodies was visualized by reaction with biotinylated anti-rabbit IgG followed by staining with streptavidin/multiple alkaline phosphatase conjugate and naphthol AS-MX phosphate (BioGenex Laboratories, CA) according to the manufacturer's procedures.

Liposome Preparation

All lipids used in this experiment were purchased from Avanti Polar Lipids, Alabaster, AL. Each lipid was stored in chloroform in sealed ampules under argon at -70°C. Lipid phosphorus was determined by the method of Bartlett (1959).

Small (<100 nm) positively charged liposomes were prepared by Dr. K. Hong (University of California, San Francisco) according to reverse phase evaporation (Szoka and Papahadjopoulos, 1978) and extrusion procedures (Olson et el., 1979). Briefly, phosphatidyl-ethanolamine (transphosphorylation of egg phosphatidyl-choline), 1,2-

dioleoyltrimethylammonium propane, and lissamine rhodamine-labeled phosphatidylethanolamine (100:50:1 ratio) were mixed in chloroform solution. The solvent was then removed under reduced pressure. Aqueous buffer (0.4 ml per 10 μmole lipid) was added to the ether solution of the dry lipid mixture (1 ml ether per 10 μmole lipid) and the sample was sonicated in a bath sonicator for 3 min under argon gas at room temperature to obtain a stable emulsion. The ether was removed slowly with a rotor evaporator and the sample was vortexed when necessary to break up gel formation. When lipid gel particles disappeared, an equal volume of aqueous buffer was added to the liposome suspension. The remaining ether was removed under reduced pressure. Liposomes were subsequently extruded under high pressure argon through polycarbonate membranes (Poretics, Livermore, CA) of a final pore size of 0.05 μm. Liposomes were prepared in 0.1 M potassium phosphate buffer, pH 7.3.

Liposome Treatment

The medium over the neuronal cultures (3-5 weeks old) was replaced with serum-free medium (Straubinger et al., 1983) followed by the addition of rhodamine-labeled liposomes (150-300 μM) diluted in MEM. Cultures were then incubated at 37°C for 45-120 min after which medium containing the liposomes was removed and cells were rinsed with MEM several times. For liposome application during extracellular recording, the native medium was also replaced with MEM followed by a 30 min recording period to determine stability of the spontaneous activity under MEM. Liposomes diluted in MEM were added at concentrations of 150-300 μM.

Fluorescence Microscopy

Rinsed or fixed cells were observed under a Zeiss Axiophot microscope equipped with phase and epifluorescence optics. For rhodamine labeling, observations were made

with a 546 nm excitation and a 590 nm barrier filter (Zeiss, FT580), and for fluorescein using a 450-490 nm excitation and a 515-565 nm barrier filter (Zeiss, FT510). Photographs were made with 35 mm Kodak 1600-gold color print film.

Extracellular Recording

Constant bath (vol: 1 ml) chambers (Gross and Schwalm, 1994) were used for extracellular recording with photoetched multielectrode arrays. The chamber consisted of an aluminum base holding the MMEP, and a stainless steel chamber with a microscope port. Two zebra strips (carbon-filled silicone elastomere, Fujipoly, Cranford, NJ) were pressed between the amplifier circuit board and the MMEP to provide electrical contact with the indium-tin oxide conductors (Gross and Kowalski, 1991).

Multielectrode recording was performed with a computer-controlled 64 channel amplifier system (Spectrum Scientific, Dallas, TX). Preamplifiers were positioned on the microscope stage to either side of the recording chamber. The amplifier band width was usually set at 500 Hz to 6 kHz. Activity was displayed on oscilloscopes and recorded on a 14 channel Racal analog tape recorder. Spike data from active channels were also integrated (rectification followed by RC integration with a time constant of 300 ms) and displayed on a 12 channel Graphtek strip chart recorder.

Encapsulation of Antibody in Positively Charged Liposomes

Anti-protein F1 antibodies or rabbit IgG (Sigma) were encapsulated in liposomes by Dr. Keelung Hong (UCSF) according to the protocol described below. Dioleoylphosphatidyl-ethanolamine (20 μmole), 1,2-dioleoyltrimethylammonium propane (10 μmole), and 0.8% rhodamine-labeled phosphatidyl-ethanolamine in chloroform were mixed and the solvent was removed under reduced pressure at 30°C.

Low ionic strength aqueous media (10 mM HEPES, pH 7.0) containing 5% dextrose (wt/v) was added to the lyophilized antibody (2 mg) to make a solution that was added to the dry lipids. The mixture was then vortexed to disperse the lipids and 300 µl of a concentrated solution (800 mM) of octylglucoside were added to 1 ml of the lipid-antibody suspension and clarified the mixture. The final volume of the lipid-antibody detergent sample was maintained to less than 2 ml. The detergent was then removed through dialysis (3 changes with 10 mM HEPES-5% dextrose at 4°C).

The antibody-containing liposomes were formed after most detergent was removed. The preparation was then filtered through an Acrodisc 0.2 µm filter (Gelman Sciences, Ann Arbor, MI) before use.

Cell Culture of PC12 and Liposome Treatment

PC12 (rat adrenal pheochromocytoma) cell line (ATCC, Rockville, Maryland) were grown in RPMI 1640 medium (Sigma) supplemented with 5% fetal bovine serum (Gibco BRL, Gaithersburg, MD) and 10% heat-inactivated horse serum (Gibco). Cells were seeded on Falcon Primaria petri dishes (35 x 10 mm, Fischer, Pittsburgh, PA) at a density of 2 x 10^6 cells/ml. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Streptomycin (50 µg/ml, Sigma) and penicillin (50 U/ml, Sigma) were added to the medium. After 1 or 24 hr of seeding, and after cells have adhered to the dish, cells were rinsed 4 times in serum-free RPMI then incubated with 1 ml of RPMI containing 40 µl of rabbit IgG-containing liposomes for 50 min at 37°C. At the end of incubation, cells were rinsed four times with RPMI to remove extraneous liposomes and examined under fluorescence microscopy. PC12 cultures were provided by Dr. Scott Norton.

Selection and Synthesis of Oligodeoxynucleotides

Unmodified antisense oligodeoxynucleotide (ODN, -9+9) , with the sequence of GCACAGCATGGTGGTATC, is the inverse complement of the nucleotides -9+9 of murine protein F1 cDNA (Cimler et al., 1987) and overlapping the start codon ATG. This oligonucleotide sequence was chosen for its possible interruption of translation initiation and synthesized (Biosynthesis Inc., Lewisville, TX). The corresponding sense oligonucleotide has an inverse complementary sequence to that of the antisense one. Labeled oligonucleotides were synthesized by adding fluorescein residues to the 5' end and purified on Sephadex G-25 column to remove any free fluorescein. Final concentrations of oligomers were determined spectrophotometrically.

Another thio-modified antisense ODN (18 mer) with the sequence ACAGCACAG-CATGGTGGT, that is the inverse complement of the nucleotides -6+12 of murine protein F1 cDNA, was synthesized by National Biosciences (Plymouth, MN).

Purification of Oligodeoxynucleotides on RP-HPLC

Thio-modified oligodeoxynucleotides (1.5 mg each) were purified on RP-HPLC (Gilson Medical Electronics Inc, Middleton, WI) using a C18 column (250 x 10 mm, HiPore RP-318, Bio-Rad). Purification of antisense ODN was carried out using 0.1 M ammonium acetate, 5% acetonitrile, pH 7.0, as buffer A, and 100% acetonitrile as buffer B (Synchrom Inc, Lafayette, IN). ODN were eluted with 60 min linear gradient at 0.5% per min at a flow rate of 0.7 ml/min. Fractions enriched in ODN (determined by absorbance at 260 nm) were concentrated and analyzed with gel electrophoresis.

Gel Electrophoresis of Antisense Oligodeoxynucleotides

ODN were analyzed on 0.4 mm thick 20% acrylamide gel and 5% cross link using a gel apparatus (with two 20 x 20 cm glass plates one of them being notched with 0.4 mm-

thick spacers). After polymerization, the gel was pre-electrophoresed for 30 min at 750 mA using TBE (0.89 M Trizma base, 0.89 M boric acid, 20 mM EDTA, pH 8.0) as running buffer. Sample solution was prepared by mixing 0.5 A260 units of oligonucleotides solution with an equal volume of stop solution (95% formamide, 20 mM EDTA, pH 8.0, 0.05% xylene cyanol, 0.06% bromophenol blue, US Biochemicals Corp., Cleveland, OH), the mixture was heated for 2 min at 80°C (to disrupt any secondary structure) and centrifuged briefly. Immediately before loading the sample, the wells were rinsed with TBE and the supernatant was heat-denatured (to avoid self-annealing or concatemer formation that will change migration rate of the oligonucleotides) at 90°C for 5 min then cooled quickly in an ice bath. The gel was run at 750 mA until the oligonucleotides had run two thirds of the length of the gel as estimated from the positions of the tracking dyes. Gels were then covered in Saran Wrap, placed on a thin layer chromatography plate containing a fluorophor that absorbs at 254 nm (Bio-Synthesis Inc). The ODN bands were visualized by UV shadowing and photographed using a Polaroid Type 55 N/P film.

Application of Oligodeoxynucleotides to Cell Culture

Thio-modified oligonucleotides (National Biosciences), purified on RP-HPLC using a C18 column, were initially applied at a final concentration of 4 μM to neuronal cultures maintained in MEM10 4 hr after seeding and every 24 hr for 5 consecutive days without medium change.

In order to avoid serum nucleases, neurons were seeded initially in MEM10/10 then maintained in defined neural basal medium (Gibco) with supplements. Four hours after seeding, and after neurons had adhered, purified phosphorothioate ODN (final concentration of 4 μM) were added to spinal neurons. Application of ODN continued every 24 hr for 5 consecutive days without medium change.

In another experiment, oligonucleotides were introduced into neurons using the trituration method described by Borasio et al. (1989). Six to eight spinal cords were isolated from mouse fetuses (E14), minced, then treated with papain in D1SGH for 20 min at 37°C. Spinal neurons were then washed in MEM 10/10, centrifuged for 4 min, then dissociated in MEM10/10 using a transfer pipette. The medium was then centrifuged for 4 min and the supernatant was aspirated leaving 0.45 ml of medium containing neurons. Oligodeoxynucleotides dissolved in water (0.5 mg/50 µl, final concentration of 200 µM) were then added and spinal neurons were dissociated by trituration through a Gilson yellow tip (20 cycles). Neurons were then incubated with ODN for 60 min at 37°C. The volume was then increased to 1 ml with MEM 10/10. Neurons were then plated on PDL-coated coverslips at a density of 300,000 cells per culture.

In a different set of experiments, Transfectam (Promega, Madison, WI) was used to facilitate the introduction of antisense ODN into spinal neurons and resulted in extensive cell death. Transfectam (0.5 mg) was prepared by dissolving in 20 µl of 96% ethanol for 5 min then adding 180 µl of deionized water. About 50 µl of the reagent were mixed with 50 µl of ODN solution (50 µg of ODN/1µl water) and incubated with neurons for 60 min before plating.

CHAPTER III

RESULTS

1. Synthesis and Novel *In Vitro* Phosphorylation of F1 Peptide

Purification of Protein Kinase C from Rat Brain

Phosphorylation of protein F1 has been shown to regulate several proposed molecular functions of the protein including calmodulin binding and LTP. The important phosphorylation reactions of protein F1 were catalyzed by PKC. Therefore PKC was partially purified from rat brain to study the phosphorylation of protein F1 as well as the selected peptide.

The PKC was purified from rat brain homogenate by DEAE-Sepharose column chromatography (Fig. 5). Fractions 16-20, containing high PKC activity were pooled and concentrated, then further purified by gel filtration. Fractions 19-21 eluted from Ultrogel AcA-44 (Fig. 6) showed high activity of the enzyme and low absorbance at 280 nm. These fractions were pooled, concentrated, and stored in glycerol at -80^{o}C. Assay of the partially purified enzyme showed no activity in absence of the histone substrate indicating that there was no endogenous substrate of the enzyme (Fig. 7, column 1). To determine that the phosphorylation observed was enzyme-dependent, the enzyme was omitted and did not result in any phosphorylation (Fig. 7, column 2). Column 3, Fig. 7, shows the amount of ^{32}P incorporated in histone by PKC in absence of phosphatidyl-serine/OAG. OAG, produced *in vivo* by phospholipase C-activated hydrolysis of phosphatidylinositol in response to several extracellular messengers including growth factors and peptide

hormones, is considered to be a second messenger for the calcium and phospholipid-dependent PKC leading to its activation. In presence of phosphatidyl-serine and calcium, OAG is proposed to cause a confirmation change in PKC that moves the pseudosubstrate out of the catalytic site allowing access of Mg-ATP and substrate thus activating the enzyme (for a review see: Soderling, 1990). Presence of PKC, the histone substrate, and phosphatidylserine/OAG in the reaction mixture (Fig 7, column 4) resulted in maximal PKC activity that was 7-times higher than that observed in absence of phosphatidyl-serine/OAG. Because the activity observed was phospholipid-dependent, the enzyme purified was shown to be PKC. The typical specific activity of purified PKC was 11.7 pmol/min/μg protein and was stable for several months at -70°C following purification.

Phosphorylation of Synthetic F1 Peptide by PKC

In order to test whether Ser-210 at the C-terminus of protein F1 is a substrate for rat brain PKC, a 21 amino acid polypeptide was selected from the primary sequence of protein F1 (from position 205-225) and synthesized (Fig. 8). This peptide is located within the negatively charged extended rod at the C-terminus of protein F1 (Fig. 8). The peptide analyzed 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 shorter retention time (Fig. 9*A*) relative to the native peptide. This shift is consistent with the introduction of a negatively-charged phosphate group into the peptide. Liquid scintillation spectrometric analysis of eluted fractions confirmed that this peptide with earlier elution time corresponded to the radioactive peak (Fig. 9*B*).

The radioactive fractions (#16-18, Fig. 9*B*) 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. 10*A*-1). By autoradiography (Fig. 10*B*-1), it was determined that all radioactivity was associated with this band which co-migrated with purified non-phosphorylated F1 peptide (Fig. 10*A*-2). These data suggest that F1 peptide is a substrate for protein kinase C.

Concentration-Dependent Phosphorylation of F1 Peptide by PKC

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. 11, the phosphorylation of F1 peptide by PKC was linearly dependent on F1 peptide concentration. A Km of approximately 0.25 μM was estimated when F1 peptide was assayed with PKC. This is consistent with the proposal that F1 peptide is a good substrate for PKC.

2. Production and Characterization of Anti-Protein F1 Antibodies

Immunoblotting of Protein F1

The synthetic F1 peptide was then used to generate polyclonal antibodies (see Materials and Methods). Using dot-blot analysis, these antibodies were shown initially to have strong reactivity with purified F1 peptide, and F1 peptide conjugated to either BSA or KLH but not with BSA by itself. In order to test the ability of these antibodies to recognize intact protein F1, SDS-PAGE analysis of protein F1 obtained from various stages of purification (ammonium sulfate precipitate, 3 fractions eluted from hydroxylapatite column, and one fraction from phenyl Sepharose column, Fig. 12*A*-*E*) was performed. A protein

band of Mr about 43,000 was enriched in the first two fractions eluted from hydroxylapatite column (Fig. 12*B*, *C*) and in the void volume eluted from phenyl Sepharose column (Fig. 12*E*). A single strong band (Mr 43,000) can be seen on immunoblotting of protein F1 eluted from phenyl Sepharose column (Fig. 12*F*) revealing that anti-F1 peptide antibodies can recognize the intact protein F1 from rat brain. Immunoblot analysis of crude brain homogenate also revealed strong immunoreactivity to a protein of molecular weight similar to protein F1.

Phosphorylation and Immunoprecipitation of Protein F1

In order to show that protein F1 can serve as a substrate for PKC, a partially purified preparation of rat brain homogenate was 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. 13*C*). In a different experiment, the antibodies were used to immunoprecipitate protein F1, phosphorylated by PKC in presence of radiolabeled ATP, from partially purified brain homogenate. The reaction mixture was analyzed on SDS-PAGE and gels were stained, dried and exposed to X-ray film. Protein F1 band was detected at Mr~43,000 (Fig. 14*C*). Another band was detected at Mr~32,000 (Fig. 14*C*) which is probably a proteolytic product of protein F1 (McMaster et al., 1988). A protein band with Mr of 45,000-50,000 (Fig. 14*B*) is probably the heavy chain of the antibodies. These results indicate that anti-protein F1 antibodies may recognize both the phosphorylated and unphosphorylated forms of protein F1.

3. Localization of Protein F1 in Cultured Mammalian Neurons using LM and EM

Immunolocalization of Protein F1: Light Microscopy

Cultured spinal neurons form a single layer of neural network growing on a carpet of glial cells underneath as shown in Fig. 15*A*. Three-week old cultures of mouse spinal neurons (dissociated from 14-day old mouse embryos) were fixed and incubated 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. 15*B*). Glial cells, however, stained weakly. In contrast, preimmune rabbit IgG treated cells did not show any immunoreactivity (Fig. 15*C*). These results clearly demonstrated that protein F1 is located primarily in neurons.

Localization of Protein F1 at the Ultrastructural Level

To further investigate the function-localization relationship of protein F1 in spinal neural networks, immunogold-labeling of protein F1 was examined. Different protocols were tested for their ability to preserve protein F1 antigenicity and cellular ultrastructure, including: cryoultramicrotomy, pre-embedding labeling, and post-embedding labeling.

In cryoultramicrotomy, aggregation/clusters of gold particles were visualized at certain areas of the sections treated with anti-protein F1 antibodies whereas much less labeling was observed in sections incubated with preimmune serum. However, the ultrastructure was completely lost and no correlation between labeling and subcellular localization could be obtained.

Although pre-embedding procedure resulted in preservation of ultrastructure of spinal neurons, gold labeling was very scarce which indicated a problem in antibody

penetration. The use of 0.1-0.3% Triton X-100 to permeabilize membranes to enhance antibody entry, resulted in loss of ultrastructural integrity.

Post-embedding labeling using gold colloidal particles provides more reliable subcellular localization of molecules under study and does not include the penetration problems encountered in other techniques. Using this protocol, the ultrastructure was maintained including: nuclear membranes, mitochondria, plasma membrane, microtubules, synapses, and synaptic terminals. In addition, gold labeling was clearly demonstrated and specific to cultures reacted with anti-protein F1 antibodies. Sections that received no primary antibody (Fig. 16*A*) or were treated with pre-immune serum (Fig. 16*B*) contained no or very few gold particles in any part of the neurons including membranes, mitochondria, presynaptic terminals or vesicles, and synapses. On the other hand, cultures treated with anti-protein F1 antibodies (80 μg/ml) were labeled on presynaptic terminals (Fig. 16*C*), on an axon-like process (Fig. 16*D*), and on microtubules (Fig. 16*E*). No labeling was noticed on parts of the grids that did not contain tissues which indicated that gold labeling was primarily associated with tissues (Fig. 16*C*). Protein F1 immunoreactivity was shown to be intracellular, but no specific subcellular localization could be demonstrated.

4. Developmental Regulation of Protein F1 in Spinal Neurons

Development of Spinal Neurons in Culture

Neurons dissociated from spinal tissues (Fig. 17*A*) and seeded on PDL substratum, extended short processes 3 hr after plating in MEM10/10 (Fig. 17*B*). Four hours later, one of the short neurites grew rapidly and became several times longer than other processes (although not fully defined, the long neurite is thought to be an axon, Fig. 17*C*). The remaining processes then started to elongate and form contacts between cells (Fig. 17*D*).

Small neural networks formed after 10 hr in culture (Fig. 17*E*) and growth cone-like structures were very obvious during that period. By 3-4 weeks in culture, these cells formed mature neural networks consisting of a monolayer of neurons overlaying a carpet of glial cells (Fig. 17*F*). Many studies have shown that these networks are electrically active and responded to specific pharmacological agents in a manner similar to that of their original tissue (Gross et al., 1985). It was noticed that spinal neuronal development was quite fast. The use of heat-inactivated sera and lower cell density considerably decreased the growth of spinal neurons.

Assay of Protein F1 Levels in Spinal Neurons *in vitro*

Protein F1 has been shown by several investigators to be highly expressed in developing neurons during axonal growth and regeneration with its levels dropping as neurons become mature. The anti-protein F1 antibodies were used to evaluate protein F1 levels in developing cultures of mammalian neurons. Neurons dissociated from embryonic spinal tissues were plated in PDL-coated 96-well microtitre plates and maintained in MEM10/10. At different points of time, cells were fixed then assayed for protein F1 using an enzyme-linked immunoadsorbent assay (ELISA). The effective concentrations of the primary and secondary antibodies and other conditions were established prior to the experiment. The standard deviation of the mean of sample to sample was 0.03 (i.e., separate cultures in wells of microtitre plates) and the assay was shown to be specific.

ELISA assay of protein F1 in growing neurons at 4 different time points over a period of 35 days (Fig. 18*A*) revealed high levels of the protein in one day-old cultures followed by a steady decrease of protein F1 levels as cells become mature. To further investigate protein F1 levels during the early stage of neuronal development and before the establishment of synaptic contacts and mature networks, assay of protein F1 over a shorter time period (1-4 days, Fig. 18*B*) was performed and revealed a peak at 48 hr post-seeding

followed by a gradual decrease in the protein levels after 4 days of plating. Fig. 18*B* shows that spinal neurons after 24 hr of seeding contain double the amount of protein F1 expressed by spinal neurons of similar age as shown in Fig. 18*A*. This is because neurons assayed for protein F1 in Fig. 18*A* were seeded at double the density of neurons assayed for protein F1 in Fig. 18*B*. Therefore, neurons seeded at higher density did not have to extend longer neurites to from synaptic contacts and consequently are thought to express lower levels of protein F1 than those seeded at low density. Furthermore, protein F1 expression was reported to be regulated by interneuronal contacts (Van der Neut et al., 1990). The high levels of protein F1 expressed at early stages by cultured neurons may correlate with the injury caused during dissociating the neurons from the spinal cords. The assay was shown to be specific as the readings were greatly reduced when anti-protein F1 antibodies were replaced with preimmune serum (Fig. 18*C*). Omission of the primary or secondary antibodies further reduced the absorbance at 415 nm (Fig. 18*C*). Microscopic observations indicated that neurons were alive and protein assay performed on the cultures following the ELISA assay showed a linear increase in protein concentrations over time (Fig. 18*A*, *B*) which indicated neuronal growth. Assay of total protein concentration of cultured neurons indicated that the growth of spinal neurons stopped after 21 days in culture. Data obtained in this study that indicate expression of high levels of protein F1 during neurite extension by newly seeded neurons are in accord with previous reports (Skene, 1989; Van der Neut et al., 1990) and further support the role of protein F1 during neuronal development.

Attempts to modulate protein F1 levels in growing neurons with different effectors were performed. Briefly, epidermal growth factor (50 ng/ml, Sigma), nerve growth factor (12.5 ng/ml, Sigma), and interleukin 1-β (10 ng/ml, Chemicon Intl.) were incubated with newly seeded neurons for 1, 3, 7, and 14 days. Using ELISA assay, no differences in protein F1 levels were observed compared to non-treated cells.

Comparison of Protein F1 levels *in vivo* versus in Culture:

To examine whether spinal neurons develop in culture in a manner similar to that *in vivo*, protein F1 levels were also estimated in spinal tissues from embryonic and postnatal mice and compared to those of spinal cultures. Spinal tissues were isolated from fetal (E15-E17) and postnatal (P1-P18) mice, homogenized and assayed for protein F1 content. Protein F1 increased as fetuses approached birth (at the end of gestation) and reached maximal levels by postnatal day 1 (Fig. 19*A*). Levels then dropped gradually and reached minimal levels by postnatal day-18. Although high levels of protein F1 were observed at P1 *in vivo* , cultured spinal neurons of similar age (day 7 after seeding) expressed low levels of the protein.

The specificity of the assay, at every time point, was tested by omitting the primary antibody, omitting the secondary antibody, omitting the protein substrate, or using rabbit IgG as primary antibodies. In all cases, absorbances at 415 nm were 5-6 times lower than that observed in the presence of anti-protein F1 antibodies (Fig. 19*B*).

5. Effect of Anti-Protein F1 Antibodies on the Physiological Functions of Protein F1

In order to elucidate the function of a certain cellular macromolecule, specific antibodies against the molecule in question can be applied to living cells. Binding of the antibodies to their target antigenic proteins may selectively interfere with different processes of the protein under investigation including molecular functions, translocation, and enzymatic activities.

The presence of protein F1 on the cytoplasmic face of plasma membranes constitutes a major technical difficulty to the study of physiological functions of this protein. In this study, several procedures were performed in order to introduce anti-protein F1 antibodies into cultured spinal neurons to elucidate their potential effect on the

development and electrical activity of developing and mature cultured mammalian neurons, respectively.

Direct Application of Anti-Protein F1 Antibodies to Cell Culture

To investigate the direct effect of the antibodies on the development of cultured neurons, anti-protein F1 antibodies in PBS, were added at a high concentration of 400 μg/ml to spinal neurons (500,000 cells/culture) 24 hr after seeding. The antibodies were applied at the same concentration two more times to the cultures after 3 and 5 days of plating and cells were fed twice weekly with MEM 10 and grown at 37°C in a humidified atmosphere containing 10% CO_2. Treatment with high antibody concentration was expected to cause introduction of some antibody molecules into the cells. Three controls received an equal volume of the carrier medium (PBS). No effect was observed on the growth of antibody-treated neurons as compared to controls. However, by the end of first week, clusters of neurons were observed in antibody-treated cultures. Four weeks later, three cultures treated with anti-protein F1 antibodies showed massive aggregation (Fig. 20, upper panels). Fiber and cell density in these cultures were obviously higher than those of control cultures (Fig. 20, lower panels). In another experiment, treatment of 2 cultures with anti-protein F1 antibodies (160 μg/ml) resulted in a lesser aggregation. IgG molecules added to the cultures might have served as adhesion molecules for neurons and resulted in extra aggregation and survival of more cells. To further investigate the effect of antibodies on neuronal aggregation, newly seeded cultures were treated with IgG molecules purified from rabbit, mouse, or protein F1 immune sera (400 μg/ml), however, neuronal aggregation was not observed.

In order to examine the possible effect of anti-protein F1 antibodies on the electrical activity of murine neural networks, the antibodies (400 μg/ml) were added directly to mature (4-5 weeks) spontaneously active spinal neural networks seeded on MMEPs and

maintained in MEM (Fig. 21*A*). The treatment resulted in a slow down of the electrical activity within 5 min of the antibody application (Fig. 21*B*) then a complete shut down of the activity after 12 min (Fig. 21*C*). The activity was recovered following a medium change (Fig. 21*D*). This interesting observation was non-specific as application of rabbit IgG or mouse IgG (at a similar concentration of 400 μg/ml) resulted in a similar effect of activity reduction and complete shut down within 10-15 min of the application. Several applications of the carrier medium (PBS) alone did not result in any reduction of the original activity.

Permeabilization of Spinal Neurons

The next step was to attempt to deliver the antibodies across the neuronal membranes. Shea and Beermann (1991) have previously reported methods to deliver antibodies into NB2a/d1 cells by permeabilization of membranes at low temperature in medium containing high glycerol concentration (1.2 M). Internalization of the antibodies was facilitated by incubating the cells in the presence of phospholipids. Mature neuronal cells (4 weeks old) were permeabilized according to the previous protocol in presence or absence of antibodies then subjected to immunocytochemistry. Neurons permeabilized in presence of antibodies were heavily stained (Fig. 22*A*, *B*). Glial cells also showed some staining. Only background staining of glial carpet in cultures permeabilized in absence of antibodies was observed (Fig. 22*C*, *D*). These observations indicated that antibodies can be delivered into neurons using this method. However, excessive death of neural network and detachment of the glial carpet was observed in large areas of the cultures and precluded the use of this method to deliver antibodies into neural networks.

Interaction of Positively Charged Liposomes with Cultured Neurons

Liposomes were finally attempted as a vehicle to deliver the antibodies inside neurons. Empty unilamellar positively charged liposomes (consisting of

phosphatidylethanolamine, 1,2-dioleoyltrimethyl-ammonium propane, and lissamine rhodamine-labeled phosphatidyl-ethanolamine; 100:50:1 ratio) were prepared by Dr. K. Hong (University of California, San Francisco) using reverse phase evaporation and extrusion procedures (Olson et al., 1979). These liposomes were diluted in serum-free MEM and applied to mature neurons for periods of 45-120 min in order to test neuronal interaction with positively charged liposomes prior to antibody encapsulation.

Figure 19 shows a network region exposed to the high liposome concentration (300 μM total phospholipid) and fixed with Gregory's fixative (Gregory, 1980) after two hours in liposomes. The labeling of spinal neurons with rhodamine linked covalently to the liposomal membrane (Fig. 23*B*) is remarkably uniform and intense. Phase contrast microscopy (Fig. 23*A*) shows neuronal somata and neurites residing on a carpet consisting primarily of flat quasi-hexagonal glia. The large neuron in the center is 25 μm in diameter. The fluorescence micrograph (Fig. 23*B*) shows that both somata and neurites are intensely stained with the glial carpet underneath showing much less staining. A dead neuron (arrow) is also stained.

Fig. 24.I shows the response of a spontaneously active network to a high concentration of liposomes (300 μM) over a 60 min period. As serum is thought to interfere with liposome fusion, liposomes were suspended in MEM. This carrier medium alters the native spontaneous activity by generating rapid, uncoordinated bursting with short burst durations, but allows recovery of the native activity with time or upon return to the original medium. Fig. 24.I-*A* shows this response to MEM (arrow), and a similar response to MEM with liposomes (Fig. 24.I-*B*, arrow). Although all channels are active 10 minutes after liposome application, a reduction of integrated activity is seen on 7 of the 12 channels at 30 min (Fig. 24.I-*D*), with further activity loss at 60 min (Fig. 24.I-*E*). In this experiment, the native activity could not be recovered after a medium change. This network behavior, coupled with microscope observations of approximately 50% cell death after two

hours in liposomes, clearly demonstrated liposome toxicity. However, those cells which survived revealed remarkably uniform and bright staining with rhodamine-labeled phospholipid.

The reduction of liposome concentration by 50% to 150 μM and the limiting of the exposure time to 45 min also resulted in staining of neurons but without any visual signs of cell death. Multichannel, extracellular electrophysiological recording revealed that spontaneous bursting activity was retained on all channels (Fig. 24.II-*A*). A medium change at 45 minutes recovered the network activity to levels close to the original activity (compare Fig. 24.II-*A* with -*E*). It appeared that, under these experimental conditions, molecular transfer via liposomes could be achieved without any cell death and without seriously interfering with the delicate spontaneous activity of the network.

Encapsulation of Antibodies in Liposomes

Anti-protein F1 antibodies were encapsulated in liposomes consisting of 20 μmole dioleoylphosphatidylethanolamine (DOPE), 10 μmole 1,2-dioleoyltrimethylammonium propane, and 0.8% rhodamine-labeled phosphatidylethanolamine. The latter was included to indicate whether or not liposomes fused with the cells and consequently might suggest delivery of liposomal contents into neurons.

Liposomes containing antibodies were tested for their interaction with spinal neurons of different ages (4 days and 4 weeks old cultures). Incubation of neurons with liposomes (0.9 μM total phospholipid, and containing 80 μg of antibodies) diluted in 1 ml of MEM for 45 min resulted in a uniform labeling of neuronal membranes with rhodamine while glial cells underneath showed little or no staining (Fig. 25*B* and 21*D*). Phase contrast microscopy of the same cultures showed no signs of cytotoxicity (Fig. 25*A*, *C*). Uniform labeling of neurons, preferential labeling of neurons over glia, and absence of cytotoxicity,

after treatment with antibody-containing liposomes, were observed in both young and mature cultures.

Effect of Anti-Protein F1 Antibodies on the Development of Mammalian Neurons *in vitro*

Anti-protein F1 antibodies or rabbit IgG (80 and 160 μg/ml) encapsulated in positively charged liposomes (dioleoylphosphatidyl-ethanolamine and 1,2-dioleoyltrimethylammonium propane, 2:1) diluted in 1 ml of serum-free medium (0.9 μM total phospholipid), were added to spinal neurons 3 hr after seeding on PDL-coated coverslips. Incubation continued for 45 min after which cells were rinsed in MEM then either incubated in MEM10/10 or fixed in Gregory's fixative for 5 min. Examination of neurons (fixed at different points of time: zero, 4, 10, 48 hr after liposome treatment) under phase contrast microscopy revealed no cell death. No changes in morphological characteristics (in particular cell shape and neurite length and shape) were observed between neurons treated with anti-protein F1 antibodies (Fig. 26*A*, *C*, *E*, *G*) or with rabbit IgG up to 48 hr after liposome treatment. Moreover, neuronal development in antibody-treated cultures was comparable to that of control cultures, not treated with antibodies. Fluorescence microscopy examination of neurons (Fig. 26*B*, *D*, *F*, *H*) revealed that liposome interaction with neuronal membranes was uniform and age-independent.

Interaction of Liposomes with PC12 Cell Line

As the liposomes used in this study did not interact with other cell lines including CHO, CV1 (an African green monkey kidney cell line), and breast cancer cells (personal communication with Dr. Keelung Hong, UCSF), liposomes were further characterized by interaction with PC12 neuroblastoma cells. Empty or antibody-containing liposomes, diluted in serum-free medium, were added to PC12 cells after growing in RPMI 1640

medium supplemented with 5% fetal bovine serum and 10% heat-inactivated horse serum for 1 or 24 hr at 37°C in a humidified incubator containing 5% CO_2. Cells were incubated with liposomes for 50 min after which cells were rinsed and examined. Phase contrast microscopy demonstrated no cytotoxicity of PC12 treated with empty liposomes (Fig. 27*A*) or with antibody-containing liposomes (Fig. 27*C*). Fluorescence microscopy revealed no uniform rhodamine-labeling of the cells (Fig. 27*B*, *D*). Scattered spots of empty liposomes were visualized on PC12 cells (Fig. 27*B*) which might indicate that liposomes were taken up by PC12 cells via endocytosis. According to these observations, the diffused non-toxic interaction of positively charged liposomes, made of dioleoylphosphatidyl-ethanolamine and 1,2-dioleoyltrimethylammonium propane (2:1), might be specific to spinal neurons.

6. Effect of Antisense Oligodeoxynucleotides (ODN) on the Development of Spinal Neurons in Culture

Selection and Synthesis of Antisense ODN

Fig. 28 shows the sequences of two pairs of antisense ODN selected from the cDNA sequence of murine protein F1 and used to investigate the effect of inhibiting protein F1 mRNA translation and/or processing on the development of cultured mammalian neurons. Sense and antisense ODN of the first pair (National Biosciences) consisted of 18 bases and had modified phosphodiester backbone (Fig. 28*A*) in which one of the non-bridging oxygens is replaced by a sulfur (Fig. 28*C*). Sulfur modification of the internucleotide bond reduces the action of several nucleases and increases the potential for crossing the membrane lipid bilayer (Connolly et al., 1984; Stein et al., 1991). The antisense phosphorothioates had an inverse complementary sequence to nucleotides -6+12 of protein F1 cDNA (Fig. 28*A*).

Another pair of unmodified ODN was synthesized (Bio-Synthesis) which had unmodified backbone and consisted of 18 bases each. The antisense ODN had an inverse complementary sequence to nucleotides -9+9 of protein F1 cDNA (Fig. 28*B*). Sequences of both antisense ODN pairs overlapped the start codon and were selected for their potential of interrupting translation initiation.

Purification of Antisense ODN on RP-HPLC and Analysis by Gel Electrophoresis

Synthesized sense and antisense phosphorothioates (about 1.5 mg each) were purified on RP-HPLC using a C18 column and eluted with 0.1 M ammonium acetate, pH 7.0, and 5% acetonitrile as buffer A, and 100% acetonitrile as buffer B. Absorbance of the fractions eluted was measured at 260 nm and revealed very similar profiles for both sense and antisense ODN. Two major peaks (fractions 10-20, and 76-79) and a minor peak (fractions 40-45) were observed on the HPLC chromatogram (Fig. 29). Fractions representing each peak were pooled and concentrated then analyzed on 20% acrylamide gel electrophoresis. Upon ultraviolet shadowing of the gel at 254 nm, ODN (18-mer) bands appeared as dark bands against a fluorescent background of the chromatographic plate. These bands (Fig. 30.I-*C*, *D*) were shown to exist in the last major peak (fractions 76-79) of sense and antisense ODN chromatograms. These fractions were dried, dissolved in water, filtered, and used to apply to tissue cultures.

Unmodified sense, antisense, and fluorescein-labeled antisense ODN (18-mer) were analyzed on 15% gel electrophoresis and showed strong bands (Fig. 30.II-*A*, *B*, *C* respectively) that migrated faster than xylene cyanol (a dye marker with molecular weight equivalent to 30-mer on 15% gels, upper arrow in Fig. 30.II).

Cytoplasmic Delivery of Antisense ODN into Cultured Spinal Neurons

As an alternative to microinjection, which is unsuitable for the injection of a large number of neurons and causes irreversible damage to neurons, cytoplasmic delivery of antisense ODN was achieved during tissue dissociation before seeding (Borasio et al., 1989). Spinal neurons were isolated from mouse embryos and exposed to papain treatment, after which antisense ODN were introduced into the neurons by simply exposing the cells to a relatively high concentration of ODN (200 and 300 μM) during mechanical dissociation. In order to verify the internalization of antisense ODN into neurons, fluorescein-labeled antisense ODN were purified on Sephadex G-25 column (to remove any free fluorescein) and added to neurons at a final concentration of 40 μM. Cells were then mechanically dissociated by trituration (20 cycles) using a transfer pipette or a yellow tip. One hour following seeding of cells on PDL-coated coverslips, neurons were washed in MEM and fixed in Gregory's fixative then examined under the fluorescence microscope. Fig. 31*A* shows a phase contrast micrograph of neurons dissociated in the presence of fluorescein-labeled antisense ODN. Fluorescence microscopy examination of the same area revealed fluorescein labeling of neuronal cell bodies (Fig. 31*B*) that indicated successful introduction of antisense ODN into neurons.

Effect of Antisense ODN on the Development of Spinal Neurons *in vitro*

Trial #1 (4 different experiments, 9 cultures per experiment: 3 cultures treated with antisense ODN, 3 cultures treated with sense ODN, and 3 controls). Thio-modified ODN, purified on RP-HPLC, were applied at a final concentration of 4 μM to neuronal cultures maintained in MEM10/10 4 hr after seeding. Further application of antisense ODN were made every 24 hr for 5 consecutive days without medium change. Development of sense and antisense-treated neurons was comparable to that of controls. No effect on the morphology or neurite formation by antisense ODN-treated cells was observed.

Furthermore, treated neurons were grown in culture for 4 weeks and formed normal neural networks.

Trial #2 (2 experiments, 9 cultures per experiment: 3 cultures treated with antisense ODN, 3 cultures treated with sense ODN, and 3 controls). Spinal neurons were seeded initially in MEM10/10 then maintained in defined neural basal medium (Gibco) with supplements in which no serum was added (to avoid serum nucleases). Four hours after seeding, and after neurons have adhered, purified phosphorothioate ODN (final concentration of 4 μM) were added to spinal neurons. Application of ODN continued every 24 hr for 5 consecutive days without medium change and did not result in any morphological differences between experimental and control cultures.

Trial #3. (using 12 cultures: 3 cultures treated with antisense ODN and Transfectam, 3 cultures treated with sense ODN and Transfectam, 3 cultures treated with Transfectam only, and 3 controls). Transfectam (0.5 mg, Promega) was used to facilitate the introduction of unmodified antisense ODN into cultured spinal neurons. Transfectam (50 μl) was mixed with 50 μl of oligonucleotide solution (10 μg/1μl water) and incubated with neurons for 60 min before plating. Following that treatment, cells did not attach to the culture plates, and excessive cell debris were observed with an estimated cell death of more than 85%.

Trial #4 (3 different experiments, 9 cultures per experiment: 3 cultures treated with antisense ODN, 3 cultures treated with sense ODN, and 3 controls). Oligonucleotides were introduced into neurons using the trituration method described by Borasio et al. (1989). Spinal cords isolated from mouse fetuses (E14) were dissociated in 0.45 ml of MEM10/10. Antisense ODN solution (0.5 mg/50 μl H_2O, final concentration of 200 μM) was then added and spinal neurons were dissociated by trituration. Neurons were then incubated with ODN for 60 min at 37°C then plated on PDL-coated coverslips at a density of 300,000 cells per culture. Development of control, sense, and antisense-treated cultures

was monitored at different time points: 3 hr (Fig. 32*A*, *B*, *C*), 10 hr (Fig. 32*D*, *E*, *F*), and 20 hr (Fig. 32*G*, *H*, *I*). Neurons in the experimental and control cultures developed similarly and started to form normal neural networks by 20 hr (Fig. 32*G*, *H*, *I*).

Fig. 5. Purification of PKC, step 1: DEAE-Sepharose column. Rat brain homogenate supplemented with cAMP (40 μM) was applied to a DEAE-Sepharose column. Elution was achieved using 0.0-0.3 M NaCl gradient in 20 mM Tris, pH 7.5, containing 10% glycerol, 1 mM EDTA, 1 mM EGTA, and 2 mM DTT. Fractions collected were assayed for PKC as described in Materials and Methods. Protein concentration was determined by absorbance at 280 nm.

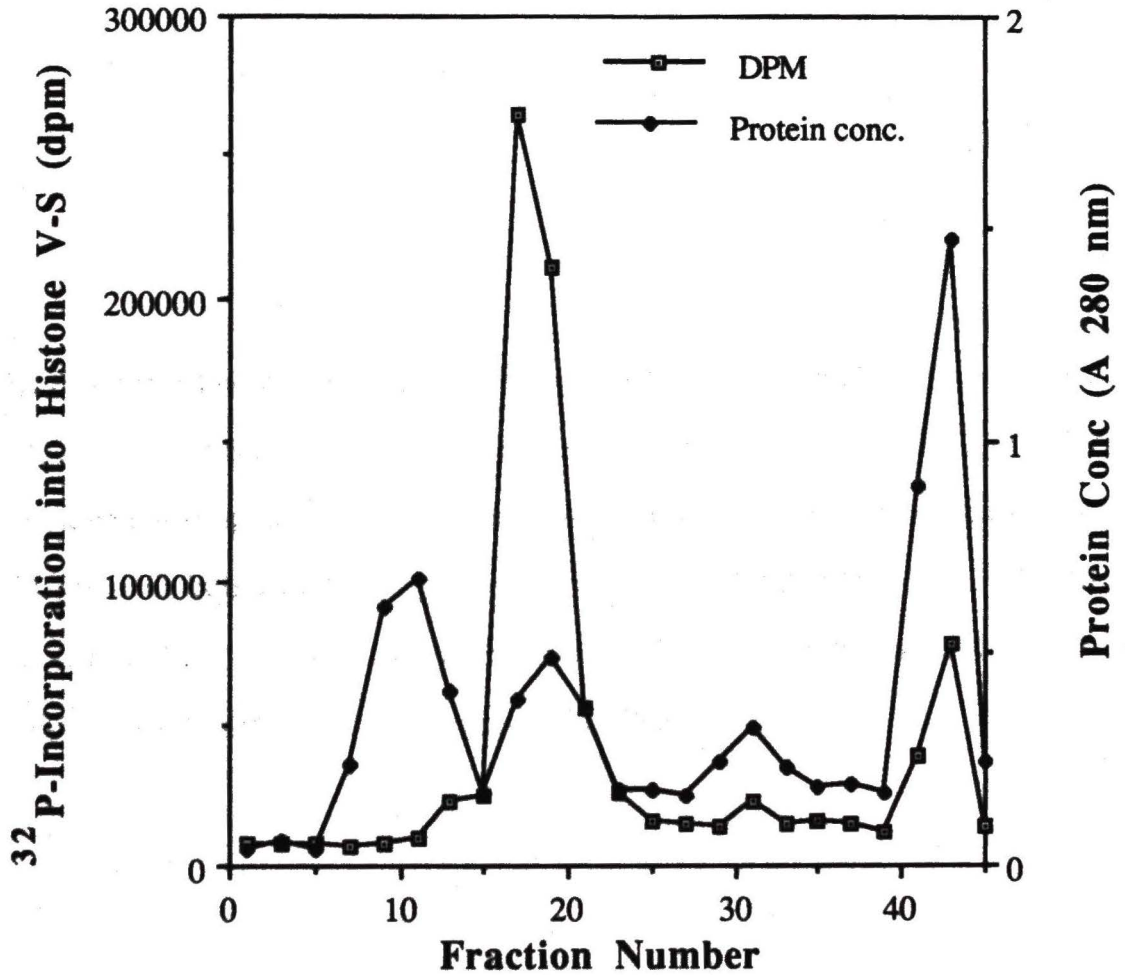

300000
200000
100000
0
2
1
0
DPM
Protein conc.
0
10
20
30
40
Fraction Number
32P-Incorporation into Histone V-S (dpm)
Protein Conc (A 280 nm)

Fig. 6. Purification of PKC, step 2: Ultrogel AcA-44 gel filtration. Fractions possessing PKC activity collected from the DEAE-Sepharose column were pooled and concentrated. The sample was applied to an Ultrogel AcA-44 column (2.5 x 112 cm) and eluted with 20 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol, 0.5 mM EGTA, 0.5 mM EDTA, and 2 mM DTT, at a flow rate of 0.5 ml/min. Fractions with high PKC activity were pooled, concentrated, and stored in glycerol (1:1) at -80^{o}C.

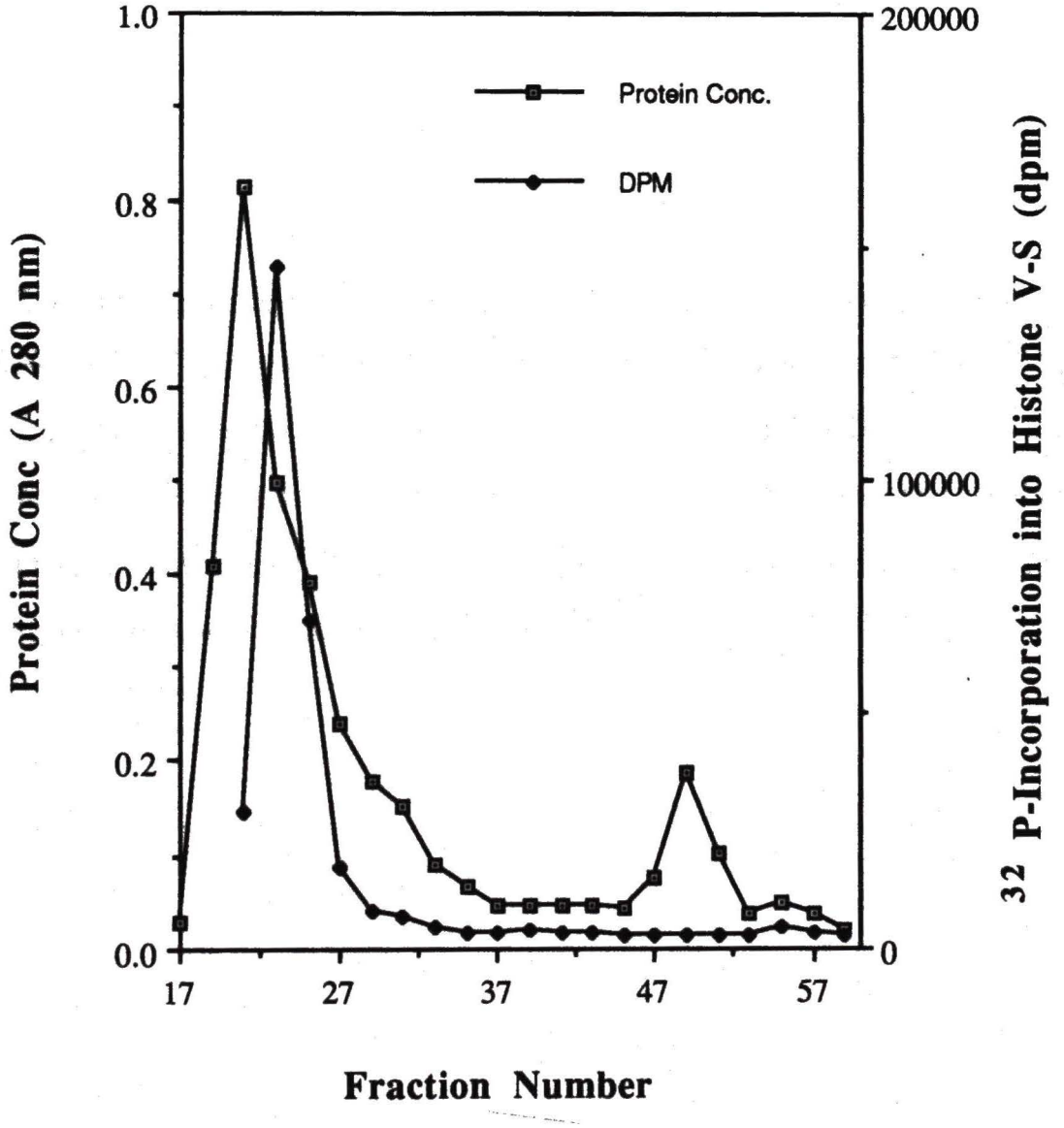

1.0
0.8
0.6
0.4
0.2
0.0
200000
100000
0
17
27
37
47
57
Protein Conc.
DPM
Protein Conc (A 280 nm)
32 P-Incorporation into Histone V-S (dpm)
Fraction Number

Fig. 7. Assay of purified rat brain PKC. Pooled fractions from AcA-44 gel filtration were assayed for PKC in the presence of [γ-^{32}P] ATP, 40 μg histone, 5 mM Mg^{2+}, 0.75 mM Ca^{2+}, 25 μg phosphatidylserine and 3 μg of OAG in 20 mM Tris-HCl, pH 7.6 (column 4). The typical protein kinase activity of the partially purified PKC was 11.7 pmol/min/μg of protein. Columns represent PKC activity in reaction mixtures lacking: histone (column 1), enzyme (column 2), or phosphatidylserine/OAG (column 3).

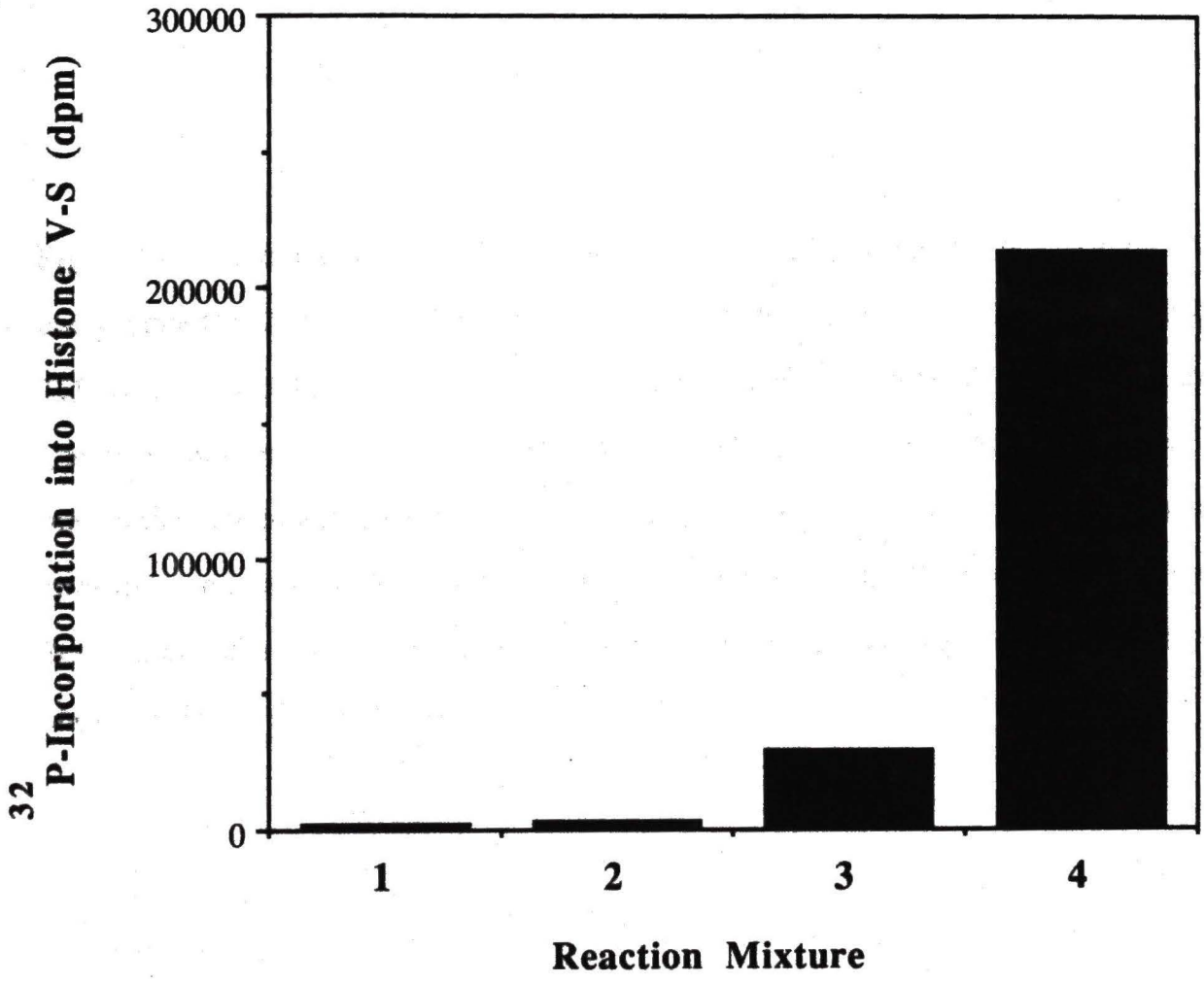
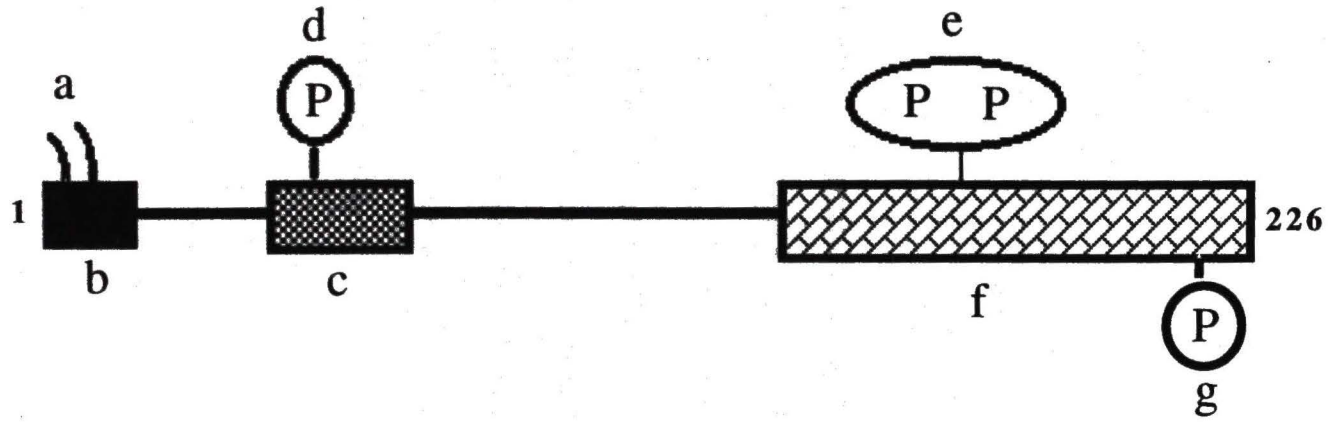

32 P-Incorporation into Histone V-S (dpm)
300000
200000
100000
0
1
2
3
4
Reaction Mixture

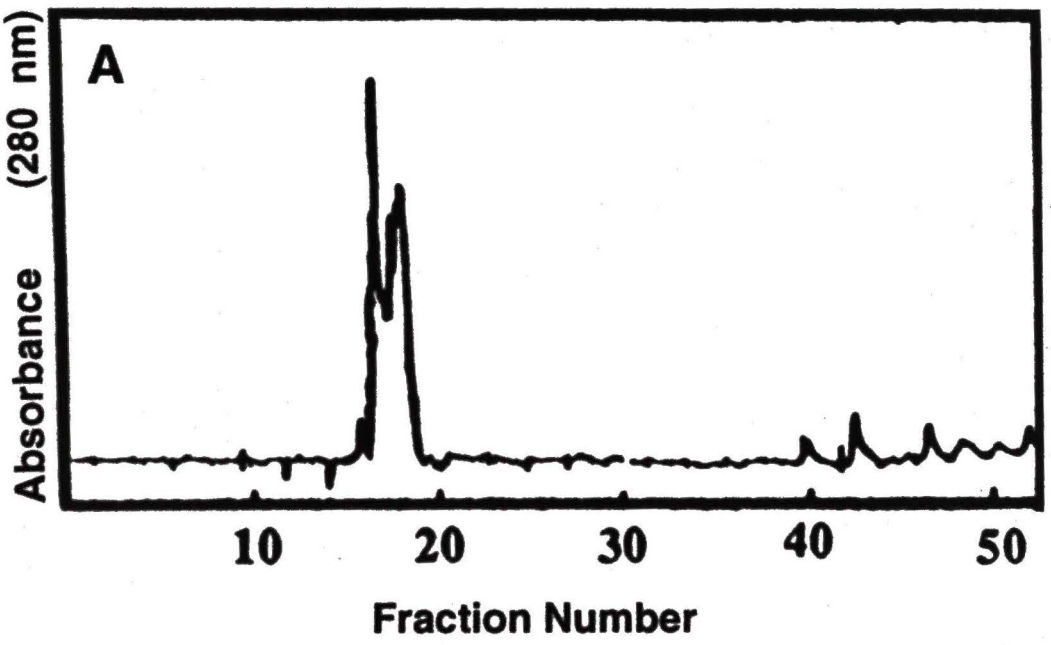
Fig. 8. Domain structure of protein F1 and synthetic peptide sequence. A 21 amino acid polypeptide (Mr 2300), that corresponds to the C-terminus of protein F1 (amino acids 205-225) was selected based on the predicted primary structure of protein F1 and synthesized. Upper diagram (modified from Apel and Storm, 1992): the domain structure of protein F1; membrane attachment sites (a), membrane targeting domain (b), calmodulin binding domain (c), established PKC phosphorylation site, Ser-41 (d), CKII phosphorylation sites, Ser-192 and Ser-193 (e), negatively charged C-terminus (f), and Ser-210, potential phosphorylation site of PKC (g).



$_{205}$AKPKES*ARQDEGKEDPEADQE$_{225}$

* Phosphorylation Site

Fig. 9. Analysis of peptide phosphorylation 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 analyzed by reversed-phase HPLC using a Waters C18 column eluted with 0-50% acetonitrile gradient in 0.1% TFA/H2O (flow rate of 1 ml/min) as described in materials and methods. (*A*) elution profile of phosphorylated F1 peptide. (*B*) Radioactivity of the fractions of HPLC elution profile of ^{32}P-phosphorylated F1 peptide.


A
Absorbance (280 nm)
10
20
30
40
50
Fraction Number

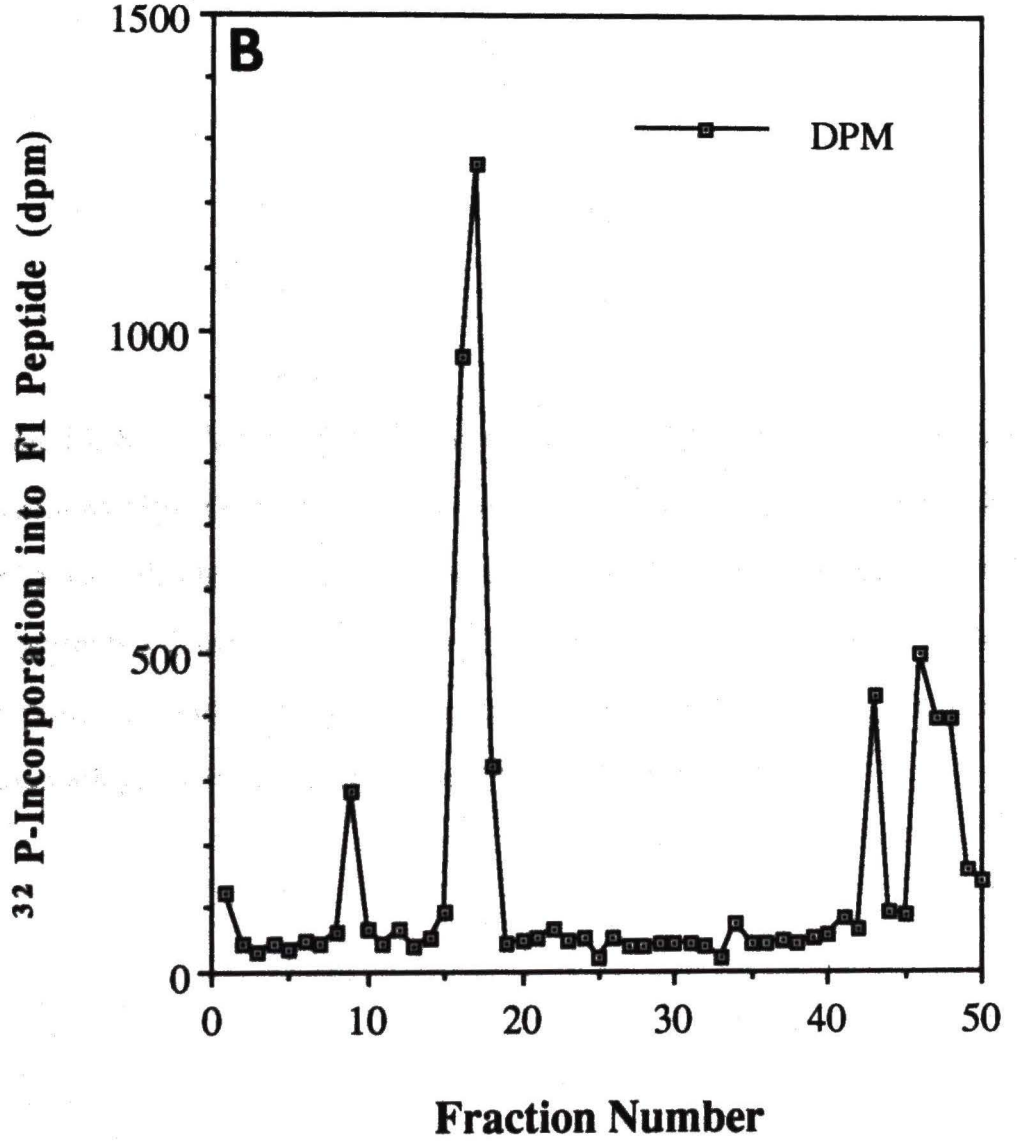

B
DPM
1500
1000
500
0
0
10
20
30
40
50
32 P-Incorporation into F1 Peptide (dpm)
Fraction Number

Fig. 10. SDS-PAGE and autoradiogram of phosphorylated F1 peptide. Radioactive fractions (16-18) from Fig. 9, were pooled, concentrated, and electrophoresed on 20% SDS-PAGE. The gel was dried and exposed to X-ray film (Kodak) at -80^{o}C. Figure 6-A 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 6-B is an autoradiogram of the phosphorylated F1 peptide (1).

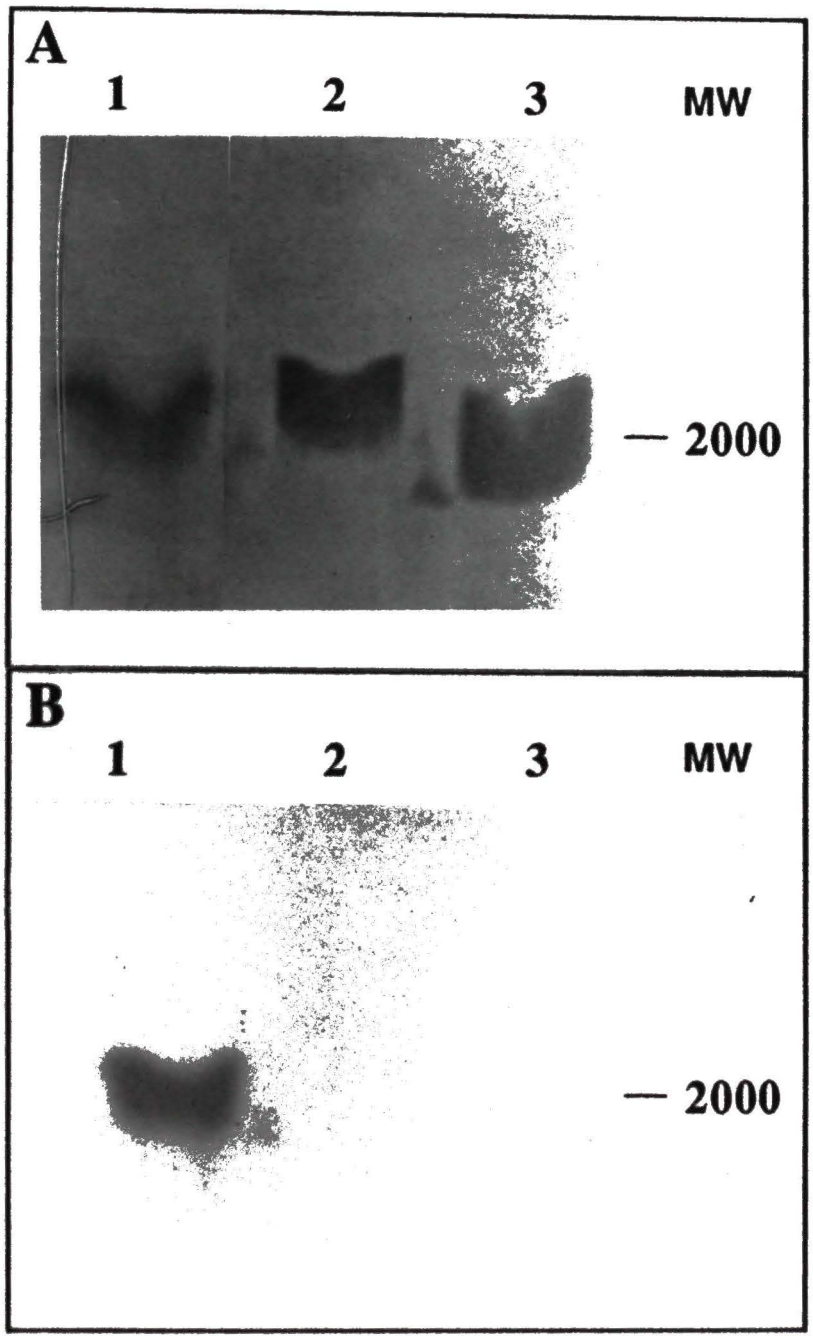

A
1
2
3
MW
— 2000
B
1
2
3
MW
— 2000

Fig. 11. Concentration-dependence of 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 phosphatidylserine, 3 µg OAG in 20 mM Tris-HCl, pH 7.6, 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 a P-81 filter. The filter was washed in acetic acid and acetone, dried and ^{32}P-incorporation quantified by liquid scintillation spectrometry.

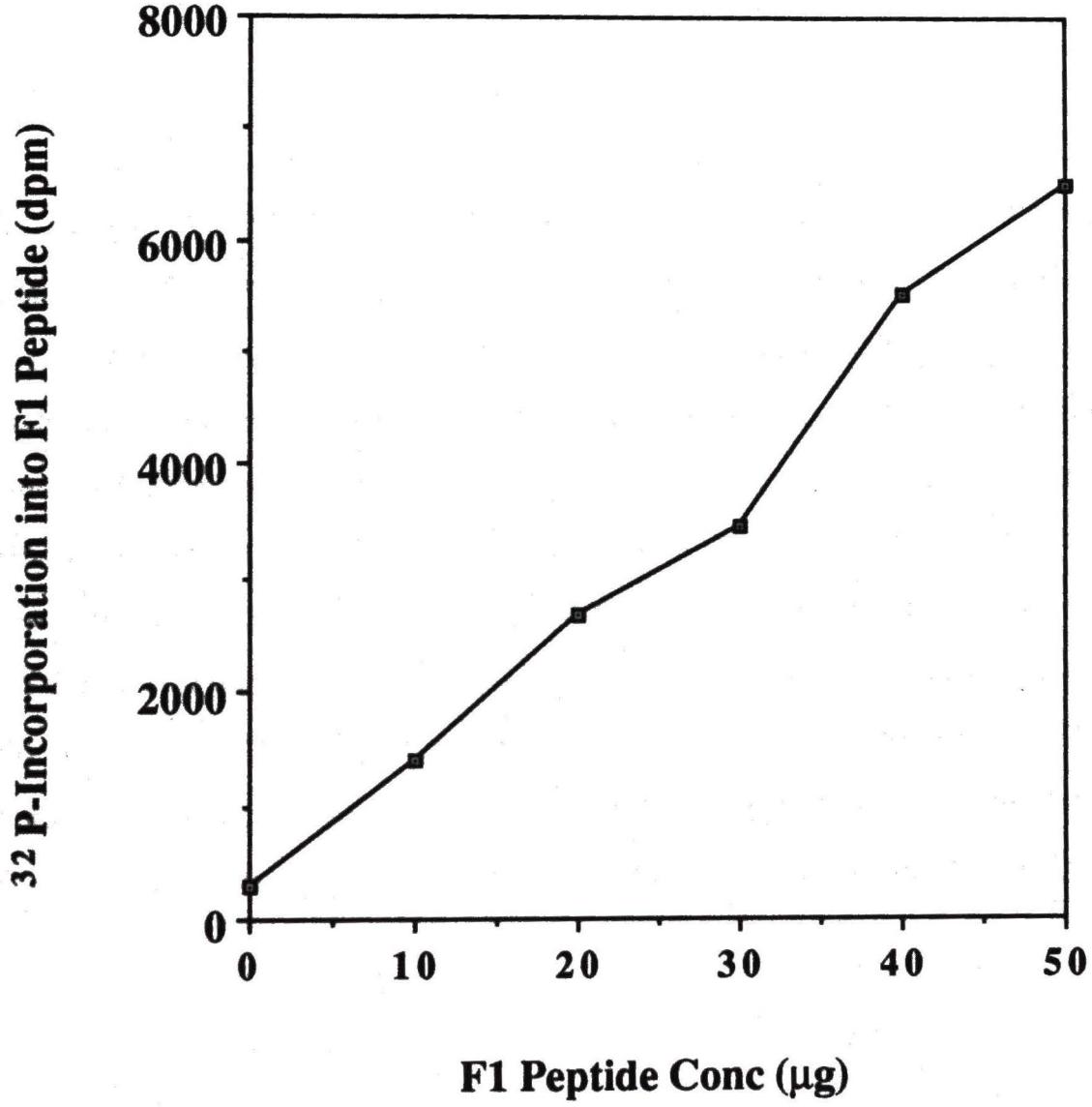

8000
6000
4000
2000
0
0
10
20
30
40
50
32 P-Incorporation into F1 Peptide (dpm)
F1 Peptide Conc (μg)

Fig. 12. SDS-PAGE and Western Blot Analysis of purified protein F1. Protein F1 obtained at different steps during purification was electrophoresed on 10% SDS-PAGE. Gels were either stained with Coomassie blue or electrophoretically transferred to nitrocellulose and immunostained with anti-protein F1 polyclonal antibodies as described in materials and methods. The molecular weight markers used were: phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soyabean trypsin inhibitor (21.5 kDa). Lanes represent Coomassie blue stained SDS-PAGE of proteins in: 40-80% ammonium sulfate precipitate (*A*), 0-30 mM (*B*), 30-75 mM (*C*), and 75-400 mM (*D*) potassium phosphate (pH 7.2) fractions eluted from hydroxylapatite column, and void volume (*E*) eluted from phenyl Sepharose column. Lane (*F*) represents immunoblotting of protein F1 purified through phenyl Sepharose column.

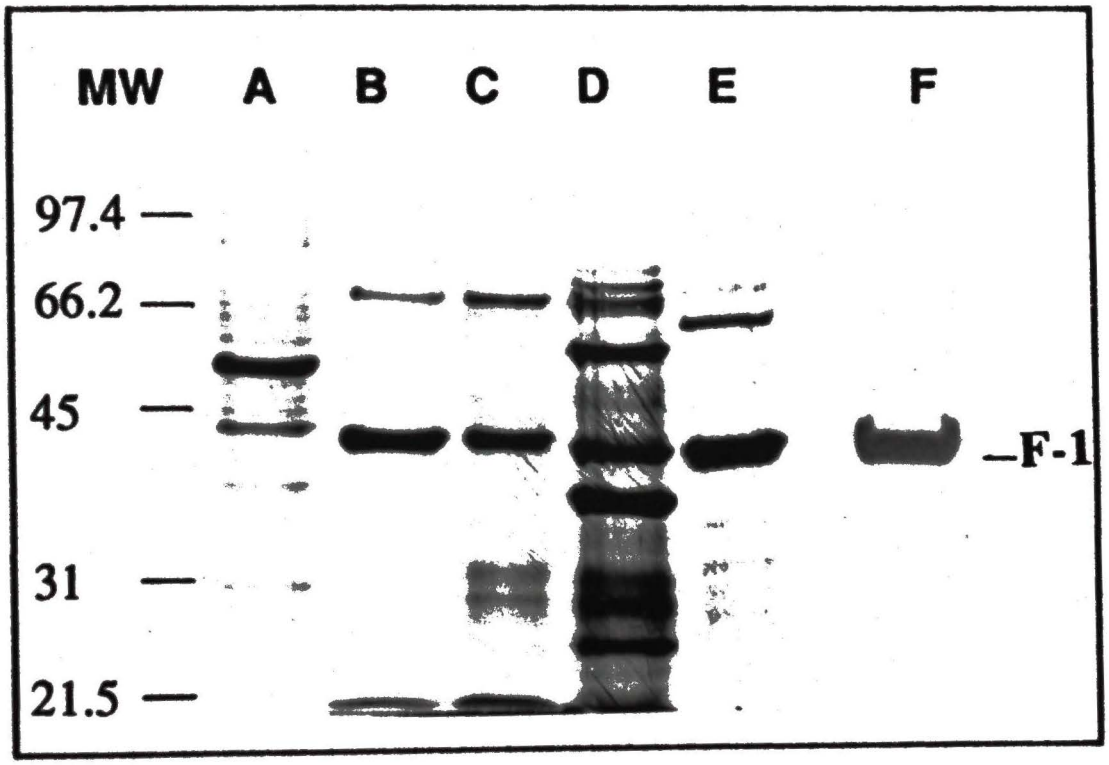

MW
A
B
C
D
E
F
97.4
66.2
45
31
21.5
F-1

Fig. 13. SDS-PAGE and autoradiogram of phosphorylated protein F1. Protein F1 in ammonium sulfate precipitate from rat brain was used as a substrate for PKC purified from the same tissue as described in materials and methods. The reaction mixture was analyzed on SDS-PAGE. The gel was then stained, dried and exposed to X-ray film. The molecular weight markers used are: phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soyabean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). (*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.

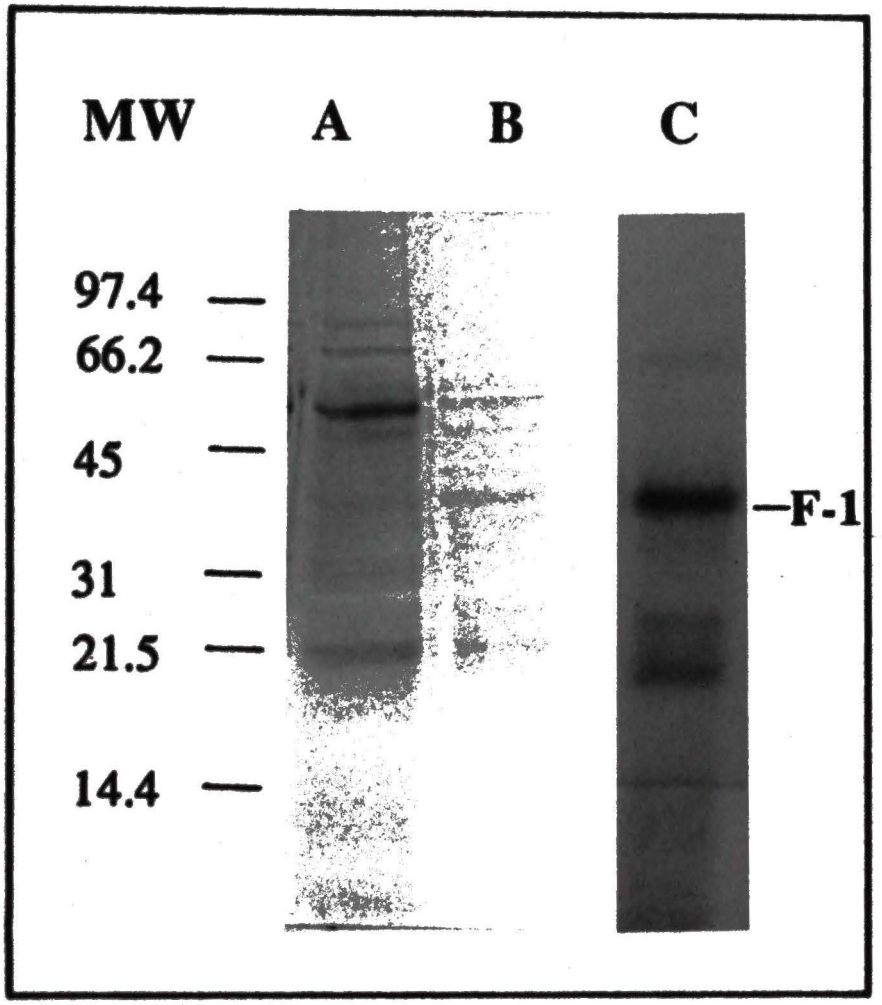

MW
A
B
C
97.4
66.2
45
31
21.5
14.4
F-1

Fig. 14. Immunoprecipitation of phosphorylated protein F1. Protein F1 in ammonium sulfate precipitate was phosphorylated by PKC in presence of [γ-^{32}P] ATP. Anti-protein F1 antibodies were added at the end of the phosphorylation and the immune complex was separated by pansorbin and the proteins were analyzed by SDS-PAGE. The gel was then stained, dried, and exposed to X-ray film. The molecular weight markers (*A*) used are: phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soyabean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). (*B*) is a Coomassie blue staining of SDS-PAGE analysis of protein F1 immunoprecipitated from partially purified brain homogenate (a strong band at Mr.~45,000 represents the heavy chain of the antibody) and (*C*) is an autoradiogram of the dried gel.

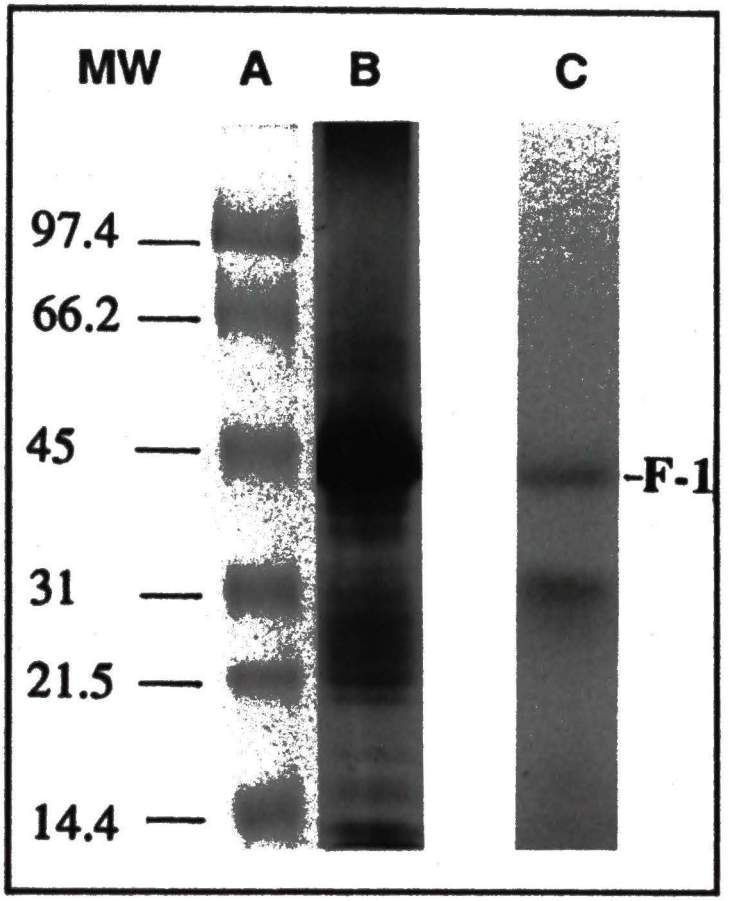

MW
A
B
C
97.4
66.2
45
31
21.5
14.4
-F-1

Fig. 15. Immunocytochemical staining of protein F1 in cultured mouse spinal neurons. Cultures of the same tissue and age (3 weeks) were treated with anti-protein F1 antibodies or rabbit IgG. (*A*) Neurons fixed with Gregory reagent and stained with Loots modified Bodian (LMB, Loots et al., 1979) stain. 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 (40 μg/ml) using StrAviGen immunostaining kit (Biogenex). Neurons showed strong reactivity to the antibodies on cell membranes and neurites. (*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 cultures on coverslips. Bar in (*A*) is 20 μm and applies to all panels.

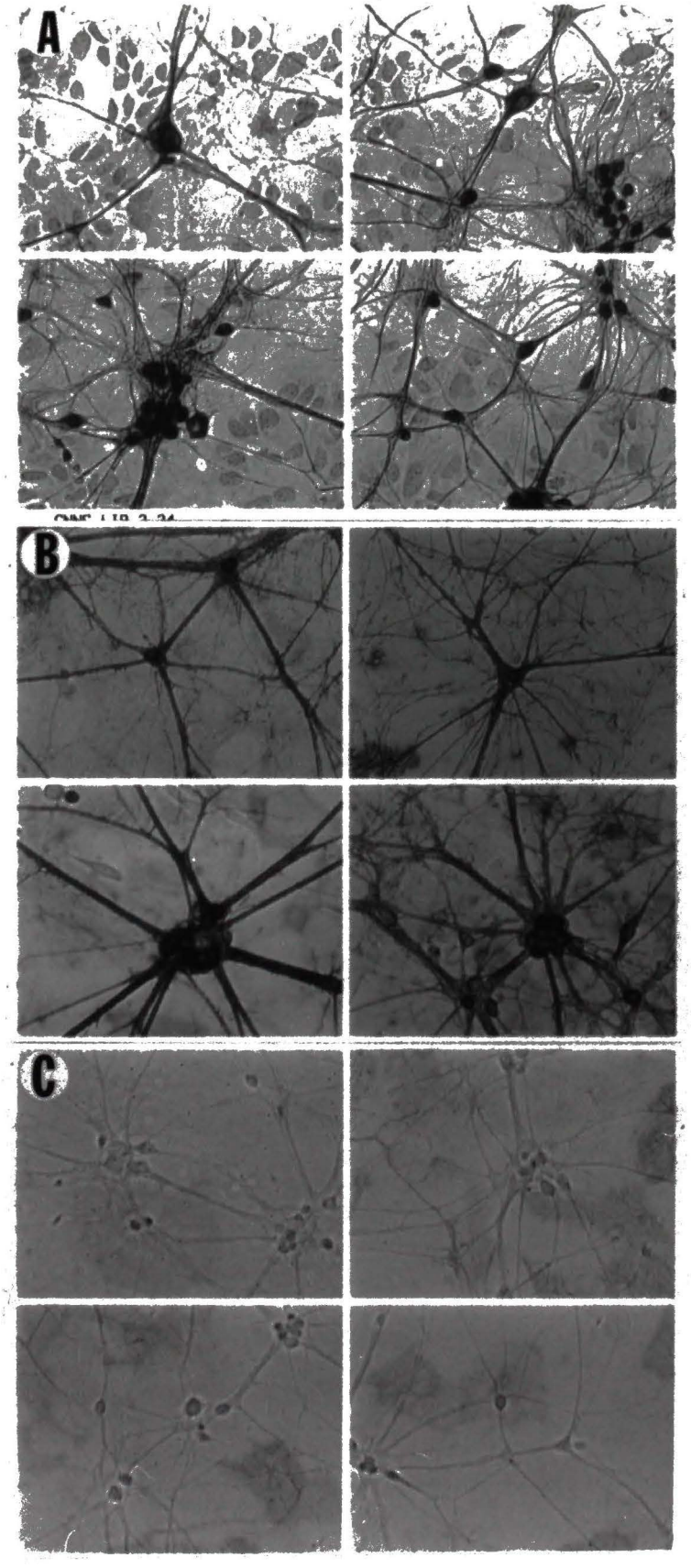
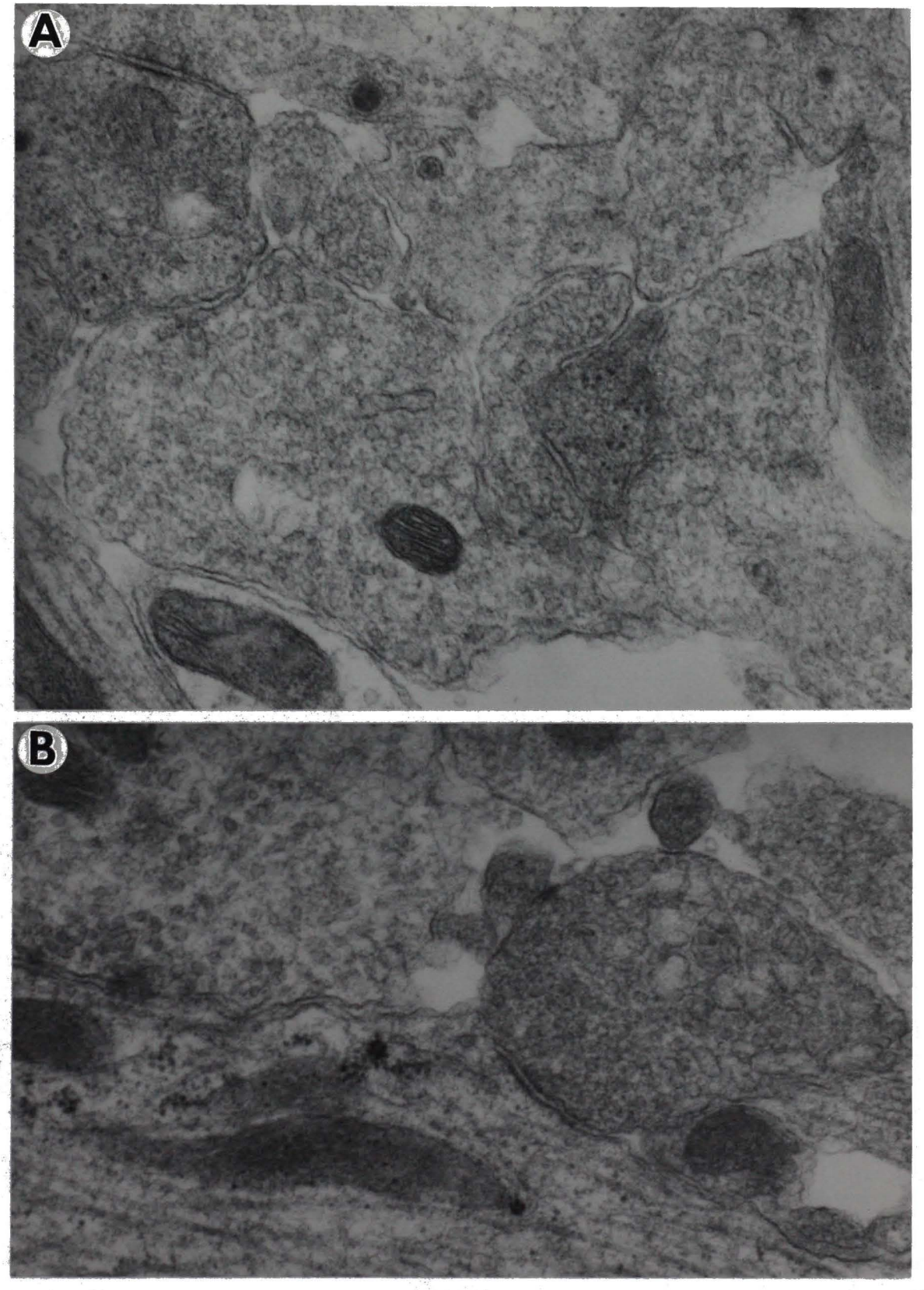
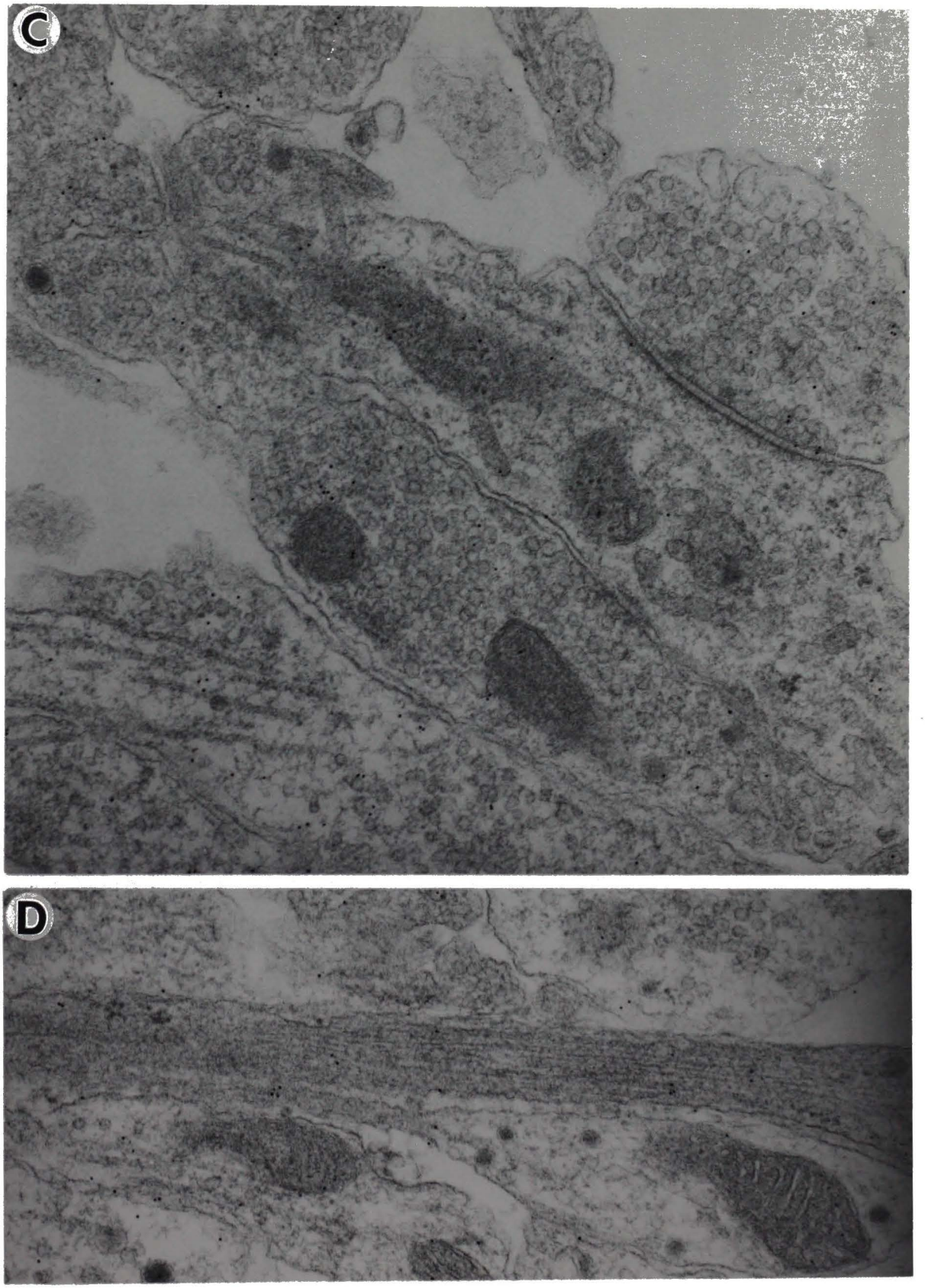

A
B
C

Fig. 16. Transmission electron micrographs of immuno-gold labeling of protein F1 in spinal neurons in cultures. Murine spinal neurons (3-4 weeks old) were fixed in 2% glutaraldehyde, 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 30 min at 4°C, postfixed, dehydrated and embedded in Epon-araldite. Longitudinal sections of neurons on nickel grids were etched, permeabilized, and blocked with 10% goat serum in BSA-Tris buffer, pH 8.0. Sections were incubated with anti-protein F1 antibodies or rabbit IgG (80 μg/ml) followed by gold-conjugated goat anti-rabbit IgG (Sigma, diluted 1:40), and finally, double stained with lead citrate and uranyl acetate. Controls included the omission of primary antibody (*A*) and the use of rabbit IgG as primary antibody (*B*) and showed no or very scarce gold labeling. Protein F1 gold labeling in synaptic bodies, an axon-like process, and on microtubules is shown in (*C*), (*D*), and (*E*) micrographs; respectively.


A
B


C
D

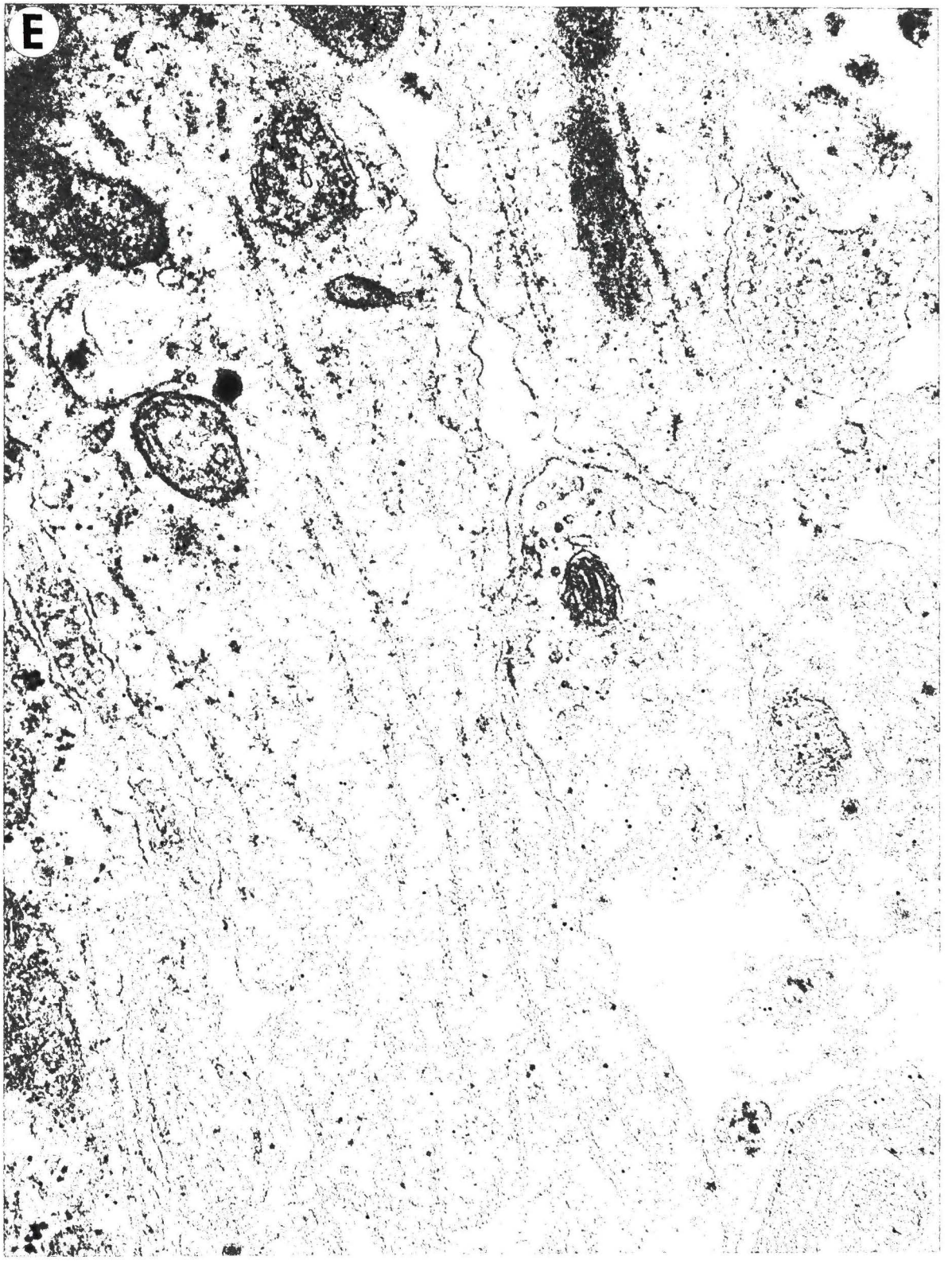

E

Fig. 17. Developmental stages of mouse spinal neurons in culture. Spinal neurons were dissociated from embryonic tissues (E-14) and plated on PDL-coated coverslips and grown in a water-saturated atmosphere of 90% air-10% CO_2 at 37°C. Cells were maintained in MEM10. (*A*) Spinal neurons at zero time in culture, (*B*) 1 hr after seeding neurons extend minor processes, (*C*) 3 hr, showing growth of a long axon-like neurite, (*D*) 7 hr, neurons extending several neurites, (*E*) 10 hr, neural networks start to form, and (*F*) 4 weeks in culture, fully established mature networks with a monolayer of neurons overlaying a carpet of glial cells. Cells in A-E were fixed in Gregory's fixative for 5 min then photographed, cells in (*F*) were fixed in Gregory's fixative then stained with LMB stain. All photographs were taken with a Zeiss microscope. Bar in (*A*) is 10 μm and applies to (*B-E*). Bar in (*F*) is 20 μm.

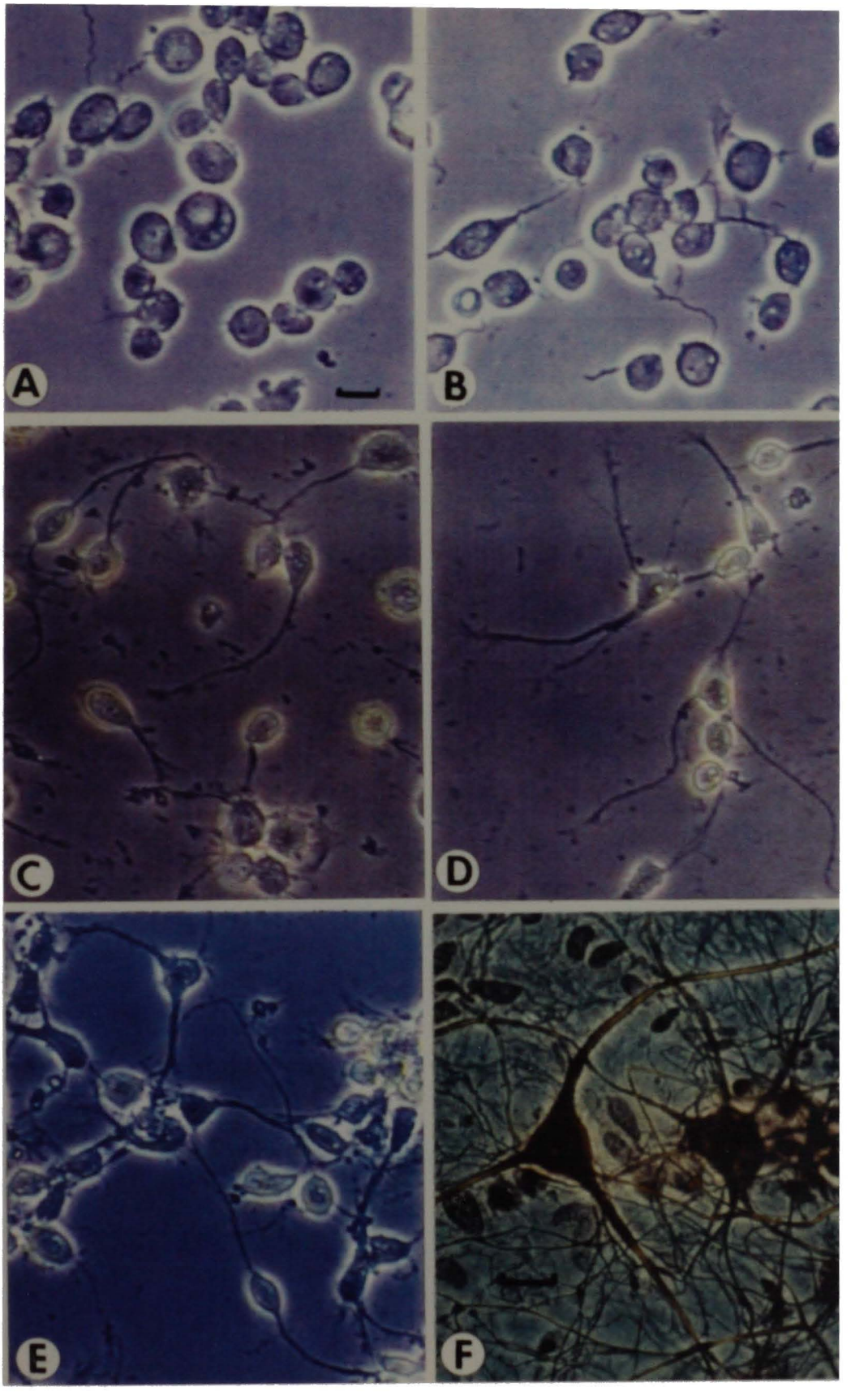
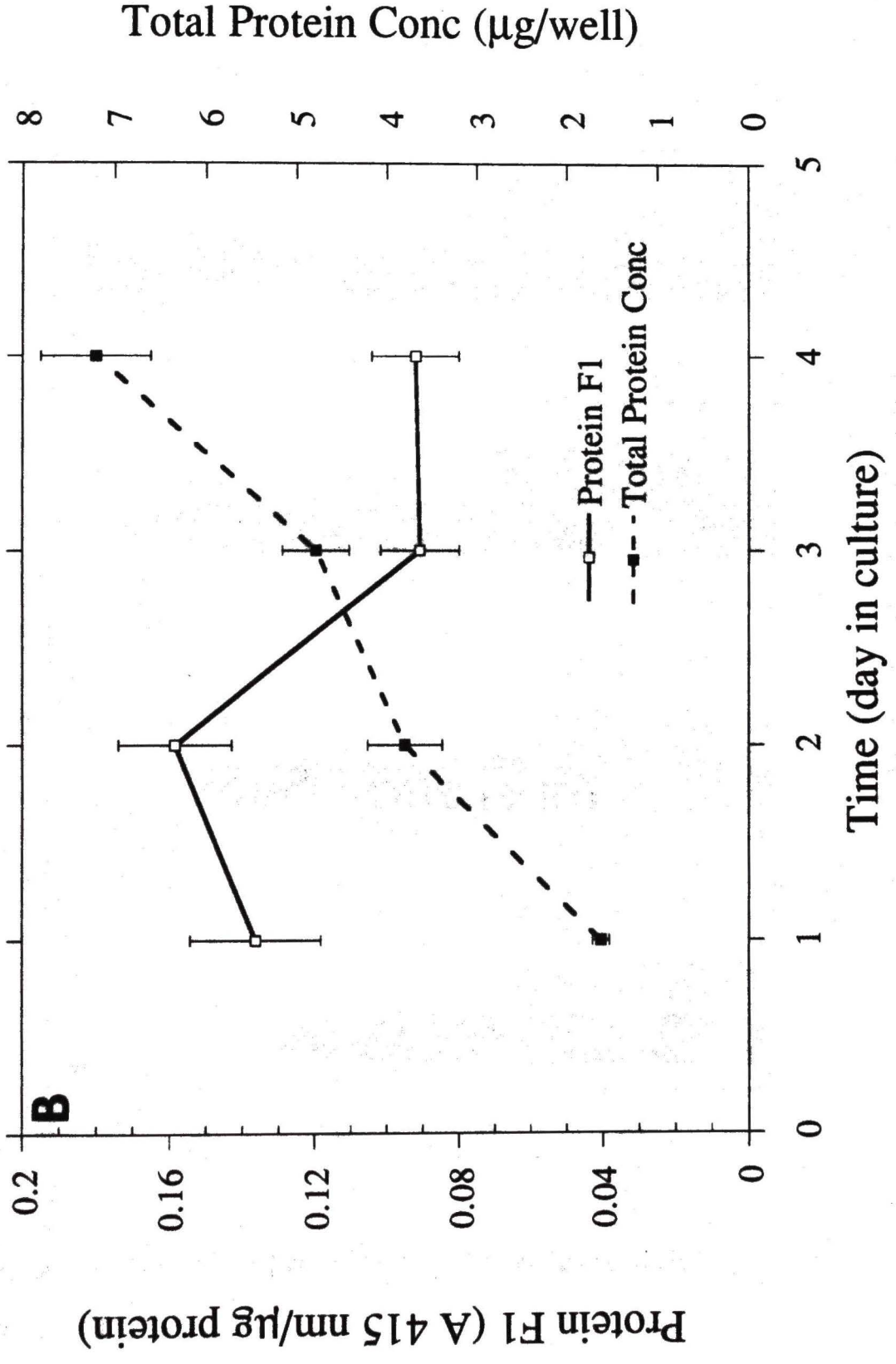

A
B
C
D
E
F

Fig. 18. ELISA assay of protein F1 levels in spinal neural networks *in vitro*. Mouse spinal neurons derived from 13-14 day old embryonic tissue were seeded in 96-well plates coated with laminin and PDL. At each time point, cultures were fixed in 4% paraformaldehyde in PBS, pH 7.4, and then stored at 4°C till a complete set was ready for further processing. Cultures were rinsed, permeabilized, and blocked with 10% goat serum in PBS for 60 min at room temperature. Cells were then reacted with either anti-protein F1 antibodies or pre-immune rabbit serum (4 μg/ml) for 60 min at room temperature, rinsed, and treated with goat anti-rabbit-HRP conjugate (Bio-Rad, 1:500) for 60 min at room temperature. Substrate solution was then added and the reaction was terminated after 15 min with 2% oxalic acid. Plates were read using a Bio-Rad plate reader. (*A*) ELISA assay of protein F1 in cultures over a 35 day period after seeding, (*B*) ELISA assay of protein F1 in cultures over a 4 day period, and (*C*) assay specificity. Data points represent the mean from readings of 9 separate wells. Error bars indicate standard deviation of the mean.

A
Protein F1 (A 415 nm/µg protein)
Total Protein Conc (µg/well)
Time (day in culture)
Protein F1
Total Protein Conc
0.1
0.08
0.06
0.04
0.02
0
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
40


Total Protein Conc (µg/well)
Protein F1 (A 415 nm/µg protein)
Time (day in culture)
Protein F1
Total Protein Conc
B
0.2
0.16
0.12
0.08
0.04
0
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5

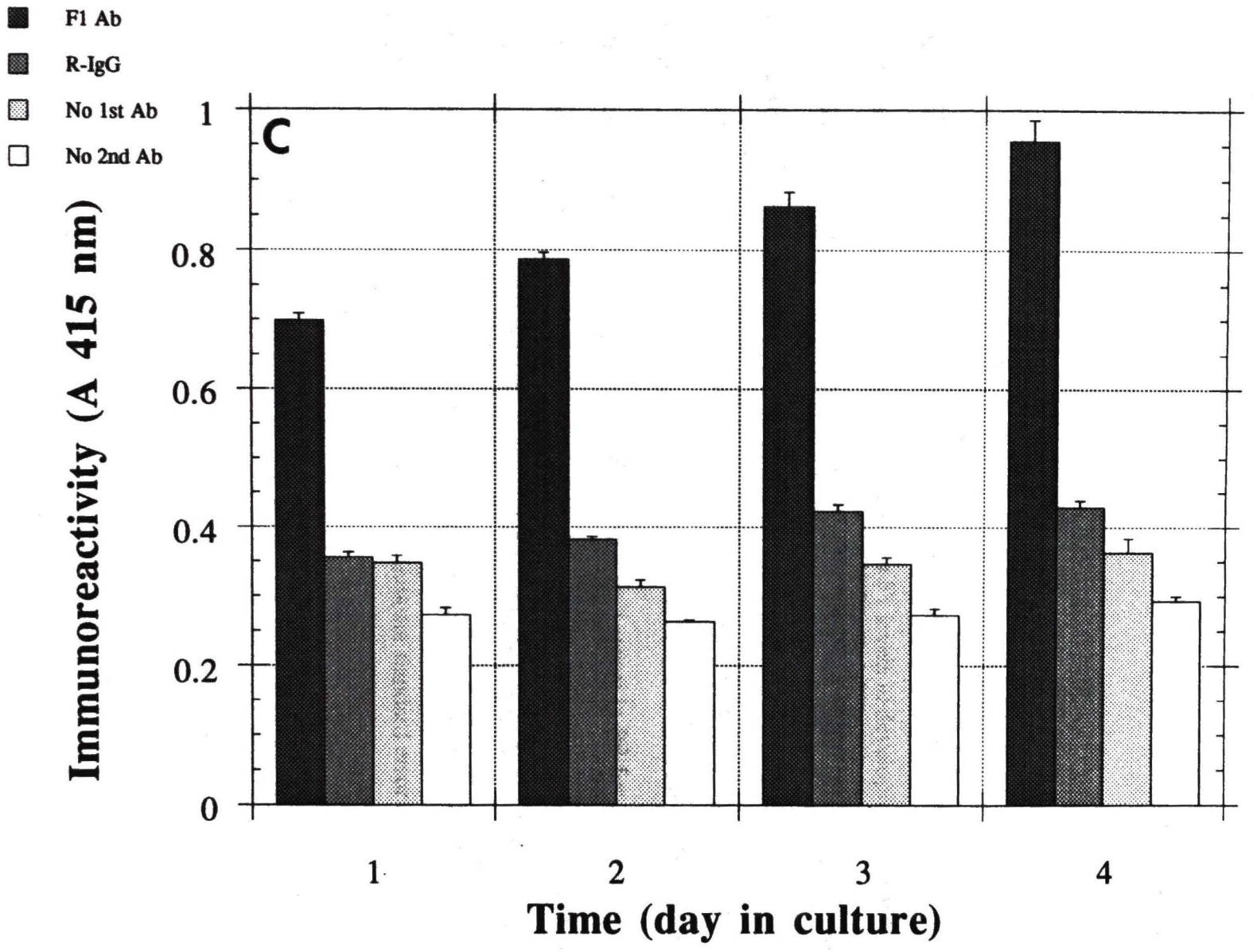
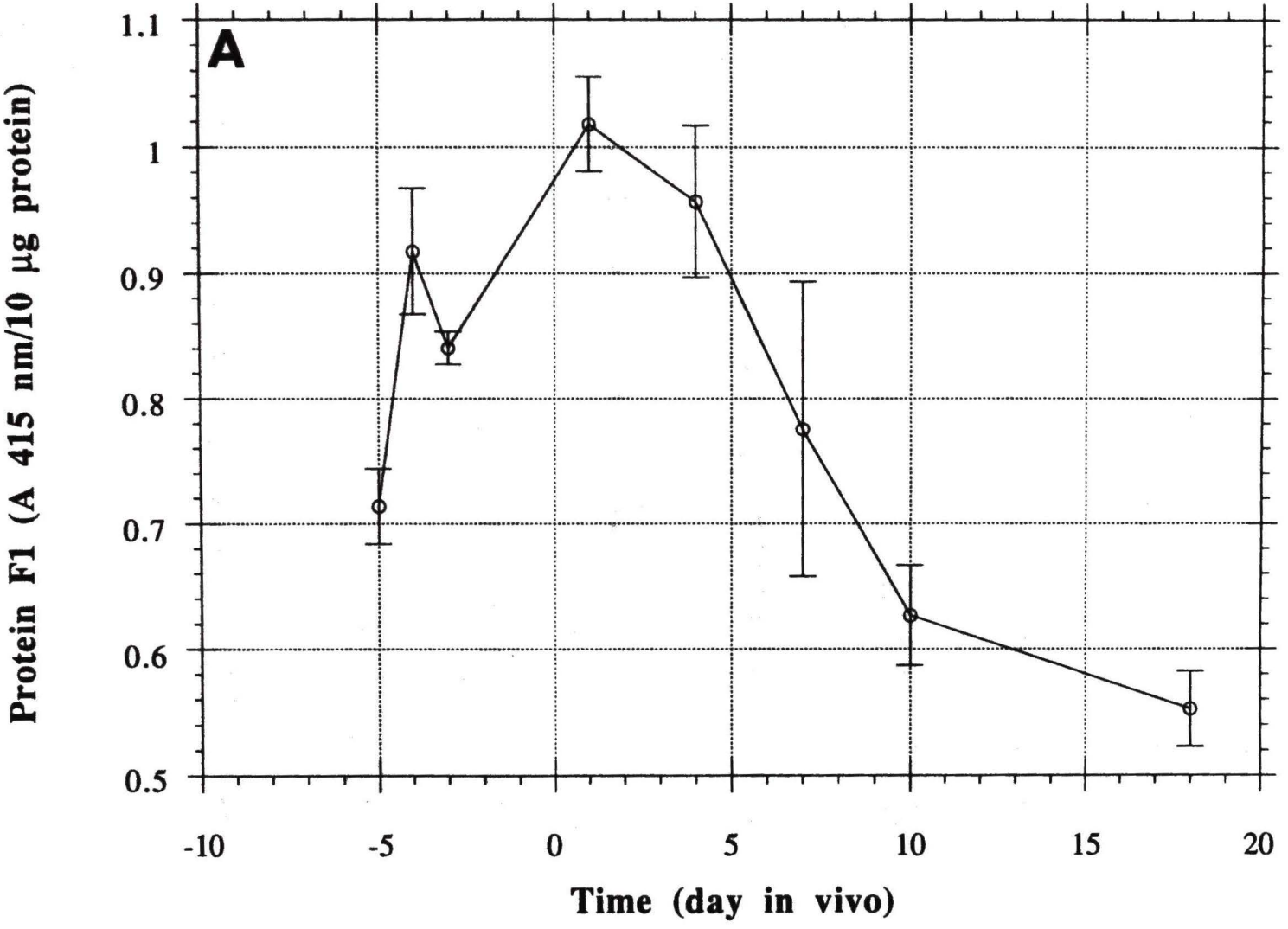

F1 Ab
R-IgG
No 1st Ab
No 2nd Ab
C
Immunoreactivity (A 415 nm)
1
0.8
0.6
0.4
0.2
0
1
2
3
4
Time (day in culture)

Fig. 19. ELISA assay of protein F1 levels in mammalian spinal cords. An enzyme-linked immunoadsorbent assay was used to estimate protein F1 levels in developing murine spinal tissues. Spinal tissues were homogenized in 50 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl, 5 mM EDTA, 2% Triton X-100, and several anti-proteases. The homogenates were centrifuged at 17,000 x g for 15 min at 4°C and the supernatants were added to a Maxisorp 96-well plate and incubated at 37°C for 3 hr. The wells were then washed in 0.1 M PB then blocked with 10% normal goat serum in PB for 1 hr and assayed for protein F1 content as described in materials and methods. The time points -5, -4, and -3 represent spinal tissues collected from mice of the following ages: E-15, E-16, and E-17. Zero represents birth (E-20). Fig. 19*A* shows protein F1 levels in spinal tissues *in vivo*. Fig. 19*B* describes the assay specificity. Error bars indicate standard error of the mean.


A
Protein F1 (A 415 nm/10 µg protein)
1.1
1
0.9
0.8
0.7
0.6
0.5
-10
-5
0
5
10
15
20
Time (day in vivo)

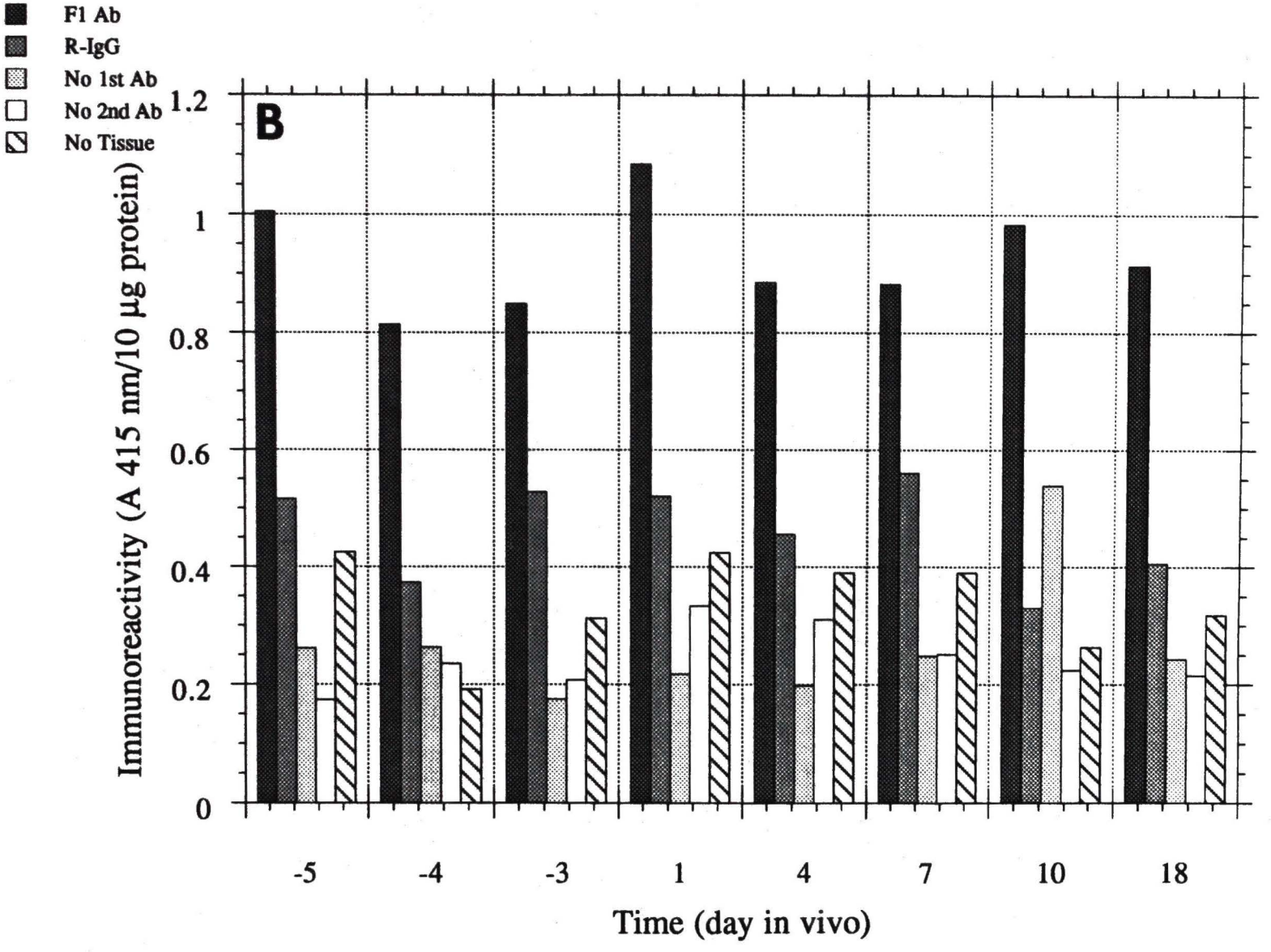

F1 Ab
R-IgG
No 1st Ab
No 2nd Ab
No Tissue
B
Immunoreactivity (A 415 nm/10 µg protein)
1.2
1
0.8
0.6
0.4
0.2
0
-5
-4
-3
1
4
7
10
18
Time (day in vivo)

Fig. 20. Effect of direct application of antibodies on spinal neurons in cultures. Anti-protein F1 antibodies were applied to spinal neurons (400 μg/ml) 1 day and again 3, and 5 days after plating on laminin and PDL substrata. The upper panels display massive aggregation of spinal neurons (4 weeks old) treated with anti-protein F1 antibodies. The effect was observed in 3 different cultures and to a lesser extent in 2 other cultures treated with 160 μg/ml of anti-protein F1 antibodies. The lower panels show control cultures treated with equal volume of PBS containing no antibodies. Cultures were fixed in Gregory's fixative and stained with LMB staining procedure. Photographs were taken with a Zeiss microscope and a Sony printer and represent different cultures. Bar is 10 μm.

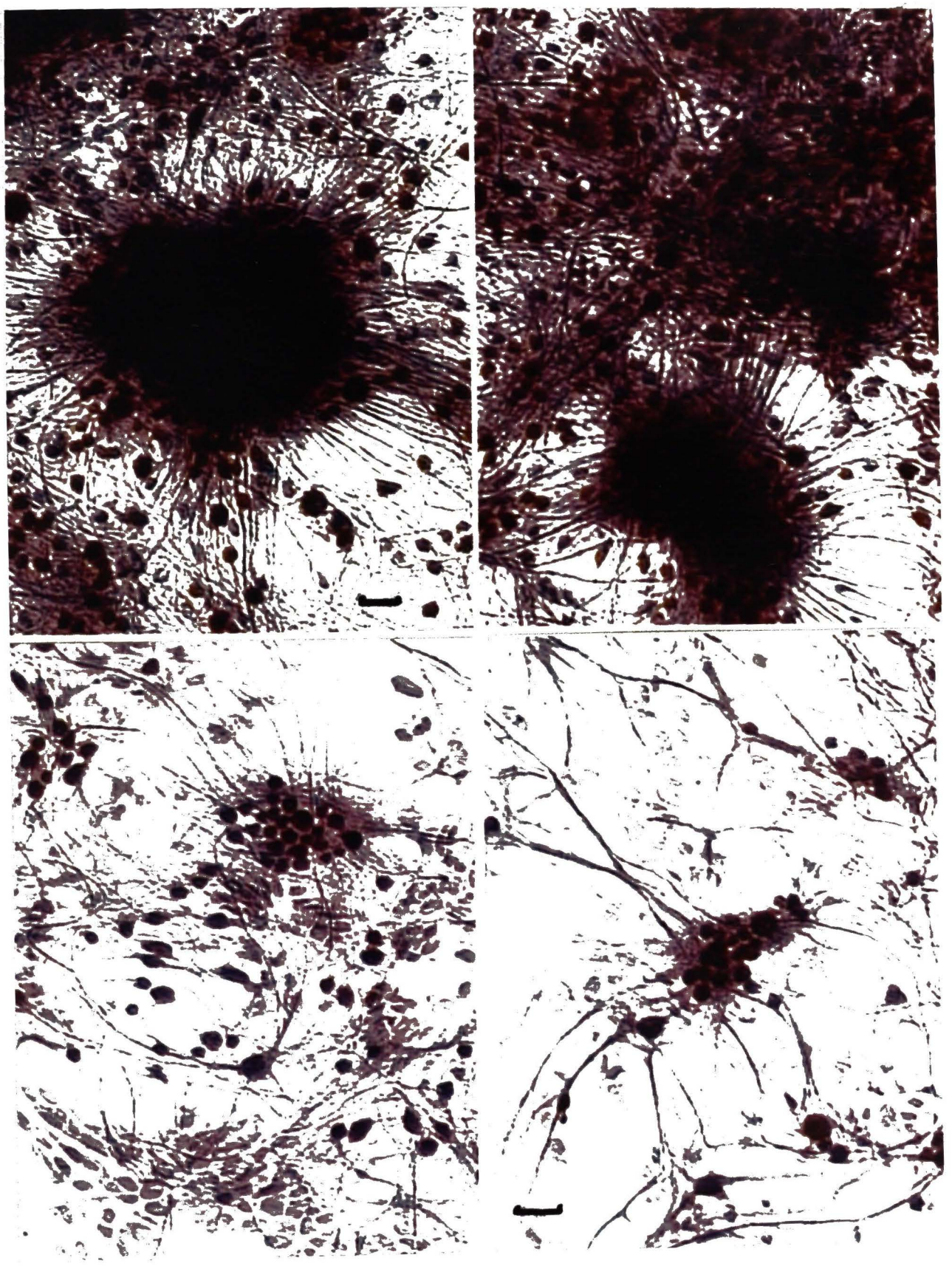


Fig. 21. Effect of IgG molecules on the electrical activity of neural networks. Anti-protein F1 antibodies, rabbit serum, and mouse serum were purified on protein A column to isolate IgG molecules. Purified IgG in PBS, pH 7.4, were applied to spontaneously active spinal neural network (55 days) seeded on multimicroelectrode plate and maintained in MEM (*A*). All IgG treatments at 400 μg/ml resulted in a decrease in the spontaneous activity of the culture (*B*) and full shut down of the activity after 10-15 min of the IgG application (*C*). The electrical activity was rapidly recovered upon medium change to MEM (arrow in *D*). The application of the carrier medium (PBS) did not have any effect on the activity. (bar: 1 min).

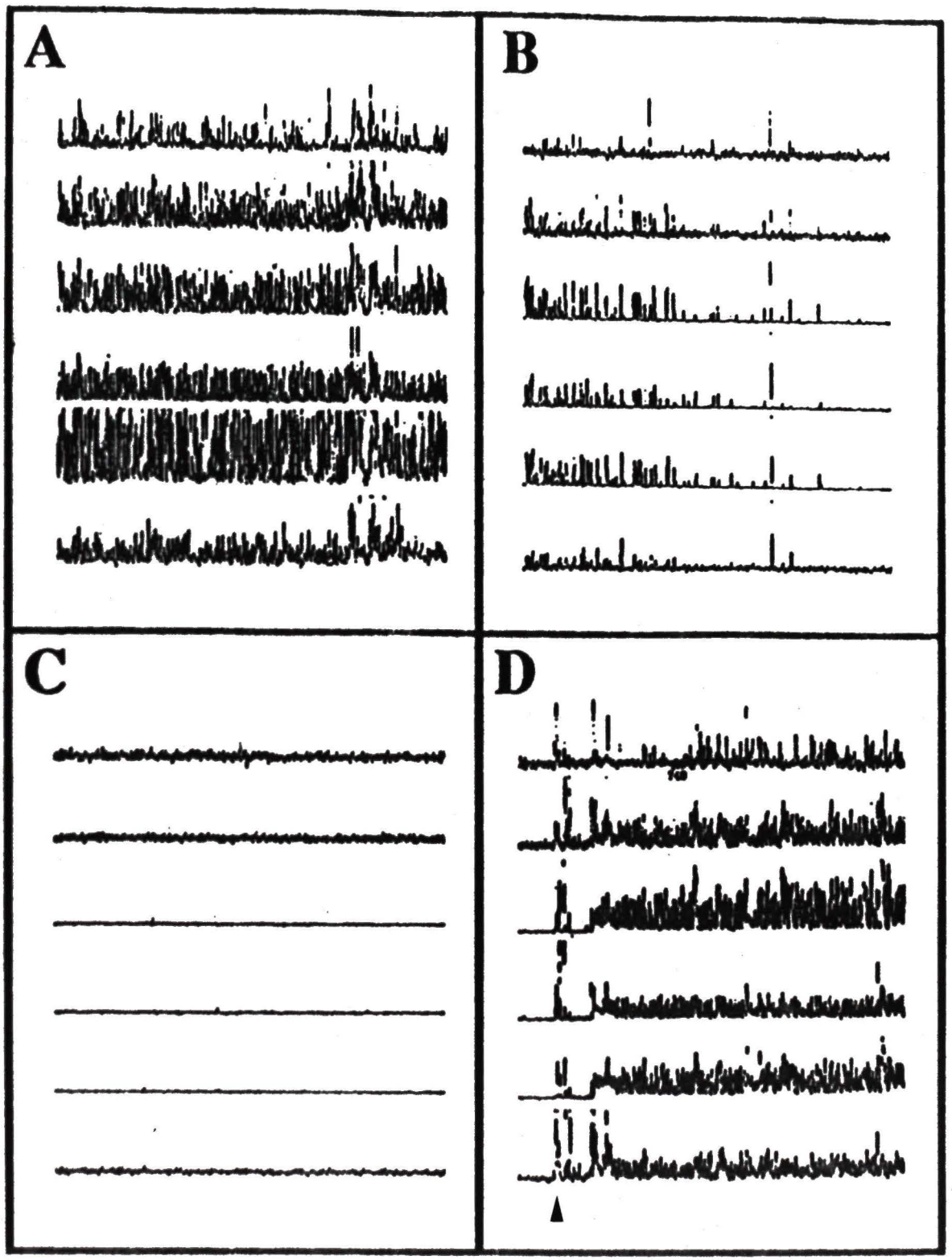

A
B
C
D

Fig. 22. Intracellular delivery of anti-protein F1 antibodies. Mature spinal neurons (3-4 weeks) were rinsed with MEM then treated with 1 ml of prewarmed PBS containing 1.2 M glycerol, pH 7.3, then incubated at 0°C for 10 min. L-α-Lysophosphatidylcholine (LPC; 40 μg/ml) was added and cultures were further incubated for 8 min. Anti-protein F1 antibodies diluted in MEM (200 μg/ml) were added to the cultures, whereas control cultures received 1 ml of MEM. Cultures were incubated at 37°C for 10 min then 1 ml of MEM containing 10% normal horse serum was added. Cultures were incubated for an additional 4 hr after which cells were fixed and intracellular delivery of the antibodies was assessed by immunocytochemistry. (*A*, *B*) and (*C*, *D*) show neurons permeabilized in presence and absence of anti-protein F1 antibodies, respectively. Background non-specific staining of the glial carpet in both panels was observed. Photographs were taken with a Zeiss microscope and represent different cultures. Bar is 10 μm.

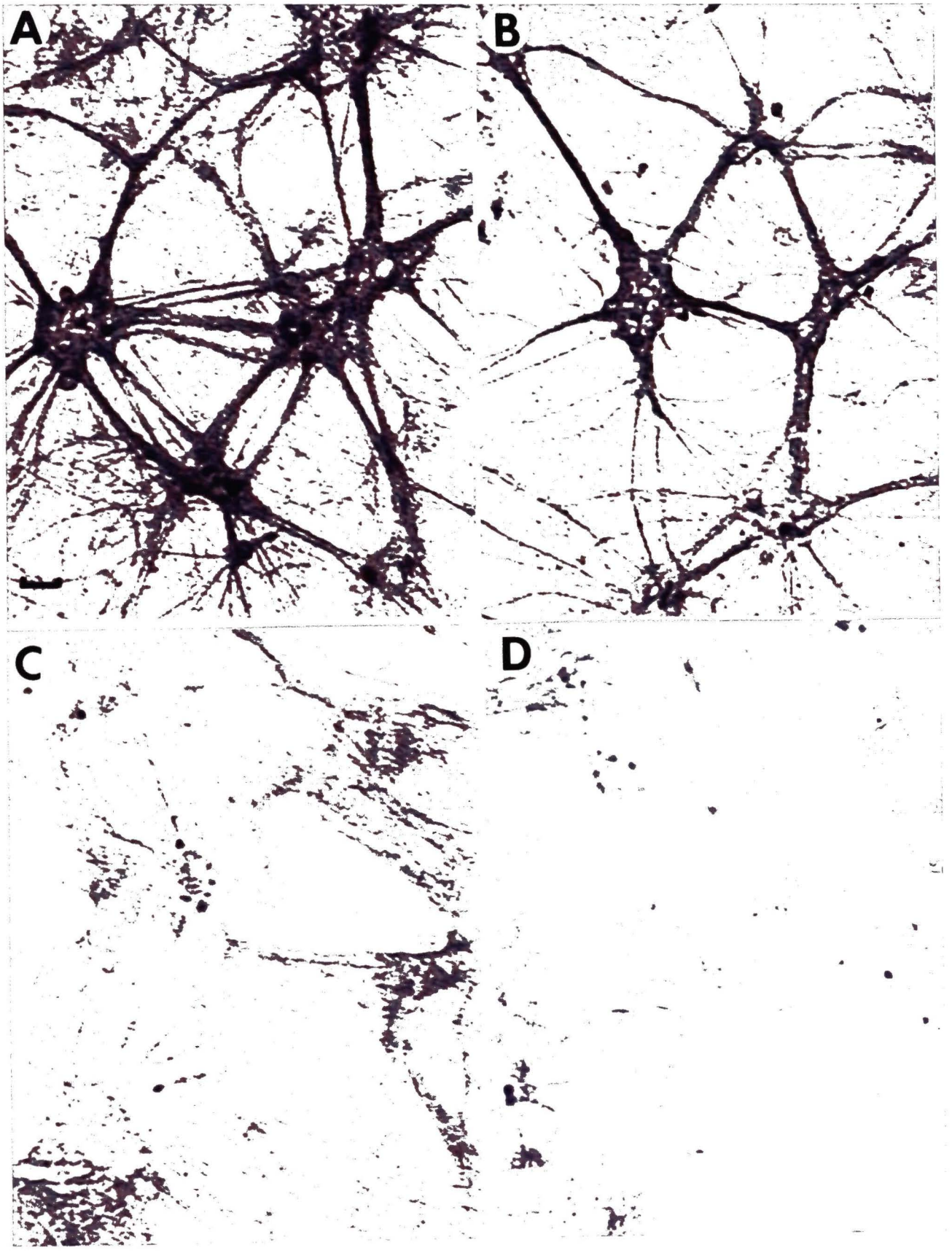

A
B
C
D

Fig. 23. Interaction of positively charged liposomes with spinal neurons. (*A*): Phase contrast view of multipolar neuron after 2 hr exposure to rhodamine-labeled liposomes (300 μM total phospholipid) and subsequent fixation. (*B*): Smooth, uniform fluorescence of somata and neurites and reduced labeling of the glial carpet. Arrows point to cell debris which is also heavily stained with rhodamine. Diameter of the large neuron in center is 25 μm.

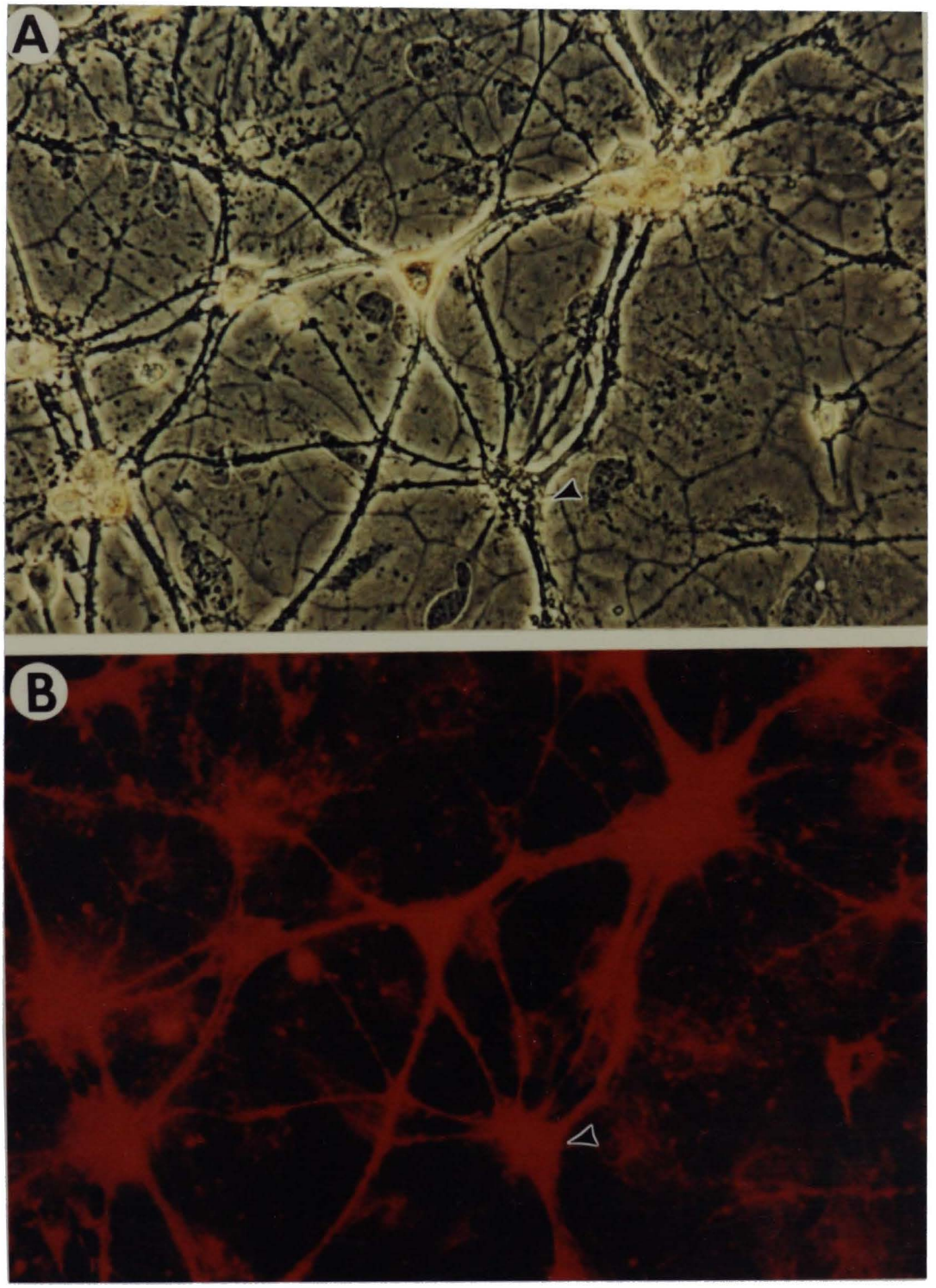
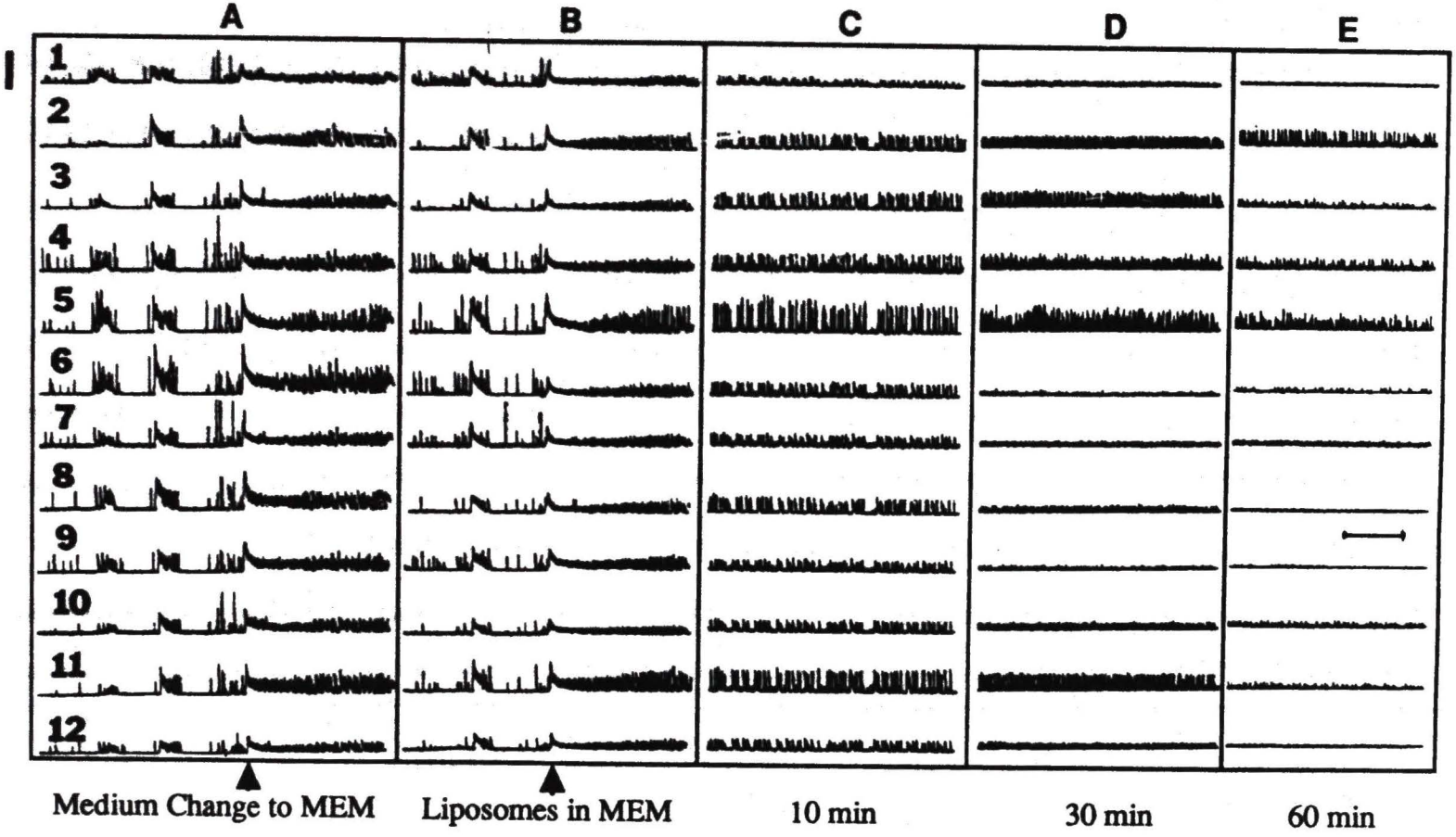

A
B

Fig. 24. Effect of liposome application on the spontaneous electrical activity of spinal neural networks. In Fig. 24.I, twelve channel recording of integrated data showing effects of switching from normal medium to MEM (*A*, arrow), the addition of liposomes in MEM (*B*, arrow), and the gradual loss of activity (*D* & *E*) 30 and 60 min after liposome application. (bar: 1 min; liposome concentration: 300 μM total phospholipid). Fig. 24.II, shows retention of spontaneous network activity over a 45 min period at liposome concentrations of 150 μM total phospholipid. (*A*) Activity in the native state, (*B*) activity in MEM, (*C*) activity upon adding liposomes in MEM, and (*D*) shows decrease in the magnitude of action potential after 45 min of liposome application. (*E*) Activity recorded 2 min after changing from liposome MEM to the original conditioned medium. (bar: 1 min).


A
B
C
D
E
1
2
3
4
5
6
7
8
9
10
11
12
Medium Change to MEM
Liposomes in MEM
10 min
30 min
60 min

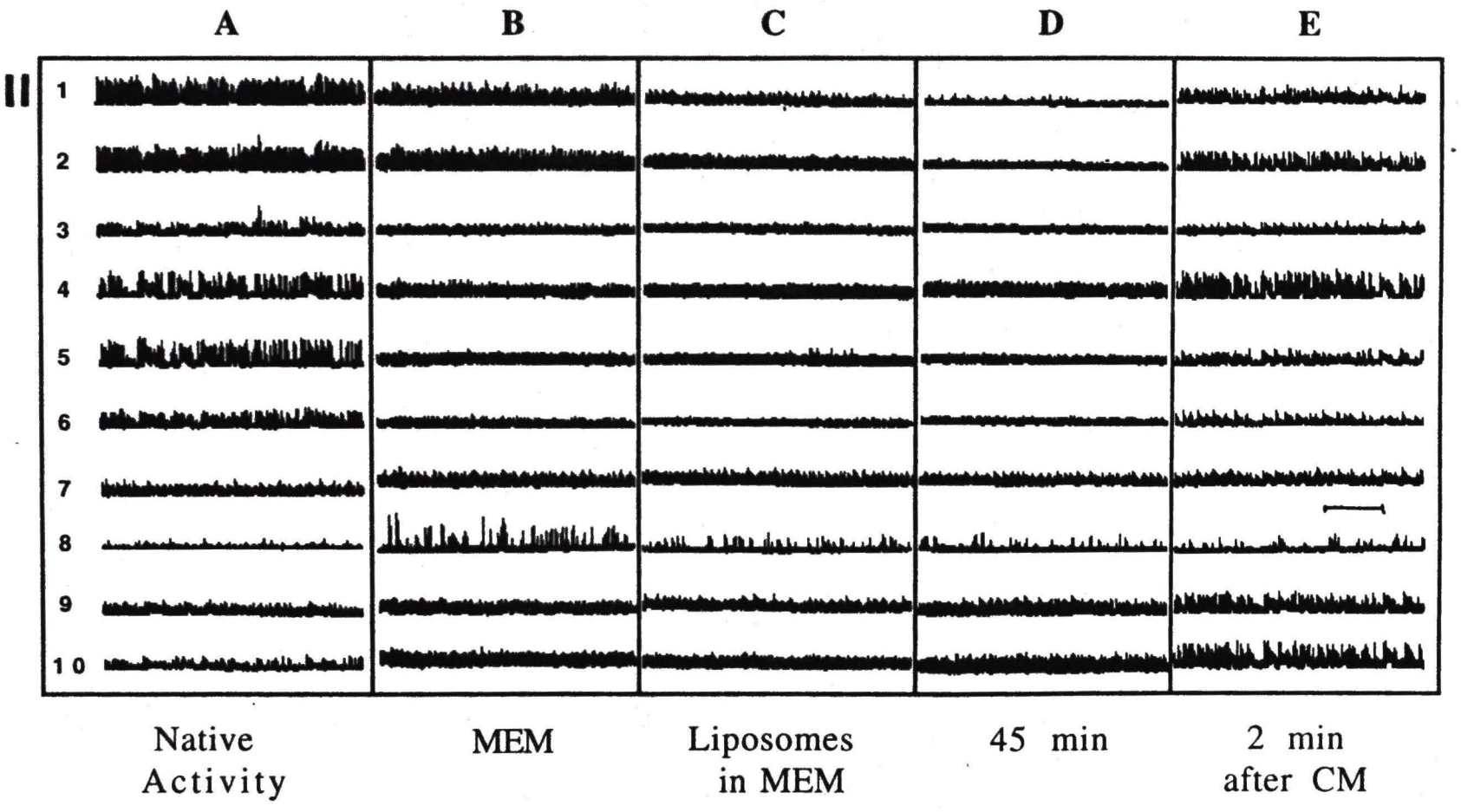
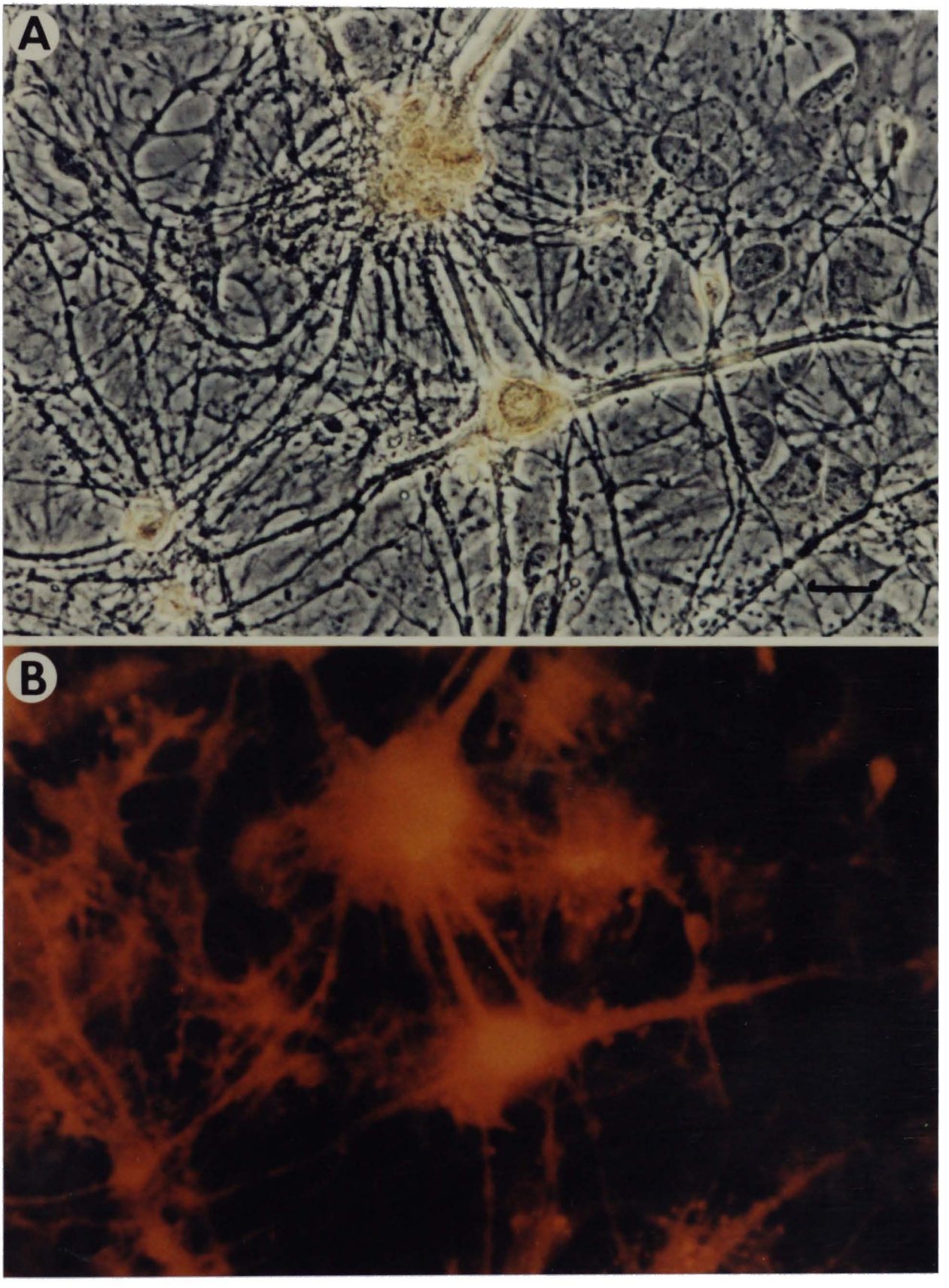

A
B
C
D
E
II
1
2
3
4
5
6
7
8
9
10
Native Activity
MEM
Liposomes in MEM
45 min
2 min after CM

Fig. 25. Interaction of liposomes containing anti-protein F1 antibodies with cultured spinal neurons. Rhodamine-labeled positively charged liposomes (dioleoylphosphatidyl-ethanolamine and dioleoyltrimethylammonium propane, 2:1) containing anti-protein F1 antibodies or rabbit IgG were tested for their interaction with spinal neurons grown *in vitro*. Liposomes (0.9 μM total phospholipids, containing 90 μg of antibodies) were incubated with 4 weeks-old (*A*, *B*) or 4 days-old (*C*, *D*) neurons in serum-free MEM for 45 min at 37°C. After incubation, cells were rinsed and fixed in Gregory's fixative then examined under phase (*A*, *C*) and fluorescence (*B*, *D*) microscopy with a 546 nm excitation and a 590 nm barrier filter (Zeiss, FT580). Photographs were taken with 35 mm Kodak Gold-1600 film. Uniform labeling of neurons, preferential labeling of neurons over glia, and absence of cytotoxicity after liposome treatment, were observed in both young and mature cultures examined. Bar in (*A*, *B*) is 20 μm and bar in (*C*, *D*) is 25 μm.


A
B

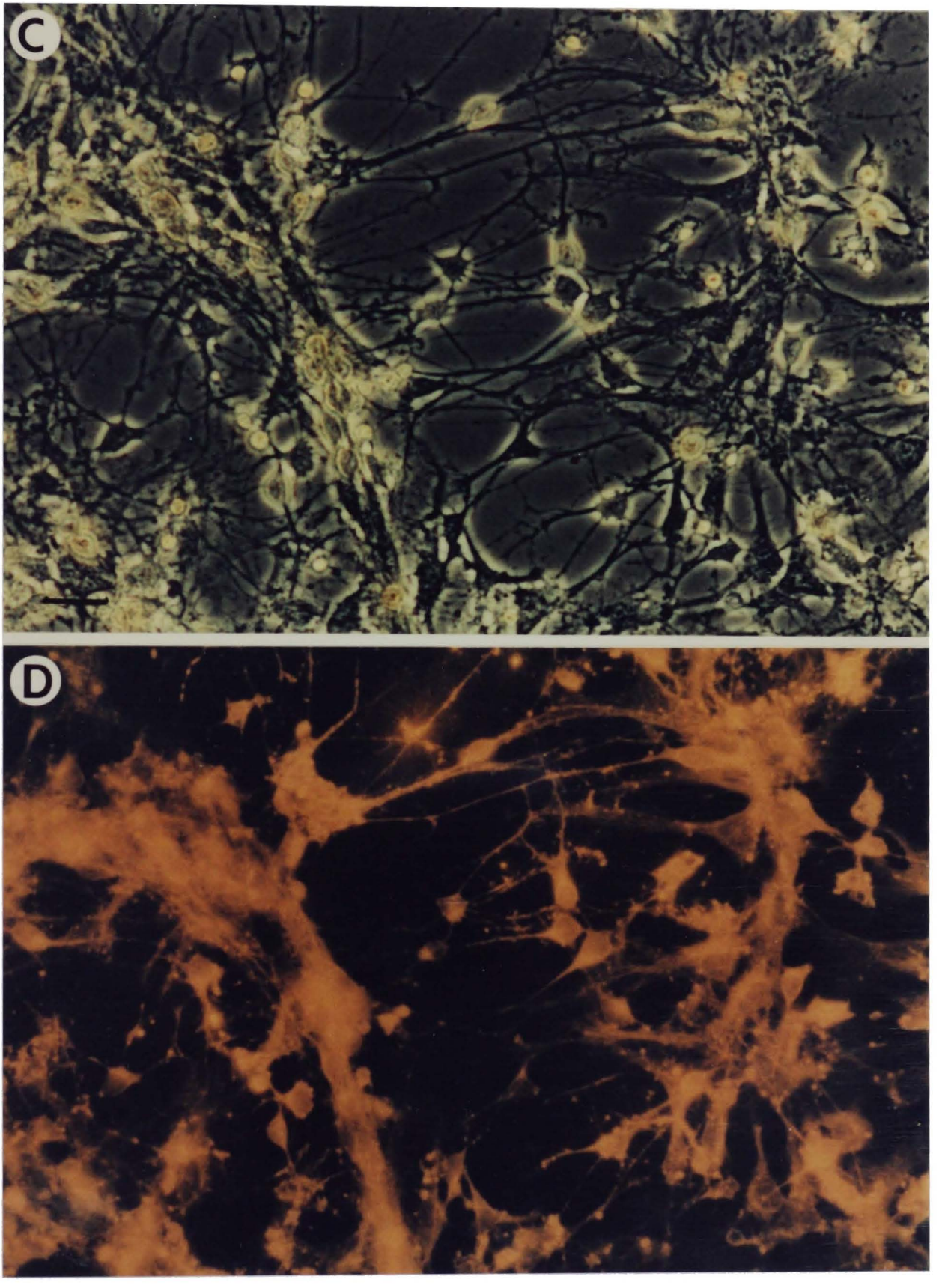
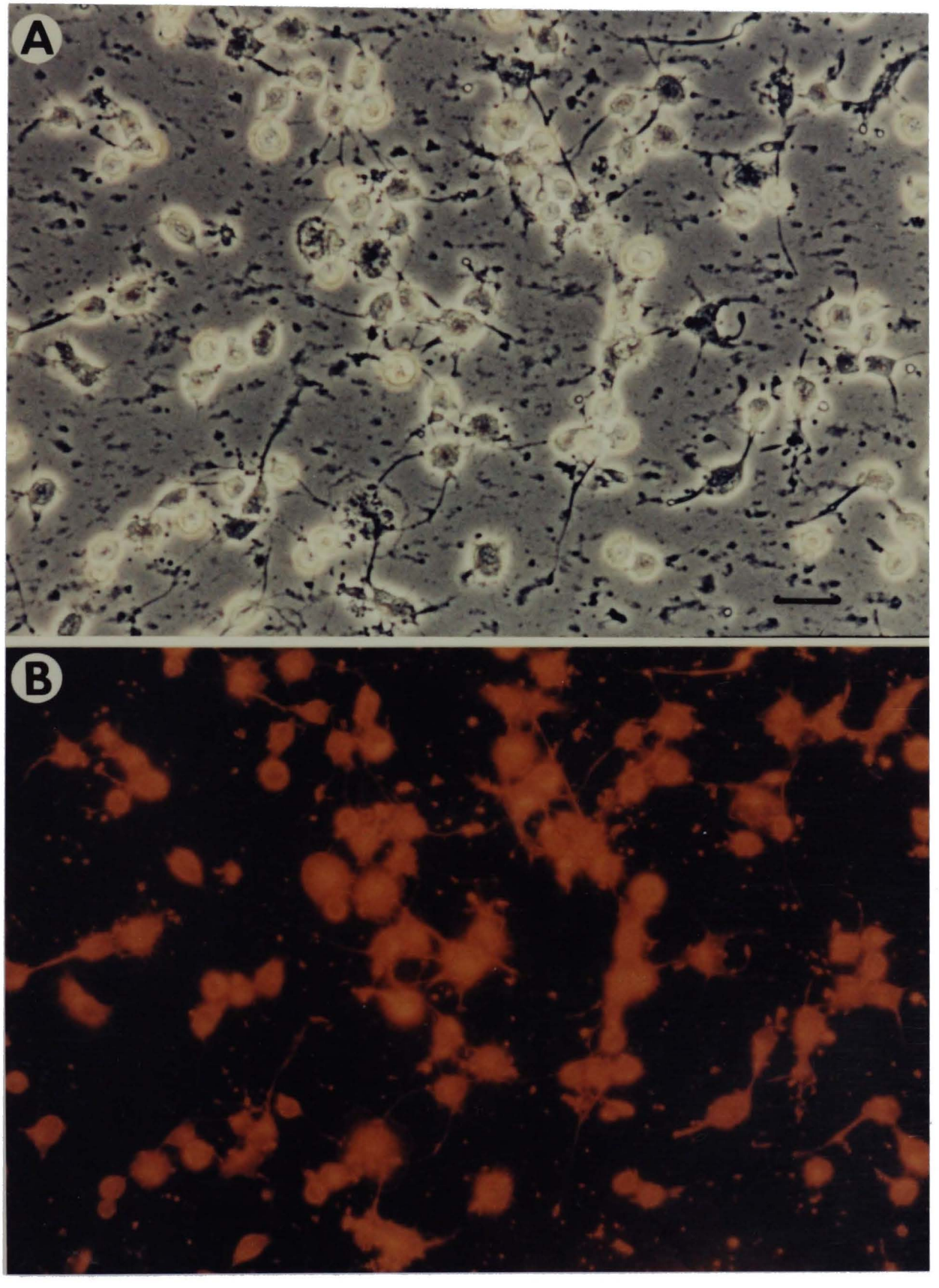
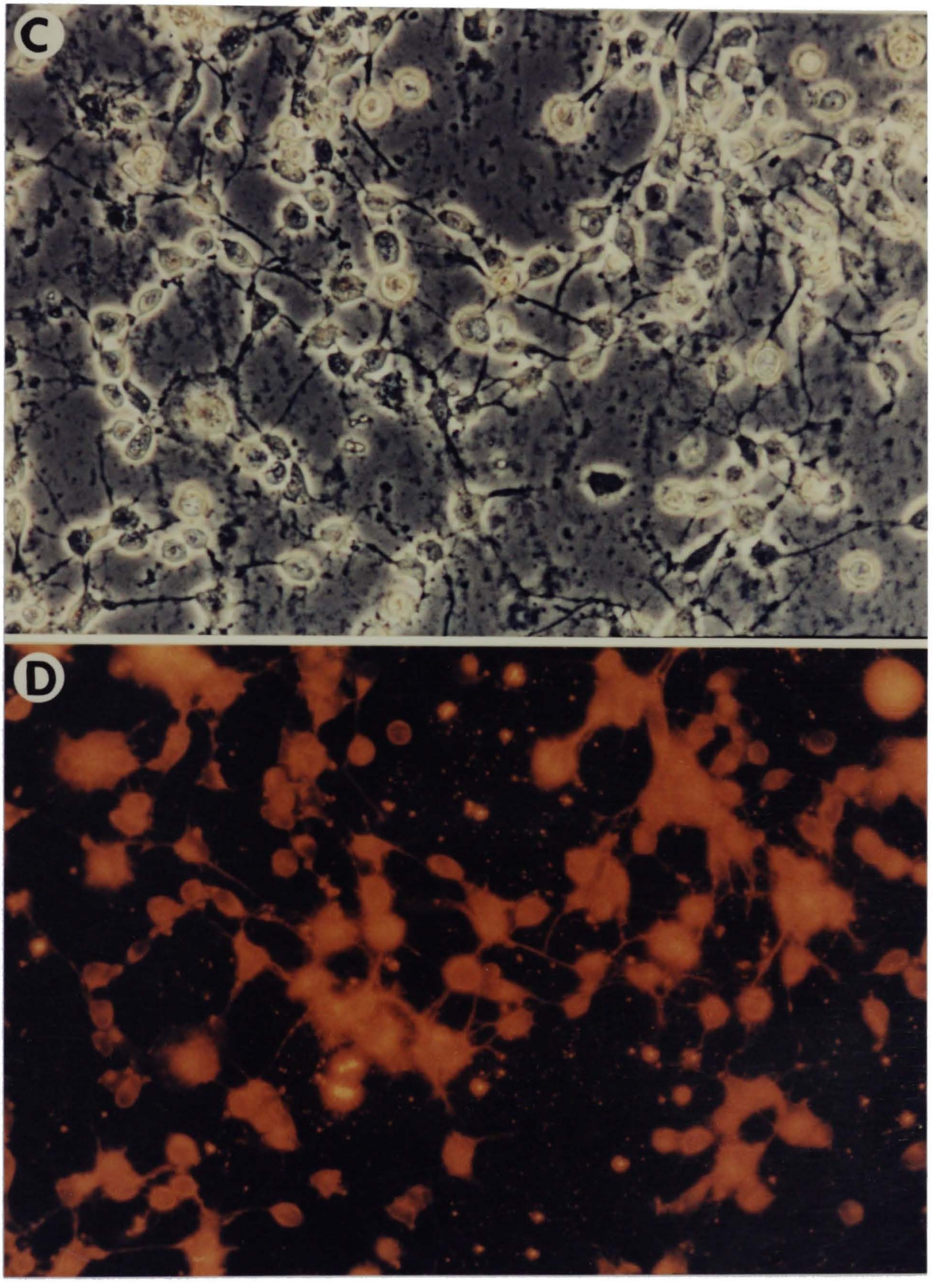
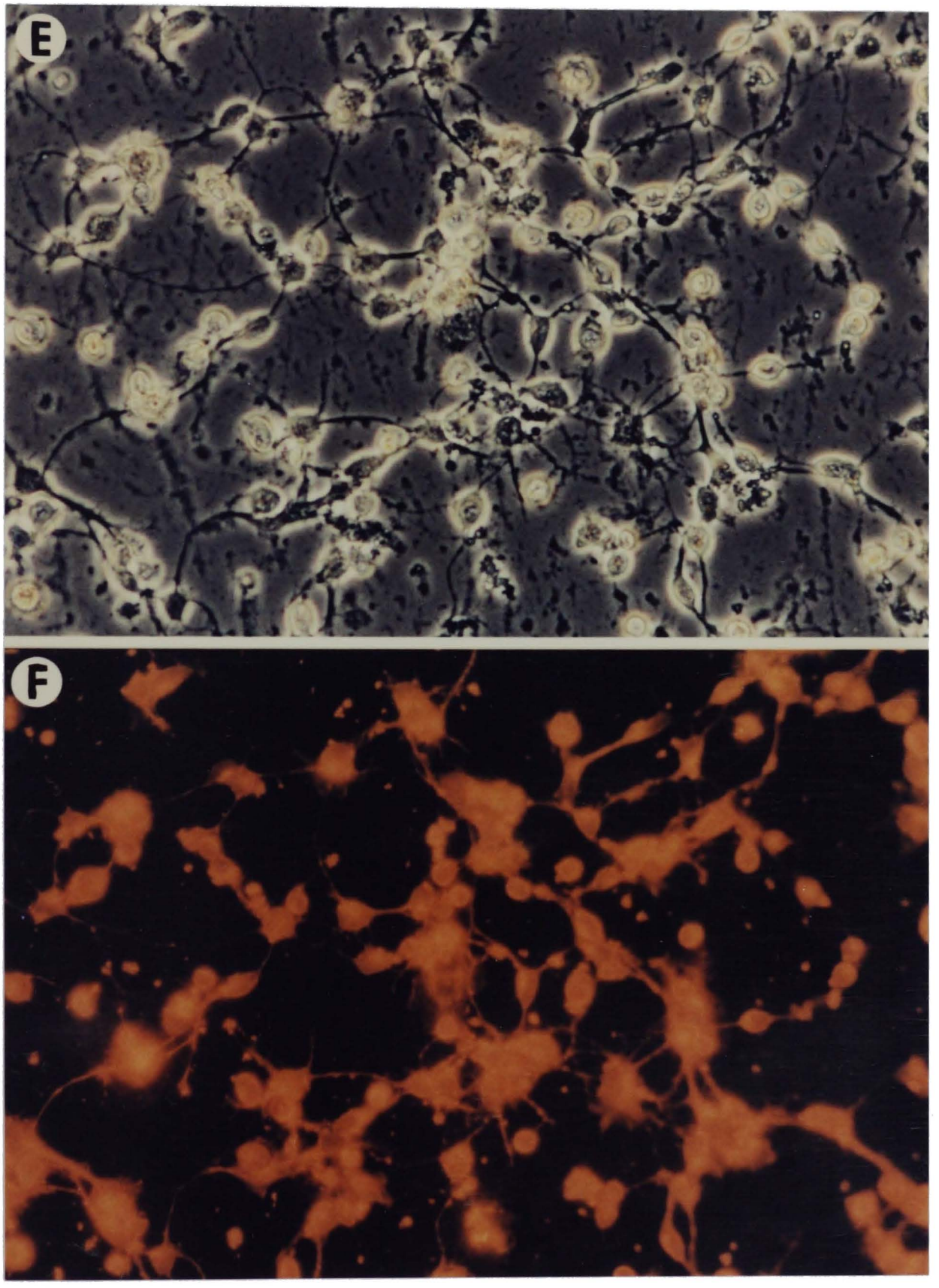
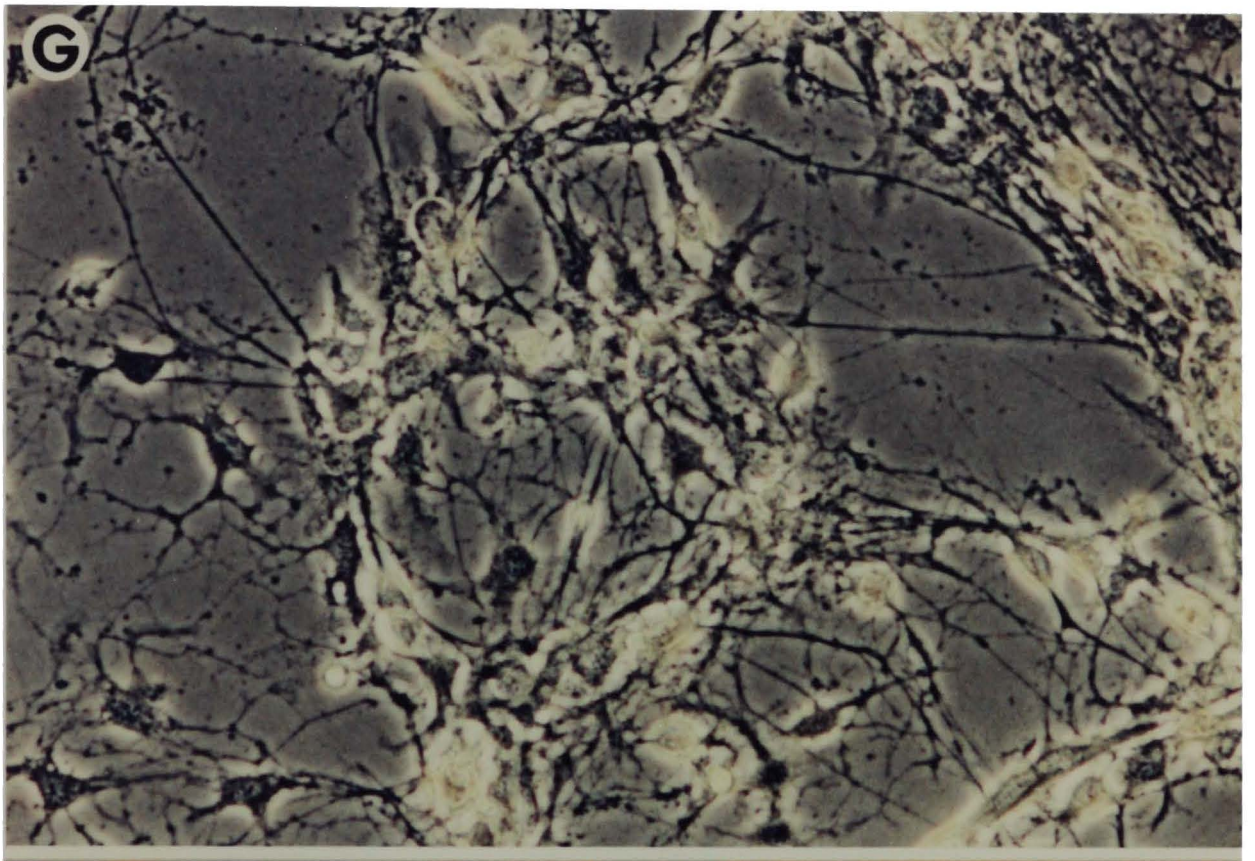

C
D

Fig. 26. Effect of anti-protein F1 antibodies on the development of spinal neurons in culture. Anti-protein F1 antibodies or rabbit IgG were encapsulated in rhodamine-labeled positively charged liposomes (20 μmole DOPE: 10 μmole cationic lipids). Three hours after plating spinal neurons in MEM10/10, cells were rinsed in MEM and incubated in 1 ml of MEM containing anti-protein F1 antibodies or rabbit IgG (final concentration 160 μg protein per ml) encapsulated in liposomes for 45 min at 37°C. At the end of incubation, cells were washed in MEM and incubated in MEM10/10. At every time point: zero, 4 , 10, 48 hr after liposome treatment; cells were rinsed in MEM and fixed in Gregory's fixative then examined with phase and fluorescence microscopy. (*A*), (*C*), (*E*), and (*G*) represent phase photographs of different cultures, and (*B*), (*D*), (*F*), and (*H*) show fluorescence pictures of the same areas at zero, 4, 10, 48 hr following liposome treatment; respectively. Bar in (*A*) is 25 μm and applies to all panels.


A
B


C
D


E
F


G

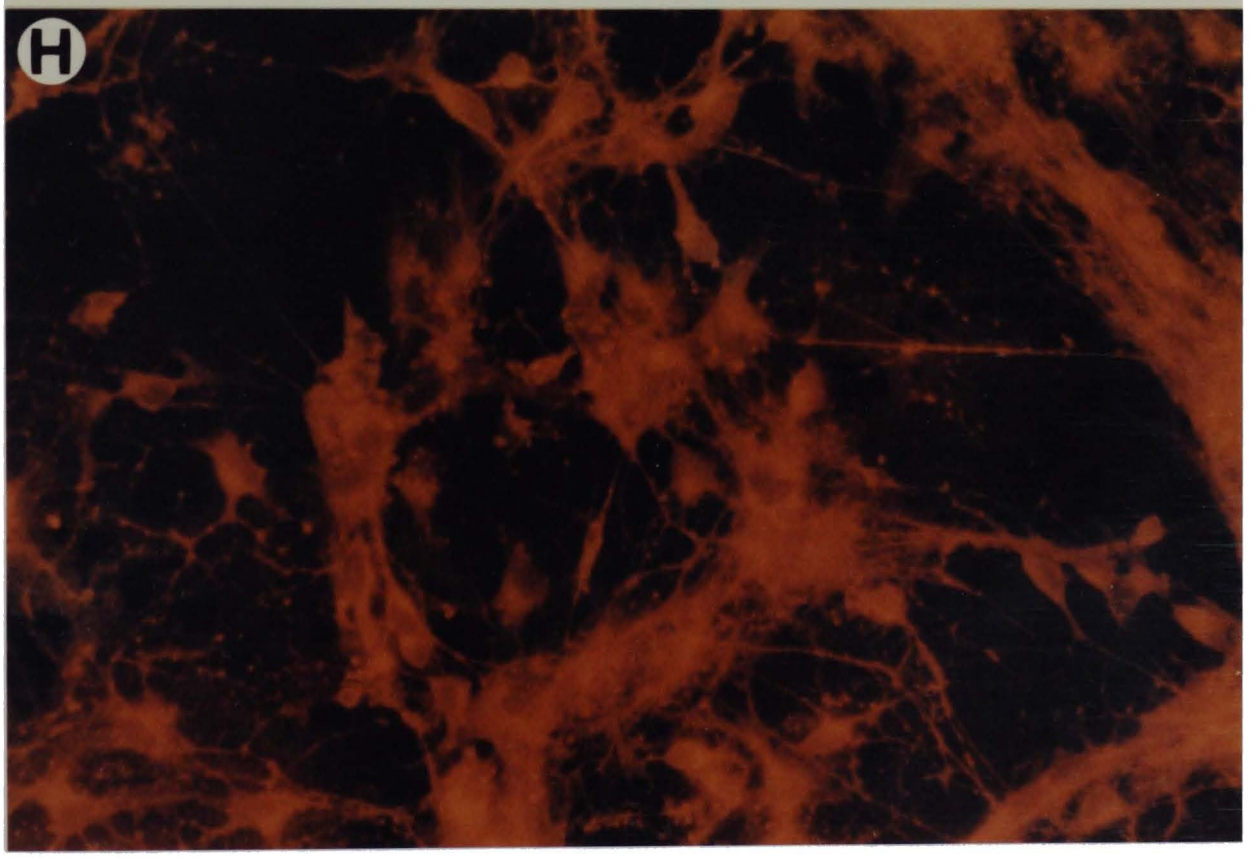
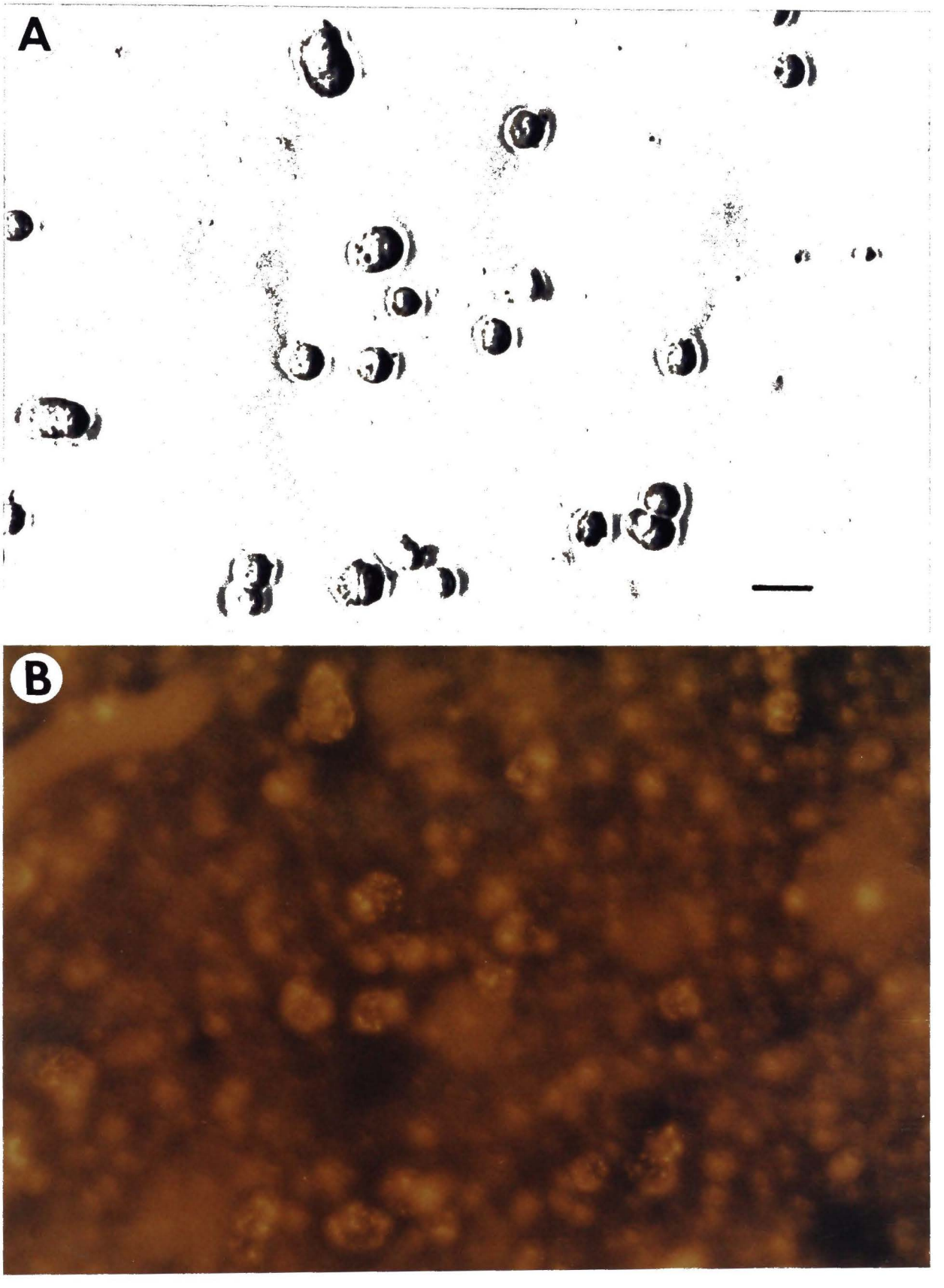

H

Fig. 27. Interaction of liposomes with PC12 cells. PC12 cells were grown in RPMI 1640 medium containing 5% fetal bovine serum and 10% heat-inactivated horse serum at a density of 2 x 10^6 cells/ml. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 1 or 24 hr, cells were rinsed 4 times in serum-free RPMI then incubated with 1 ml of RPMI containing: (i) positively charged rhodamine-labeled liposomes (0.9 μM total phospholipid) or (ii) rabbit IgG-containing liposomes (1.2 μM total phospholipid) for 50 min at 37°C. At the end of incubation, cells were rinsed 4 times with RPMI to remove extraneous liposomes and examined under fluorescence microscopy. (*A*) and (*C*) represent phase photographs of different cultures treated with liposomes, (*B*) and (*D*) fluorescent pictures of the same regions. In (*A*) and (*B*) cells, 1 hr-old, were treated with empty liposomes, (*C*) and (*D*) cells, 24 hr-old, were treated with rabbit IgG-containing liposomes. Bar is 25 μm.


A
B

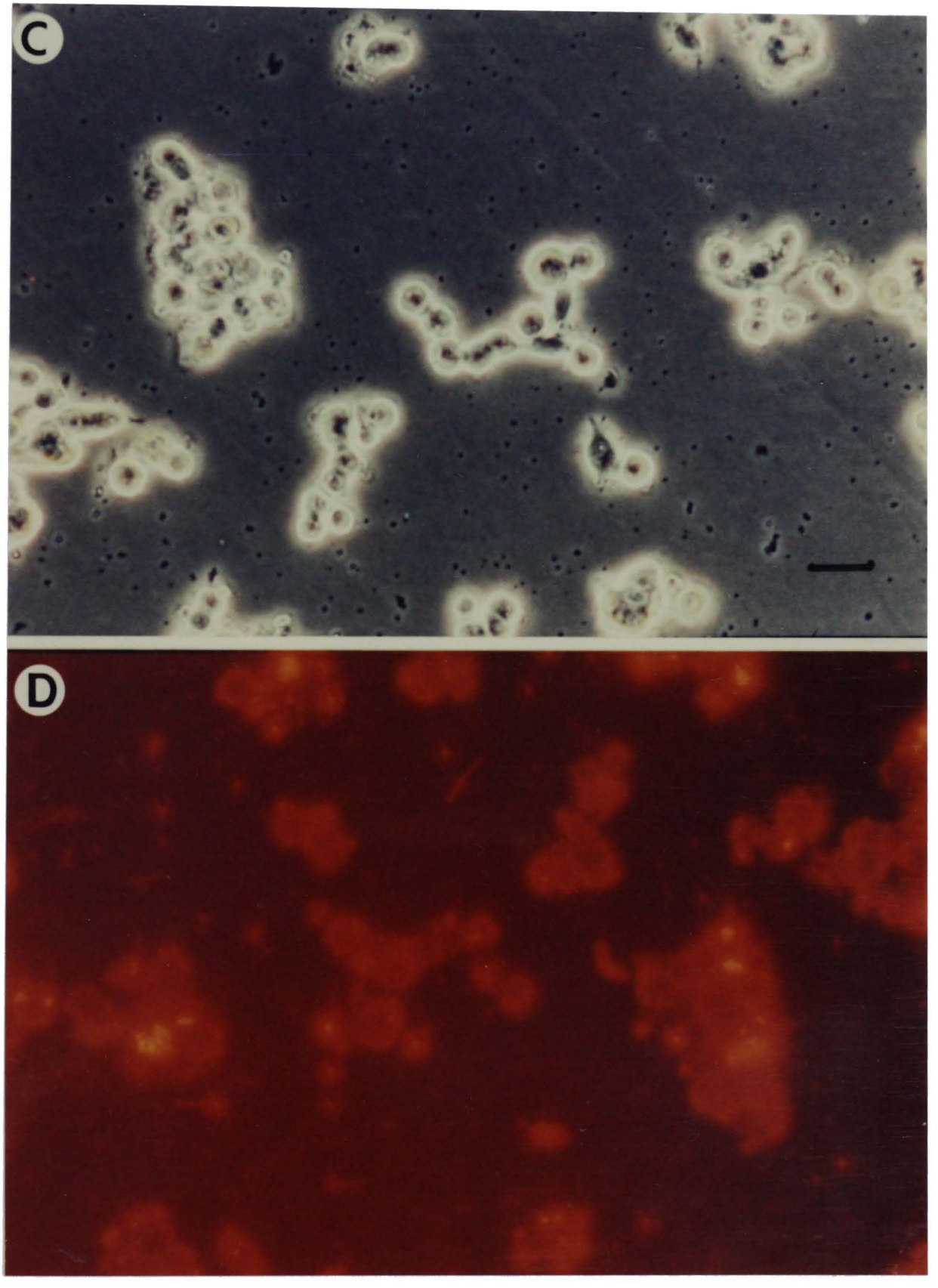

C
D

Fig. 28. Sequences and modifications of antisense ODN. Two different pairs of antisense ODN were selected from the cDNA sequence of murine protein F1 (Cimler et al., 1987). (*A*) Sequences of sense and antisense phosphorothioate ODN. The antisense ODN has an inverse complementary sequences to nucleotides -6+12 of protein F1 cDNA (National Biosciences). (*B*) Sequences of sense and antisense unmodified ODN. The antisense ODN has an inverse complementary sequence to nucleotides -9+9 of protein F1 cDNA (18-mer, Biosynthesis). (*C*) Represents the difference between phosphodiester bonds in unmodified and thio-modified ODN in which one of the non-bridging oxygens was replaced by a sulfur. Final concentrations of ODN were determined spectrophotometrically by absorbance at 260 nm.

A

F1 ANTISENSE (-6+12), PHOSPHOROTHIOATES

5' ACA GCA CAG CAT GGT GGT 3'

F1 SENSE (-6+12), PHOSPHOROTHIOATES

5' ACC ACC ATG CTG TGC TGT 3'

B

F1 ANTISENSE (-9+9), UNMODIFIED

5' GCA CAG CAT GGT GGT ATC 3'

F1 SENSE (-9+9), UNMODIFIED

5' GAT ACC ACC ATG CTG TGC 3'

C

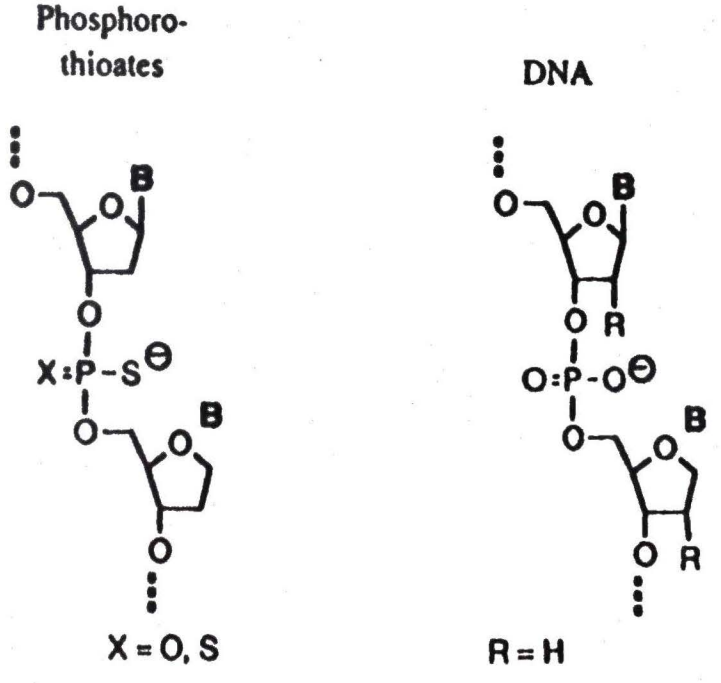


Fig. 29. Purification of phosphorothioate oligodeoxynucleotides on RP-HPLC. Sense and antisense thio-modified ODN (1.5 mg each, National Biosciences) were purified on RP-HPLC. Buffer A: 0.1 M ammonium acetate, 5% acetonitrile, pH 7.0. Buffer B: 100% acetonitrile. ODN were eluted with 60 min linear gradient at 0.5% per min and a flow rate of 0.7 ml/min. Column: C18 (250 x 10 mm, HiPore RP-318, Bio-Rad). Absorbance 260 nm. Fractions containing ODN were pooled, concentrated, and analyzed with gel electrophoresis.

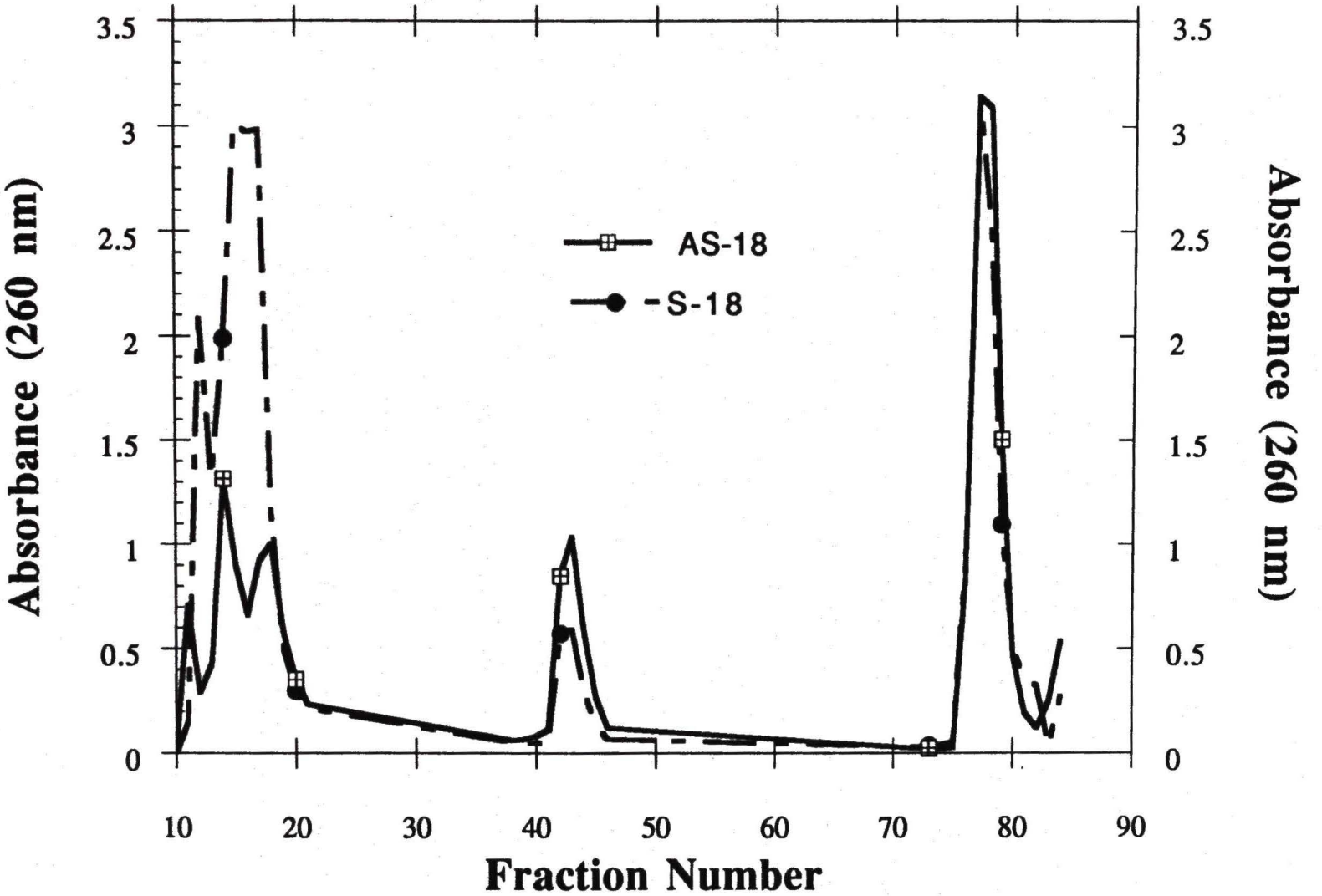
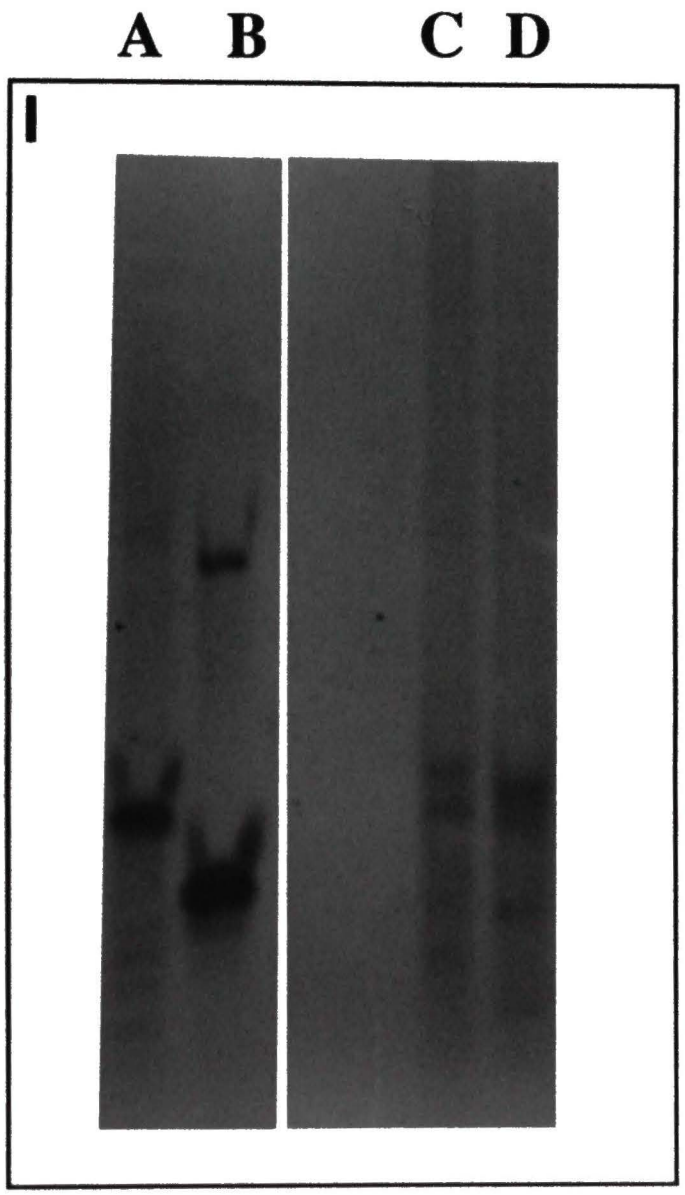

AS-18
S-18
Absorbance (260 nm)
Absorbance (260 nm)
Fraction Number
0
0.5
1
1.5
2
2.5
3
3.5
10
20
30
40
50
60
70
80
90

Fig. 30. Analysis of oligodeoxynucleotides by gel electrophoresis. HPLC fractions with high absorbance at 260 nm were pooled and concentrated by speed vacuum. Antisense phosphorothioates were analyzed on 20% acrylamide gel with 5% cross link. Prior to loading, samples containing 0.5 OD 260 units were mixed with equal volume of stop solution (95% formamide, 20 mM EDTA, pH 8.0, 0.05% xylene cyanol, 0.06% bromophenol blue, US Biochemicals Corp., Cleveland, OH), heated for 5 min, and cooled quickly on ice. The gel was run at 750 mA until the bromophenol blue and xylene cyanol had migrated to 2/3 of the gel. Gel was then placed on a fluorophore-coated thin chromatographic plate and ODN bands were visualized by UV shadowing at 254 nm and photographed using a Polaroid Type 55 N/P film. In Fig. 30.I, (*A*) and (*B*) are molecular weight markers of 18 and 15-mer length respectively. (*C*) and (*D*) represent the last peak of HPLC chromatogram of phosphorothioate antisense and sense ODN, respectively. Fig. 30.II shows the profile of unmodified 18-mer sense (*A*), antisense (*B*), and fluorescein-labeled antisense (*C*) ODN on 15% acrylamide gel (gel analysis provided by Biosynthesis). Upper arrow indicates xylene cyanol dye (of molecular weight equivalent to 30-mer ODN) and lower arrow indicates position of ODN bands.


A B C D

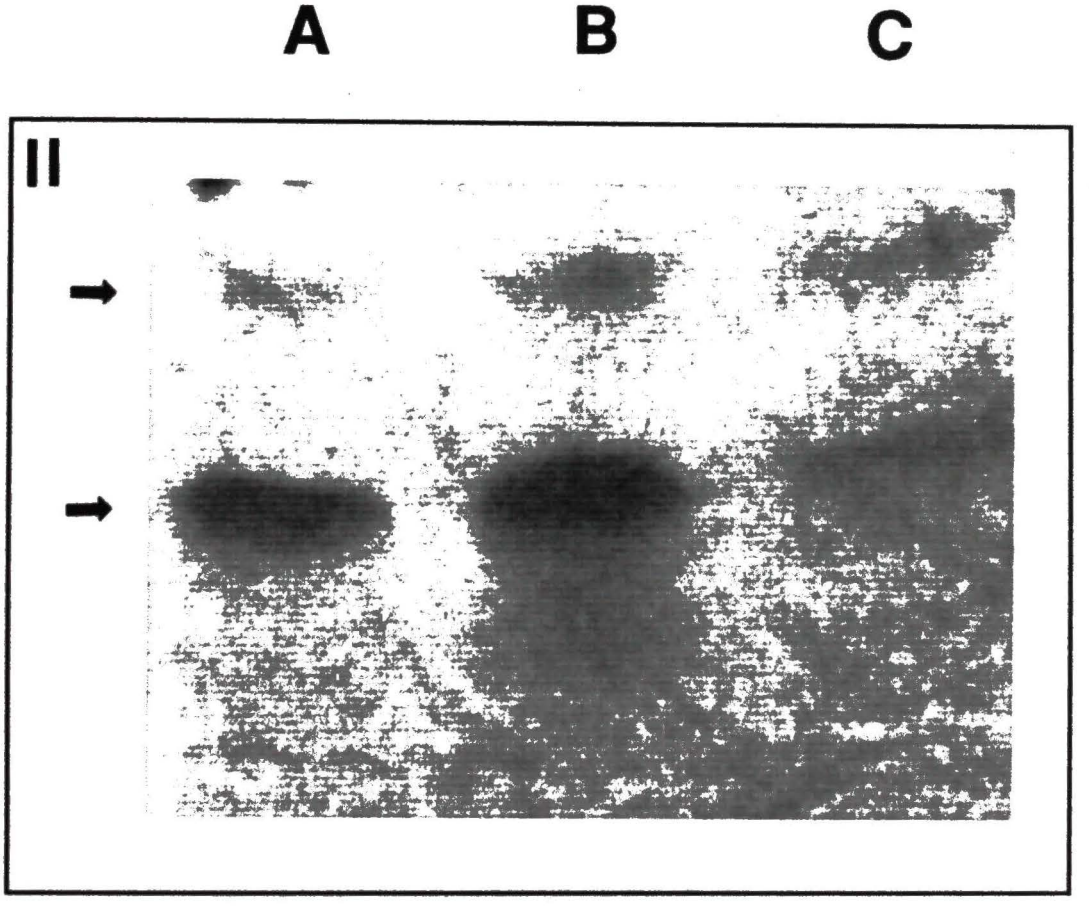

A
B
C
II

Fig. 31. Internalization of antisense ODN into cultured spinal neurons. Oligonucleotides were introduced into neurons using a trituration method. Labeled oligonucleotides were synthesized by adding fluorescein residues to the 5'end of unmodified ODN and purified on Sephadex G-25 column. Spinal cords were isolated from embryonic tissues (E14) and dissociated in MEM10/10. Cells were then centrifuged for 4 min and the supernatant was aspirated leaving 0.45 ml of medium containing neurons. Fluorescein-labeled ODN dissolved in water were then added (final concentration 40 μM) and spinal neurons were dissociated by trituration through a yellow tip (20 cycles). Neurons were then incubated for 60 min at 37°C then plated on PDL-coated coverslips at a density of 300,000 cells per culture. After 1 hr of seeding, cultures were fixed in Gregory's fixative for 5 min, rinsed in water several times then examined under Zeiss microscope equipped with epifluorescence optics. Pictures were taken with a Kodak 35 mm Gold-1600 film. Upper panel represents a phase micrograph of neurons 1 hr after seeding. Lower panel is fluorescence micrograph of the same region using a 450-490 nm excitation and a 515-565 nm barrier filter (Zeiss, FT510). Bar is 30 μm.

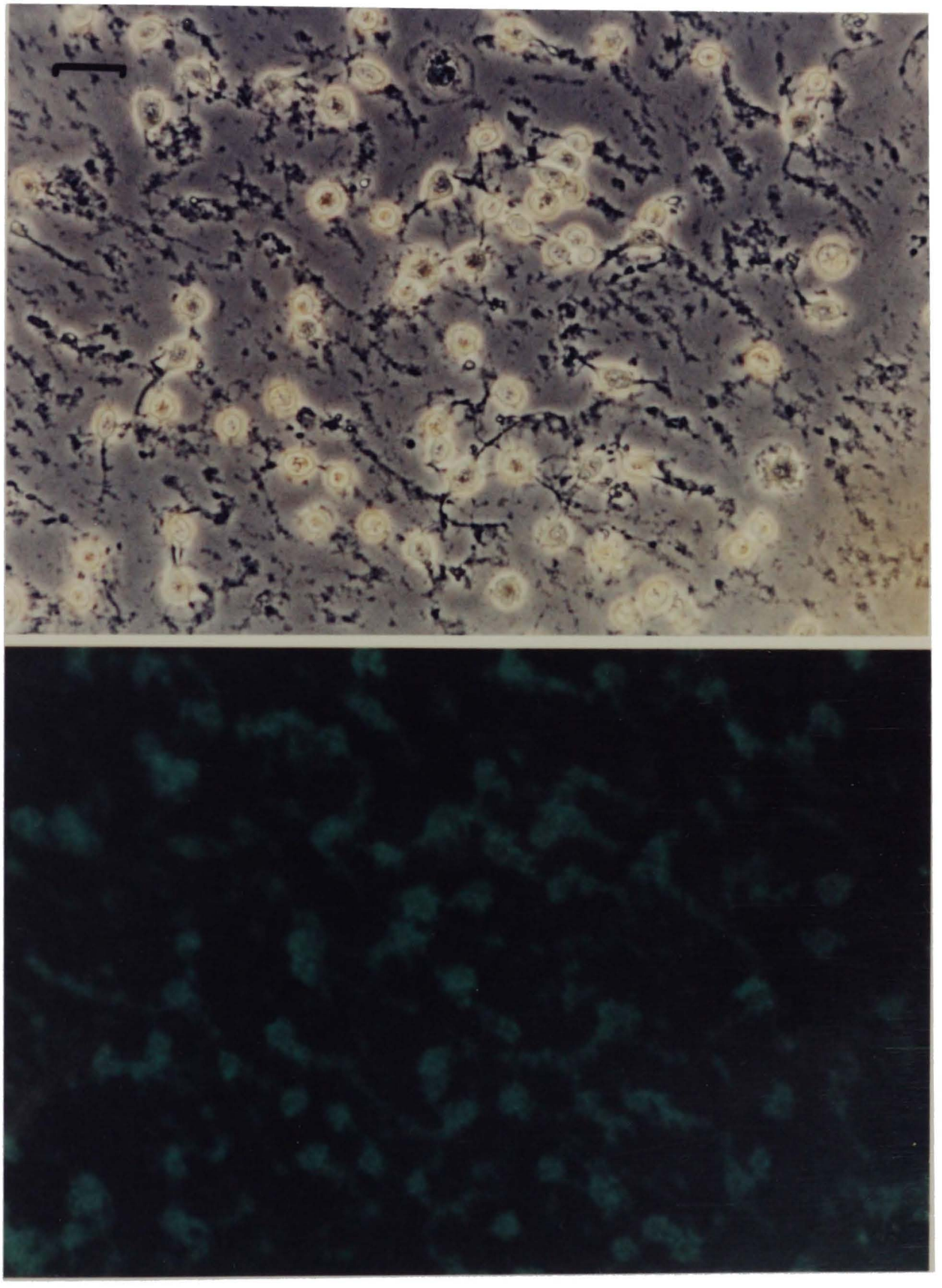
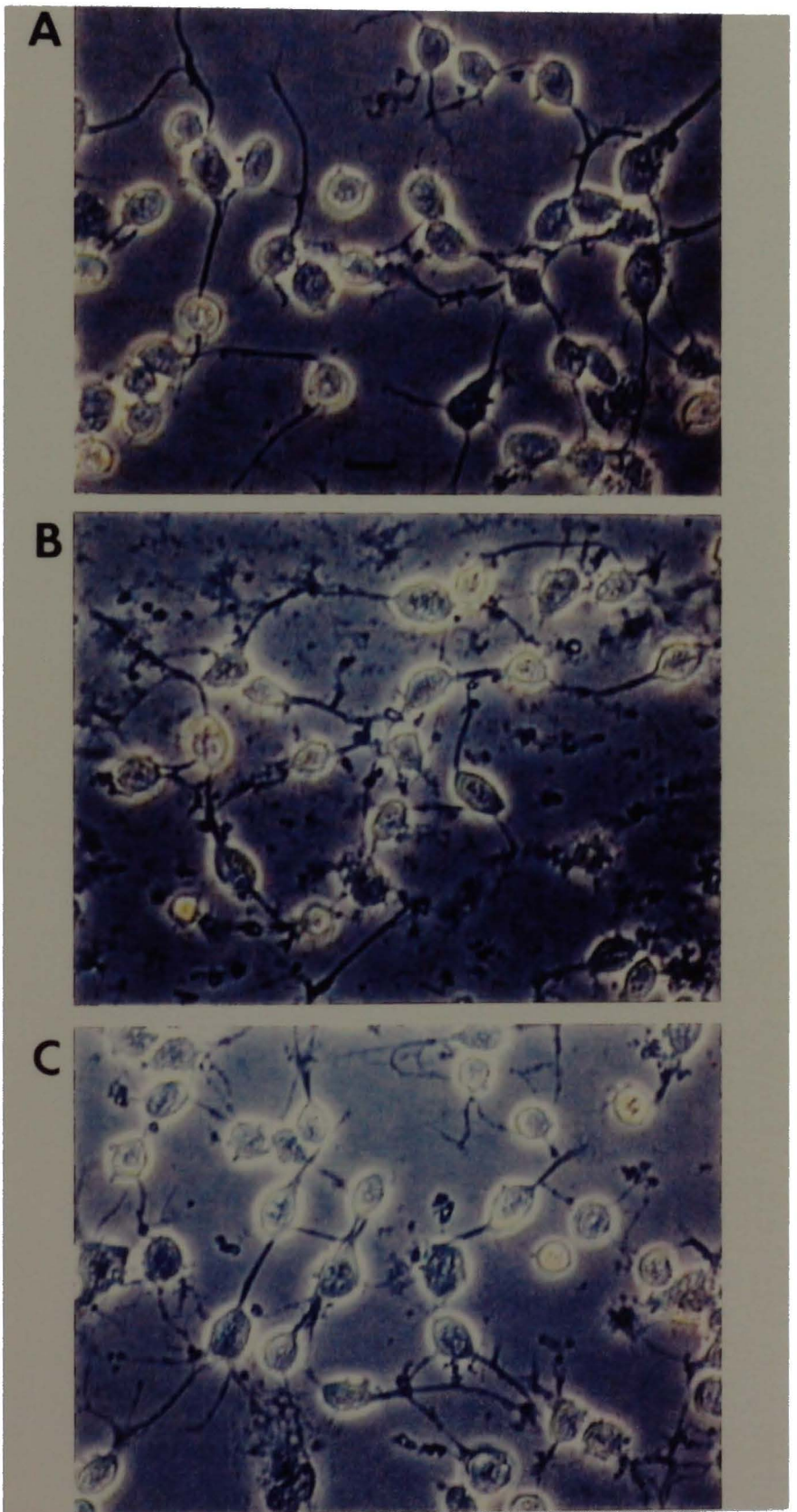
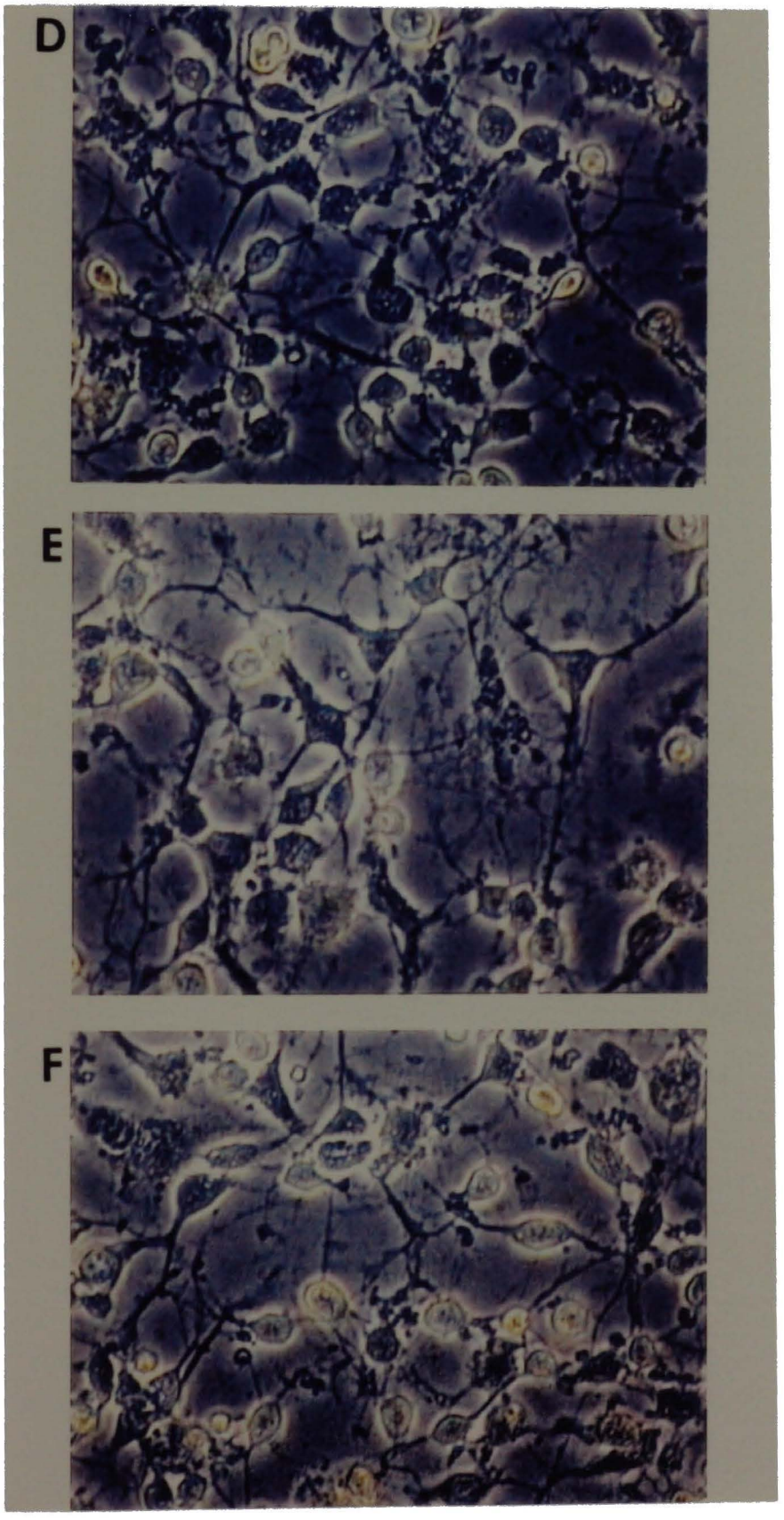
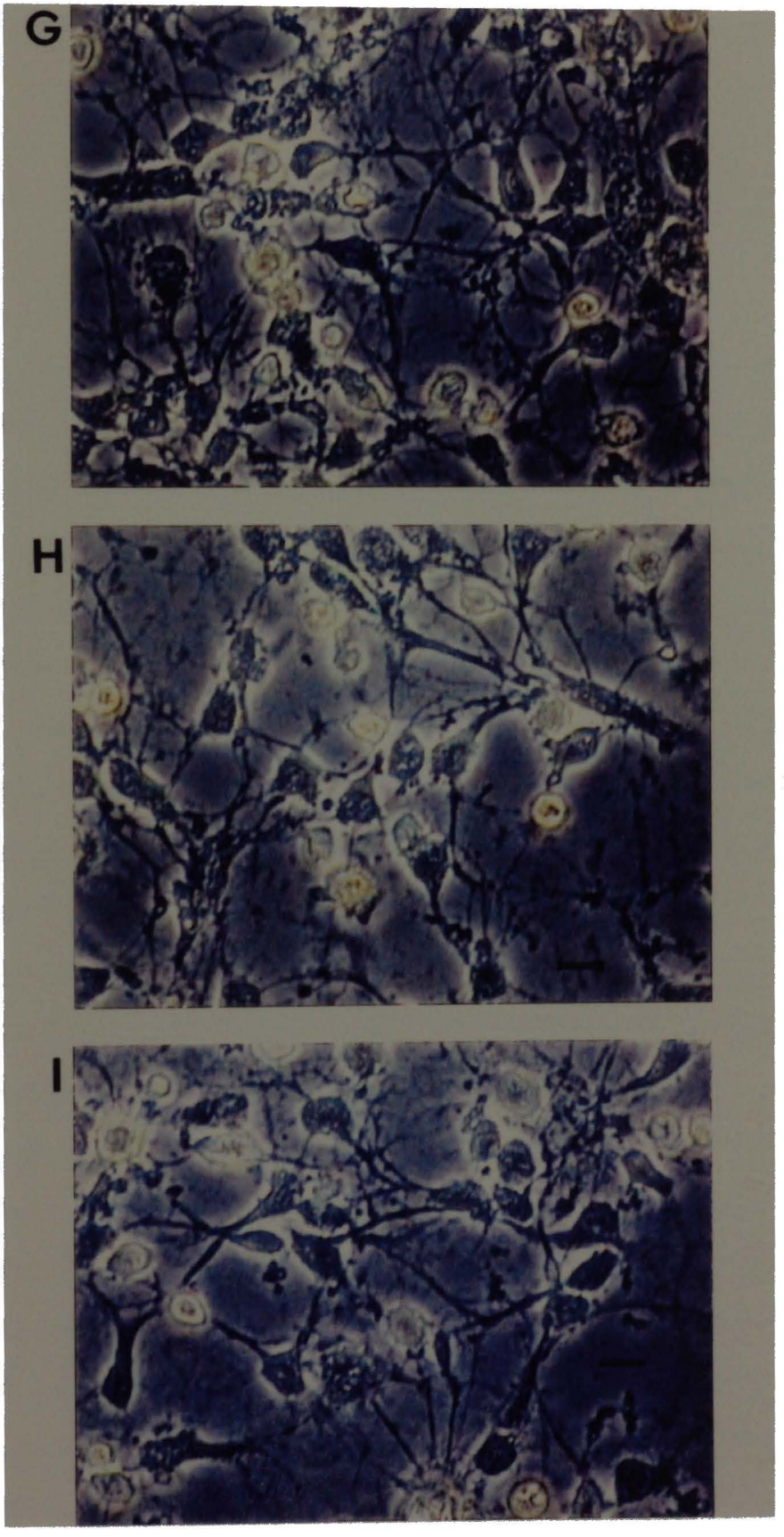


Fig. 32. Effect of antisense ODN on the development of murine spinal neurons in culture. Sense or antisense 18-mer ODN (200 and 300 μM) were introduced into spinal neurons dissociated from fetal tissues (E14) using the trituration method. Neurons were then incubated for 60 min at 37°C, the volume was made up to 1 ml with MEM 10/10, and cells were then plated on PDL-coated coverslips at a density of 300,000 cells per culture. At every time point: 3 (*A*, *B*, *C*), 10 (*D*, *E*, *F*), and 20 hr (*G*, *H*, *I*) after seeding, cells were rinsed then fixed in Gregory's fixative for 5 min. Sense treated cultures (*A*, *D*, *G*), antisense treated cultures (*B*, *E*, *H*), and controls (*C*, *F*, *I*) were examined and photographed using a Zeiss microscope equipped with a video camera. Bar in (*A*) is 10 μm and applies to all panels.


A
B
C


D
E
F


G
H
I

CHAPTER IV

DISCUSSION

Protein F1 (GAP-43) is a prominent member of a family of growth-associated proteins that are expressed during axonal development and regeneration. Protein F1 has been proposed to play an essential role in the growth process (Skene, 1989). Several investigators have correlated protein F1 expression to neuronal development and regeneration; however, the precise role of this protein is still unknown (for a review see: Benowitz and Perrone-Bizzozero, 1991).

The F1 peptide synthesized in this study (residues 205-225 of the intact protein F1) was selected because of shared sequence homology in this region of the protein between human, bovine, rat, and mouse species (Benowitz and Perrone-Bizzozero, 1991). Furthermore, it contains a potential PKC phosphorylation site (Ser-210), the function of which is not known. It was reasoned, therefore, that antibodies raised against this sequence may provide tools for the determination of the function of this phosphorylation site and/or functions of protein F1 in neurons.

Data from this study indicate that Ser-210 near the C-terminus of protein F1 can be phosphorylated by PKC *in vitro* in a concentration-dependent manner. Other peptides that are known to serve as substrates for PKC have Km range of 25-400 μM (Hassel et al., 1988). Compared to the previous peptides, the synthesized F1 peptide is considered a good substrate of PKC (Km= 0.25 μM) and therefore raises the possibility that Ser-210 on the intact protein may be a substrate *in vivo*. On the other hand, the PKC phosphorylation of Ser-210 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 Ser-210 *in vivo* is yet to be determined.

Several groups have reported that PKC phosphorylation of protein F1 occurs exclusively at serine residues (Chan et al., 1986; Oestreicher et al., 1989; Benowitz and Perrone-Bizzozero, 1991). Subsequent studies using site-directed mutagenesis have confirmed PKC phosphorylation at Ser-41 of protein F1 (Apel et al., 1991). Although protein F1 can also be phosphorylated *in vitro* by CKII at Ser-192 and Ser-193 (major sites) and Thr-88, 89, or 95 (minor sites) (Apel et al., 1990), no biological significance of CKII catalyzed phosphorylation is known. Recently, Spencer et al. (1992) reported that protein F1 is phosphorylated in cultured neurons and rat brains on three sites: Ser-41, Ser-96, and Thr-172. These results suggest that protein F1 can be phosphorylated at different sites and that phosphorylation may play important functions in neurons. *In vitro* PKC phosphorylation of protein F1 inhibits calmodulin binding to the protein (Alexander et al., 1987) and correlates with the persistence of long-term potentiation (Lovinger et al., 1986). Inhibition of PKC phosphorylation also inhibited calcium-dependent neurotransmitter release from rat cortical synaptosomes (Dekker et al., 1989). We may speculate that possible phosphorylation of protein F1 by PKC at Ser-210 may also mediate the proposed physiological function(s) of protein F1 in neurons.

Calmodulin was shown to inhibit phosphorylation of protein F1 by CKII (Apel et al., 1991). This may indicate that the calmodulin binding domain and the CKII phosphorylation sites at the C-terminus of protein F1 are juxtaposed as a result of the minimal secondary and tertiary structures of protein F1 (Apel et al., 1991). Phosphorylation of Ser-210 may be involved, by itself or via interactions with the adjacent CKII phosphorylation sites, in the regulation of interactions of the C-terminus of protein F1 with the calmodulin binding domain on the protein or with other cytoskeletal proteins.

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 Ser-41 (McMaster et al., 1988).

Anti-protein F1 antibodies, purified on a protein A column, stained spinal neurons in culture. Neuronal membranes and neurites exhibited strong reactivity to the antibodies while glial cells did not exhibit specific immunoreactivity (Fig. 15*B*). Previous immunolocalization studies with mature neuronal cultures reported protein F1 to exist mainly in growth cones, the growing tips of axons (Meiri et al., 1986, Meiri et al., 1988). However, Burry et al. (1992) 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 (Campagne et al., 1992a, b). Our data indicated that protein F1 is localized within the soma and all neurites of mature (3-4 weeks old) cultured spinal neurons.

In the present study the antibodies were used in ultrastructure localization studies of protein F1 in cultured spinal neurons. In mature spinal neuronal cultures of 4 weeks, immunogold labeling of protein F1 was associated with several subcellular compartments including presynaptic terminals, microtubules, and axon-like processes. Gold particles were also observed in the cell bodies of neurons. These observations agree with previous reports that indicated protein F1 exists in membrane as well as in cytoplasmic fractions.

Labeling of axon-like processes may represent protein F1 being transported, via fast axonal transport, from the soma to the synaptic terminals. That label by anti-protein F1 antibodies was specific as indicated by the lack of associated gold particles when preimmune serum was used. In addition, omission of the primary antibodies did not result in significant labeling which precluded the possibility that the gold-labeled secondary antibodies recognized non-specific antigenic sites within spinal tissues. Although, anti-protein F1 antibody immunoreactivity was demonstrated within neuronal cell bodies and processes, no clusters of gold particles within certain subcellular locations could be demonstrated. Counting of gold particles per unit area did not show significant labeling of any specific subcellular compartment. Protein F1 is known to be associated with plasma membranes and is localized on the cytoplasmic surface. In this study, gold labeling of protein F1 indicated that the protein is indeed intracellular, although no intense labeling of the plasma membranes was noticed. Increasing the antibody titre used for staining resulted in an increase in the number of gold particles seen on the stained tissues, however, the distribution of the particles was similar to that observed when lower concentrations of the anti-protein F1 antibodies were used. Although protein F1 was shown to be localized at the presynaptic membranes of adult rat brains (Gorgels et al., 1989), synapses of cultured spinal neurons did not exhibit gold particle density higher than the rest of the tissue. Several method variations could be further attempted including increasing the concentration of the serum used as a blocking agent (may be up to 100%), diluting the primary antibodies, and decreasing the cross reactivity of the rabbit primary antibodies by passing them through a column of agarose beads bound to mouse serum.

Because no association of the polyclonal antibodies used in this study with particular sites in spinal neurons was observed, one might argue that these antibodies recognized other antigens in the spinal neurons in addition to protein F1. However, the antibodies were able to identify native protein F1 purified from rat brain on Western

blotting and to selectively immunoprecipitate phosphorylated protein F1 from partially purified rat brain homogenate. In addition the specificity of the antibodies was further shown by staining of neurons, but not glial cells in cultures, and by the monitoring of protein F1 levels in developing spinal neurons in culture and *in vivo*.

Protein F1 is a prominent member of fast axonally transported proteins that are synthesized in the soma and transferred to the synaptic terminals. Also, Protein F1 was reported to exist in neurons attached to plasma membranes and in the cytoplasmic pool through a dynamic palmitoylation of Cys-3 and Cys-4 at the N-terminus of the protein. Taken together, these data may explain the distribution of gold particles in several subcellular compartments of the spinal neurons including synaptic terminals, neurite processes, and the cell bodies where protein F1 is synthesized.

In future studies, additional information or data obtained in this study can be clarified by further purification of the current antibodies on an affinity column. Staining of mature spinal neurons using monoclonal antibodies against the same protein may also be compared to the staining obtained with the polyclonal anti-protein F1 antibodies used in this study. Neurons used in this study were mature and shown to have low levels of protein F1. It is possible, because of the high expression of protein F1 in neonate neurons, that similar immunolocalization studies on young neuronal cultures (10-24 hr old) might reveal more information regarding cellular localization of protein F1. It would be interesting to locate growth cones in young cultures and study their immunoreactivity to anti-protein F1 antibodies.

By using an enzyme-linked immunoadsorbent assay (ELISA, Van der Neut et al., 1990), protein F1 was employed as a developmental marker via comparisons of protein F1 levels *in vitro* with those associated with different developmental stages *in vivo*. In this study, determination of the abundance of protein F1 itself using an ELISA assay was preferred over the estimation of protein F1 mRNA. The ELISA assay allowed direct

quantification of protein F1 in neurons. Other methods that are based on quantification of protein F1 mRNA might not be a good reflection of the protein quantity. Protein F1 mRNA levels are not directly controlled by activation of transcription and their stability was shown to be independent of translation (Perrone-Bizzozero et al., 1993). In addition, phorbol esters selectively increased the half life of protein F1 mRNA by 6-fold in PC12 cells. Such an increase was abolished by the inclusion of PKC inhibitors (Perrone-Bizzozero et al., 1993). Furthermore, protein F1 mRNA was shown to bind specific cytosolic brain proteins via its 3' untranslated region (Kohn and Perrone-Bizzozero, 1993). Such protein-mRNA interactions were thought to contribute to the stabilization of protein F1 mRNA during differentiation of neurons (Kohn and Perrone-Bizzozero, 1993). In other words, existence of high levels of protein F1 mRNA does not necessarily reflect the amount of this protein in cells.

The ELISA assay was selected over Western blotting as it provided direct quantification of the protein under investigation with the sensitivity required in a small number of cells. The relative abundance of protein F1 was estimated in murine spinal neurons *in vivo* and in cultures. The assay was shown to be accurate, as the standard deviation of the mean of sample to sample was 0.03, and reproducible. In cultures, after 48 hr of seeding spinal neurons derived from embryonic tissues (E13-14), protein F1 levels reached maximal levels then declined as neurons became mature. The high levels of protein F1 observed during the first 48 hr after seeding are thought to be correlated with the regeneration of spinal neurons after the dissociation injury caused during mechanical isolation of these cells from their original tissues. Protein F1 levels decreased 48 hr after cultured neurons had extended neurites and formed synaptic contacts. Such a high decrease in protein F1 levels does not appear to be caused by neuronal death as no major morphological degeneration of neurons was seen. Also the determination of total protein concentration of assayed cultures showed a steady increase in total protein relative to time

which indicates neuronal and glial growth. These results are consistent with a previous study in which protein F1 levels in cultures of rat spinal neurons were shown to increase from 24-72 hr and then decrease (Van der Neut et al., 1990).

During differentiation of hippocampal neuronal cultures, axons were previously shown to retain protein F1 immunoreactivity that was reduced in dendrites (Goslin and Banker, 1990). It is unknown at what stage of development of cultured spinal neurons the neuritic processes differentiate into axons and dendrites. However, the results obtained in this study indicate that lower protein F1 levels may be associated with differentiation of spinal neurons in cultures.

By contrast to *in vitro* studies, *in vivo* protein F1 levels were shown to peak at postnatal day 1 then decreased as tissues become mature. Whether or not the high levels of protein F1 observed at P1 are correlated with the general injury that occurs following birth, is unknown. Although high levels of protein F1 are expressed by spinal neurons *in vivo* at P1, cultured neurons of similar age (7 days after seeding) contained low levels of the protein.

Alternatively, when protein F1 levels are expressed as absolute amount of protein F1 per spinal cord (instead of normalization per μg of protein), protein F1 levels showed a different pattern. Protein F1 amount decreased sharply from E15 to E17 then increased by P1 and reached maximal levels by P7. Protein levels then remained high until P18. These results may emphasize the significance of protein F1 after birth.

It is difficult however, to make a significant comparison between protein F1 levels *in vivo* versus *in vitro* for several reasons: (i) it is not known whether or not all neurons of the spinal tissue survive in culture or whether there is selective death of certain subpopulations of these cells; and (ii) although the spinal neuronal cultures contain neurons and glial cells (similar to the original tissue), it is unknown whether differential growth of glial cells occurs in culture, furthermore, glial growth in spinal cultures is inhibited by the

addition of FdU 5 days after seeding. It is also unknown when maximal synaptogenesis occurs in spinal tissue or in cultured spinal networks and whether or not maximal levels of protein F1 at 48 hr in culture and P1 *in vivo* occur simultaneously with maximal synaptogenesis. Answers for one or more of these questions in addition to the results observed in this study may help define the role of protein F1 during the growth of spinal tissues.

Next, the potential effects of anti-protein F1 antibodies on the physiological functions of cultured mammalian neurons were investigated. Specifically, changes in synaptic functions or effects on the spontaneous electrical activity of neuronal networks in response to anti-protein F1 antibodies were studied. Pilot experiments in this direction have shown that direct application of the produced antibodies (400 μg/ml) decreased the electrical activity of spinal neurons with complete inhibition within 15 min of the application. However, that effect was non-specific as application of mouse or rabbit serum at the same concentration resulted in similar effects. It is still unknown by what mechanism the IgG molecules inhibited the electrical activity of neurons. Moreover, the observed effect was quickly (within 1 min) reversed by washing the cells which indicates weak binding of the IgG molecules to receptors or synaptic components.

Anti-protein F1 antibodies were also applied directly to newly seeded neurons at a high concentration (400 μg/ml) but did not have any immediate effect on the development or the morphology of spinal neurons. However, cultures treated with the antibodies exhibited massive clusters of neurons by the end of the first week *in vitro*. That effect is thought to be nonspecific as IgG molecules might have been used by neurons as adhesion molecules.

After attempting to introduce anti-protein F1 antibodies into neurons by direct application into the culture medium at high concentration, phospholipids were employed to facilitate antibodies entry across plasma membranes. A protocol used by Shea and

Beermann (1991) to introduce anti-protein F1 antibodies into PC12 cells using LPC, an egg-white phospholipid derivative, was applied in this study to deliver anti-protein F1 antibodies into mammalian neurons in culture. That method resulted in permeabilization of cultured neurons and internalization of the antibodies as evidenced by the immunocytochemical stain of the permeabilized cells (Fig. 22*A*, *B*). However, because the method involved the application of high glycerol concentration and incubation of neurons on ice, phospholipid-mediated delivery of anti-protein F1 antibodies into cultured mouse spinal neurons caused excessive neuronal death.

Anti-protein F1 antibody transfer via liposomes into neurons in cultures was then attempted. First, positively charged liposomes composed of phosphatidyl-ethanolamine, dioleoyltrimethyl-ammonium propane, and rhodamine labeled phosphatidyl-ethanolamine (100:50:1 mole ratio) were prepared and added, during multichannel extracellular recording, to monolayer neuronal cultures derived from embryonic murine spinal tissue. Incubation periods of 1.5-2 hr at liposome concentrations of 300 μM resulted in some cell death and loss of action potentials on many channels (Fig. 24.I). Incubation periods of 45 min at a reduced concentration of 150 μM resulted in maintenance of activity on all channels and 100% cell survival (Fig. 24.II). Strong rhodamine fluorescence was seen on all neurons of 6 networks and was found evenly distributed throughout somata and neurites (Fig. 23*B*). Minimal fluorescence was seen in the glial carpet. At least two mechanisms are described for the interaction of liposomes with cell membranes namely, endocytosis and fusion. Since rhodamine was covalently bound to phosphatidylethanolamine, the uniform fluorescence might indicate fusion of liposomes with neuronal membranes. The uniform distribution of rhodamine in the living neurons as well as the association of this dye with membrane debris of dead neurons imply that the liposomes have fused with the neuronal membranes and have not entered these cells via endocytosis.

Preferential interaction of liposomes with neurons may be due to differences in the membrane components of neurons and glial cells. This observation is further supported by the interaction between liposomes and PC12 cells in which a spotted, not uniform, labeling of PC12 membranes was observed. It is also possible that the amount of positive charge on liposomes is a factor in controlling the interaction of liposomes with cells. Similar liposomes (made of phosphatidylethanolamine and cationic lipids, 2:1 ratio) are toxic to CHO cells but did not interact with CV1 cells (Dr. K. Hong, personal communication). Furthermore, the use of more positively charged liposomes (made of phosphatidyl-ethanolamine and cationic lipids, 1:1 ratio) caused excessive death of spinal neurons in culture. These observations suggest that neuronal and glial cells are different with respect to membrane composition and surface charge. In addition, the previous data indicate that liposomes manufactured for cell delivery purposes must be customized for each cell type used.

Previous procedures developed to deliver macromolecules into neuroblastoma cells using L-α-lysophosphatidylcholine have resulted in the death of 40-60% of the cells in 8 minutes (Shea and Beermann, 1991). In the present study, the time of liposome incubation with cells was extended to 45 min with no obvious cytotoxicity at a total phospholipid concentration of 150 μM. The ability to maintain neurons and glia in a liposome environment for this period of time together with the ability to adjust liposome content concentrations, can provide a suitably adaptable procedure for the successful delivery of bioactive molecules to neurons.

It was noticed also that the interaction observed between liposomes and spinal neurons did not occur with other cell lines including CHO, CV1, and breast cancer cells. No uniform labeling of plasma membranes of PC12 cells was observed upon application of positively charged liposomes. In one experiment, presence of rhodamine-labeled spots

within or on top of PC12 cells might indicate receptor-mediated endocytosis of liposomes by PC12 (Fig. 27*B*). However, that observation needs to be further investigated.

Uniform labeling of all neuronal cells bodies and neurite membranes, labeling of cell debris, and absence of patchy labeling of neurons (with the consequent degradation of liposomal contents within lysosomal compartments) precluded that liposomes were taken up by neurons via receptor-mediated endocytosis. Furthermore, the uniform and intense neuronal labeling observed in this study may not be explained as non-specific lipid transfer between liposomes and neuronal membranes as it did not occur with glial cells in the same cultures and it could not be repeated with several other cell types. This preliminary evidence may imply fusion of liposomes with neuronal and, to a lesser degree, glial membranes. These results are of great interest as they indicate that a direct access to the cytoplasm of neuronal cells may finally be possible and several bioactive membrane-impermeable molecules including antibodies and DNA can be preferentially delivered into primary neurons. To further prove that liposomal contents are indeed delivered to neurons, rhodamine-labeled liposomes containing a fluorescein-labeled membrane impermeable molecule such as dextran (to show delivery of liposomal contents) may be applied to neurons. This approach together with uniform labeling of neuronal membranes, would prove fusion of liposomes with neurons.

The use of multielectrode extracellular recording to characterize the spontaneous network activity before, during, and after liposome application provided a reliable and sensitive method for monitoring the functional state of the network and can reveal network damage before morphological damage is recognized. At a liposome concentration of 150 μM, the maintenance of electrical activity, 100% cell survival, and strong staining of neurons with rhodamine suggest that delivery of macromolecules to nerve cells is possible without damage to the neurons or major alterations of the associated electrophysiological

network behavior. A potential system for delivering macromolecules into cultured mammalian neurons has, therefore been established.

Based on the these observations, anti-protein F1 antibodies were encapsulated in positively charged liposomes (made of DOPE and cationic lipids). Treatment of newly seeded neurons, derived from embryonic spinal tissues, with anti-protein F1 IgG encapsulated in rhodamine-labeled liposomes did not affect neuronal development. Uniform rhodamine labeling of cell membranes was observed and was believed to imply delivery of the IgG molecules included in the liposomes. Fluorescein-tagged IgG molecules can be used to further confirm delivery of the antibodies into cells. No cytotoxicity was observed and neurons developed for at least 3 days after liposome treatment. A few suggestions can be made from this data. First, although anti-protein F1 antibodies were delivered into neurons and bound to protein F1 C-terminus, Ser-210 might not be implicated in the proposed role(s) of protein F1 in axonal growth and consequently did not affect neurite outgrowth. Conformational changes, if any, as a result of antibodies binding to protein F1 might not have affected the function(s) of the protein. Second, the concentration of anti-protein F1 IgG molecules delivered (which in turn was controlled by a certain concentration of liposomes above which signs of cytotoxicity were observed) was insufficient to bind enough protein F1 molecules to show an effect on neurite outgrowth.

Although we do not have definitive evidence for antibody incorporation into the cytoplasm of spinal neurons, diffused rhodamine labeling of spinal neurons indicates probable fusion of liposomes with neurons and may consequently indicate delivery of the antibodies into the cells. Further studies need to be performed to confirm fusion of the positively charged liposomes to spinal neurons and delivery of liposomal contents into these cells. Assuming that the antibodies have entered the cells and bound to protein F1, two possibilities may explain the absence of morphological changes upon treating spinal neurons with anti-protein F1 antibodies. First, Ser-210 at the C-terminus of the protein

might not mediate the effect of protein F1 on the morphology and/or the neurite extension of neurons. Second, although binding of the antibodies to protein F1 should affect the translocation of the newly synthesized protein from the soma to the synaptic terminals, the concentration of antibody molecules might not have been sufficient to block the protein to induce an observable effect. Based on the liposomal population and size distribution, we estimated that the incorporation capacity of antibodies into liposomes to be around 0.01% of the total antibody used for encapsulation (about 6 ng antibody were added to the cells). Although almost all neurons were stained with rhodamine, it is unknown how much of the encapsulated anti-protein F1 antibodies were actually delivered into neurons.

Although spontaneous electrical activity was not lost on the selected channels, after incubation of liposomes (150 μM) with spinal neurons for 45 min, changes in integrated amplitude (i.e., spike frequency within bursts) and in the overall burst patterns did occur. Because the application of empty liposomes changed the pattern of the spontaneous electrical activity of spinal neural networks, no attempts were performed to deliver anti-protein F1 antibodies via liposomes to mature neurons during extracellular recording. The effect of liposomes on the electrical activity of neurons should be first further characterized before any effect of the encapsulated molecules could be demonstrated. On the other hand, liposome-induced changes in electrical activity of neural networks may be further reduced by adjusting the intra-liposomal ion environment.

Inhibition of gene expression using antisense oligodeoxynucleotides can be achieved via several mechanisms including: (i) blocking new protein synthesis by translational arrest, (ii) inducing mRNA degradation by an RNase H-dependent mechanism, (iii) preventing maturation of mRNA by masking of sequences needed for spliceosomes, (iv) blocking gene transcription through the formation of triple helix structure, and (v) inhibition of transport of mRNA out of the nucleus (for reviews see: Stein and Cohen, 1988; Hélene and Toulmé, 1990).

Inhibition of gene expression by antisense oligonucleotides depends on several factors such as the target sequence on mRNA, the copy number of specific mRNA being targeted, the type of the targeted cells, and the type of ODN used. The ability of an antisense oligonucleotide to function depends on: (i) the stability of the oligonucleotides in the medium and inside the cell, (ii) the ability of the oligonucleotides to enter the cells, and (iii) the ability of the oligonucleotides to hybridize stably and specifically to the target sequence.

To further investigate the involvement of protein F1 in neurite outgrowth in cultured spinal neurons, we applied antisense oligonucleotides to newly seeded spinal neurons derived from fetal tissue (E13-14), to interfere with protein F1 mRNA processing/translation, and monitored neurite outgrowth. In the present study, two oligonucleotide pairs (18 mer each) were used with sequences that overlap the translation initiation site but one pair had a thio-modified backbone.

In this study, unmodified and phosphorothioate-substituted oligodeoxynucleotides were believed to interfere with protein synthesis via blockade of ribosomal activity or RNase H-mediated degradation of mRNA or both. Thio-modified oligonucleotides were applied directly to the medium and thought to enter the cells via receptor-mediated endocytosis (Loke et al., 1989). On the other hand, unmodified oligonucleotides were introduced into the cells by different methods.

Phosphorothioates antisense ODN are characterized by their resistance to the action of several cellular and serum nucleases and have higher affinity to cross the membrane lipid bilayer. HPLC-purified thio-modified ODN were added (final concentration of 4 μM) to newly cultured neurons seeded on laminin and PDL and did not affect neurite elongation or cell morphology.

Unmodified antisense ODN were first introduced into spinal neurons using Transfectam (0.3 mg/ml, Promega) and extensive cell death was observed. ODN were then

delivered into the cells during trituration as shown by labeling of neurons with fluorescein-labeled oligonucleotides (Fig. 31). Antisense oligonucleotides (final concentration of 200 μM) were introduced into murine spinal neurons seeded on PDL only. No effect on neurite outgrowth or morphology of primary neurons derived from fetal mouse spinal cord was noticed. Development of neurons treated with sense or antisense ODN was comparable to that of non-treated controls.

The concentration of antisense ODN used in this study might have not been enough at that particular stage (E14) to deplete the spinal neurons of protein F1 whose expression at this stage was probably very high. Aigner and Caroni (1993) reported that they had to culture neurons from E16 chicks as they could not deplete younger neurons (that have higher ability to regenerate neurites in culture) of protein F1. The presence of glial cells (in the spinal neuronal cocultures), either through cell-cell contact or through growth factors released by glial cells (as NGF) may have provided extra induction of protein F1 expression and masked the effect of protein F1 antisense ODN on neurite formation. Other experiments that correlate protein F1 to neurite outgrowth have been performed on either cell lines or primary neurons depleted from non-neuronal cells. Another possibility is that the selected ODN sequence might not have hybridized correctly or that sequence at the 5' end of protein F1 mRNA might have not been a good site to induce translational arrest. No effect on neurite formation by spinal neurons was induced upon the application of: (i) thio-modified antisense ODN (that resist nuclease action) at low concentration, and (ii) unmodified antisense ODN (that are subjected to rapid degradation by serum and cellular nucleases) at very high concentration. One remaining possibility, though very expensive, is to apply thio-modified antisense ODN at high concentrations (up to 50-100 μM) to cells in culture.

The effect of antisense ODN observed in this study might be related to the coating materials on which neurons were seeded. It has been reported that antisense ODN against

protein F1 mRNA were introduced by trituration into dorsal root ganglion (DRG) neurons of the chick and changed neurite elongation and morphology in a substrate-dependent manner (Aigner and Caroni, 1993). DRG neurons seeded on laminin only and treated with protein F1 antisense ODN, extended neurites that were thinner and less branched with smaller growth cones than neurites extended by the non-treated cells. Laminin is a strong promoter of neurite outgrowth of DRG neurons (Rogers et al., 1983) and its interaction with the growth cones was demonstrated to involve PKC activation (Bixby, 1989). On the other hand, antisense-treated DRG that were plated on polyornithine substratum failed to form neurites (Aigner and Caroni, 1993).

In one experiment, camera lucida technique was used to compare number of neurons, number of neurites per neurons, and neurite length among cultures treated with antisense or sense ODN and controls. Variations of not more than 20% were observed between cultures and could not be related to the effect of the antisense ODN as similar variations were demonstrated between control cultures due to differences in seeding procedures.

In summary, polyclonal antibodies against the C-terminus of protein F1 have been produced and characterized. The peptide was shown to be a strong immunogen and the antibodies were shown to recognize both the phosphorylated and non-phosphorylated forms of protein F1.

A new *in vitro* PKC phosphorylation of Ser-210 of protein F1 was reported. Phosphorylation of Ser-210 by PKC was shown to be concentration-dependent with Km of 0.25 μM.

The produced antibodies stained spinal neurons but not glial cells in mature cultures derived from embryonic spinal tissues (E-14). Ultrastructural localization of protein F1 in mature spinal cultures showed gold particles specifically associated with intra-cellular compartments including presynaptic terminals and axon-like neurites.

Protein F1 levels were assayed during the development of spinal neurons in culture and *in vivo*. A peak of protein F1 was shown after 2 days in cultures (derived from E14) compared to high levels at P1 in spinal tissues *in vivo*.

Positively charged liposomes were characterized as a potential delivery system of macromolecules into primary mammalian neurons. Uniform, non-toxic, and preferential interaction of liposomes with spinal neurons over glia was established. Since rhodamine was covalently bound to liposomal phospholipids, the uniform distribution of rhodamine in the living neurons as well as the association of this dye with membrane debris of dead neurons imply that the liposomes may have fused with the neuronal membranes and did not enter these cells via endocytosis. Polyclonal anti-protein F1 antibodies produced against the C-terminus of the protein and delivered into newly seeded spinal neurons (derived from E14) via liposomes, did not affect neurite elongation or neuronal development.

No interruption of neurite formation or effect on the development of spinal neurons *in vitro* were demonstrated upon treatment of spinal neurons with antisense ODN under the following conditions: (i) the cultures used contain neurons and non-neuronal glial cells, (ii) neurons were dissociated from fetal tissues (E14), (iii) cells were seeded on PDL-coated coverslips, (iv) ODN were introduced into neurons during trituration at a final concentration of 300 μM, and (v) antisense ODN had complementary sequences to murine protein F1 mRNA 5' end that overlap its start codon.

CHO cells stably transfected with a protein F1 expression vector produced long and protein F1-immunoreactive filopodial processes (Zuber et al., 1989). On the other hand, PC12 cells deficient in protein F1 and full length protein F1 mRNA were able to extend neurites in response to NGF and bFGF (Baetge and Hammang, 1991). Transfection of PC12 with a recombinant expression vector coding for antisense protein F1 inhibited the evoked release of dopamine from these cells, however, no effect on neurite formation was reported (Ivins et al., 1993). Cultured chick dorsal root ganglia neurons, depleted of

protein F1 using antisense oligonucleotides, extended longer and thinner neurites when plated on laminin and failed to form neurites when plated on polyornithine (Caroni and Aigner 1993). Failure of cultured neurons to form neurites when plated on poly-L-ornithine (compared to laminin substratum) may be explained by absence of a strong neurite outgrowth signal from the adhesion material. In this report, primary mammalian neurons treated with antisense oligonucleotides to protein F1 mRNA 5'end and plated on poly-D-lysine extended normal neurites comparable to those of control cells.

Protein F1 has been shown to interact with Go protein enriched in the presynaptic membranes and act as an intracellular effector to increase the transduction of G-protein coupled receptors (Strittmatter et al., 1993). In addition, protein F1 was shown to play an important role in neurotransmitter release (De Graan and Gispen, 1993) due to its presynaptic localization in mature brains (Campagne et al., 1990) and its correlation with several signal transducing mechanisms (for a review see Skene, 1989). Based on the previous reports and the data obtained in this study, protein F1 might not be essential for the extension and maintenance of neurites. The main physiological role of protein F1 may include modulation of growth cone and synaptic functions.

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