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**Molecular and biochemical characterization of NP185, a
neuronal-specific, synapse-enriched, and clathrin coated-vesicle
associated phosphoprotein**

Li, Shengwen, Ph.D.

City University of New York, 1994

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**MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF NP185, A
NEURONAL-SPECIFIC, SYNAPSE-ENRICHED, AND CLATHRIN COATED-
VESICLE ASSOCIATED PHOSPHOPROTEIN**

by

Shengwen Li

A dissertation submitted to the Graduate Faculty in Biomedical
Sciences in partial fulfillment of the requirements
for the degree of Doctor of Philosophy,
The City University of New York

1994

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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF NP185, A
NEURONAL-SPECIFIC, SYNAPSE-ENRICHED, AND CLATHRIN COATED-
VESICLE ASSOCIATED PHOSPHOPROTEIN

by

Shengwen Li

Advisor: Professor Saul Puszkin, Ph.D.

NP185 was previously discovered as a neuronal protein associated with brain clathrin-coated vesicles (CCVs) using two monoclonal antibodies (mAbs), 8G8 and 6G7 (Kohtz and Puszkin, 1988). Further biochemical studies displayed that the NP185 protein binds to synaptic vesicles (SVs), synaptosomal plasma membranes (SPM), decoated vesicles, clathrin light chains (CLCs) and tubulin, and its binding affinity for tubulin and CLCs are regulated by two different protein kinases (Kohtz and Puszkin, 1989; Su et al., 1991). Developmental expression of the NP185 molecules follows a pattern consistent with avian cerebellar synaptogenesis, and enriches in synaptic terminals (Perry et al., 1991).

Evidence presented in this thesis demonstrates the presence of NP185 in neuronal cells, not only in the central nervous system, but also in the peripheral nervous system,

particularly in the neuromuscular junction of avian striated muscle. By using indirect rhodamine immunofluorescence of NP185 mAb for NP185 protein localization combined with fluorescein- α -bungarotoxin to mark the postsynaptic site of acetylcholine receptors (AChRs), it was shown that the distribution of both NP185 and AChRs are correlated, but not overlapped, NP185 is in the presynaptic site of neuromuscular junction. That was further supported by SDS-PAGE and Western blot analyses using the sample of immunoprecipitation of NP185 protein from striated muscle.

Molecular analyses described here in this thesis aim to establish the identity of NP185 and its relation to other known proteins. After successful purification the NP185 protein was microsequenced. Internal peptides obtained from the purified NP185 by proteolytic digestion showed that clear homologies to internal amino acid sequences of a mouse brain phosphoprotein, F1-20. A series of experiments were performed to determine the relation between NP185 and F1-20 on the structural and functional level. The mAbs, 8G8 and 6G7, raised against NP185, as well as mAb against F1-20 react with a CCV protein with molecular mass of 185-190 kDa by immunoblot analysis of SDS-PAGE. Partial cDNA sequences from immunoscreening overlapped with F1-20 cDNA, while Northern blot analyses showed that NP185 mRNA is brain-specific, consistent with that of F1-20. While F1-20 mAb binds to immunoaffinity purified NP185; NP185 mAbs, 8G8 and 6G7, recognize the GST-F1-20 fusion protein expressed in *E.coli*, which was glutathione S-transferase (GST) fused with the

entire ORF of F1-20 cDNA obtained by reverse transcriptase PCR. Functional analyses of the bacterially expressed protein showed that it induces assembly of clathrin into basket structures (Li et al., 1993). In addition the bacterially expressed GST-F1-20 fusion protein binds to brain tubulin. The tubulin binding domain was mapped to 124 residues, and epitopes for both 8G8 and 6G7 mAbs mapped to 60 residues of deduced amino acid sequence from F1-20 cDNA by truncation and PCR domain amplification (Li et al., 1994). Based on these immunological, biochemical and functional data, NP185 is nearly identical to F1-20.

The above data and observations made by the others implicate NP185 as a multifunctional protein in synaptic terminals involved in docking, signalling, and molecular adaptor function. A testable model is introduced.

PREFACE

Doctoral Thesis---Dissertation---derived from the Latin dissertare "to discuss". The complex origin of the Latin word (dis, apart, and serere, to join) suggests that a dissertation might be visualized as "bring together, in proper order, a set of disparate ideas or facts".

.....Ebel HF, Bliefert C, and Russey WE.

Science today, is the continuation of the work that performed yesterday. When I first came here at the Mount Sinai School of Medicine, the Graduate Director of the Cellular and Molecular Pathology Program, Professor Saul Puszkin, Ph.D., said to me, "Science carries on a tradition". D. Stave Kohtz II, a gifted student in Dr. Puszkin's laboratory, now an Assistant Professor at Mount Sinai, discovered NP185 protein by accident. While making mAbs against crude extract of clathrin coated-vesicle (CCV) proteins, he noticed that two mAbs, did not recognize clathrin. Following this observation, he was able to find a new protein, named "NP185". This protein was found to co-migrate with a huge mass of clathrin heavy chain (CHC), 180 kDa, on SDS-PAGE. "NP" is the abbreviation used for neuron-specific protein, and "185" for the molecular weight 185 kDa that was determined by migration on SDS-PAGE (Kohtz and Puszkin, 1988). Further studies showed that NP185 can be induced by NGF in PC12 cells (Kohtz and Puszkin, 1988; Milbrandt, 1987); also that, it binds to tubulin in a phosphorylated manner (Kohtz and Puszkin, 1989).

Following the legacy, Borcheng Su, M.D., found that NP185 was associated with clathrin light chains (CLCs) during

purification of the NP185 protein through immuno-affinity chromatography. He was carefully following up the observation that NP185 protein was always co-eluted with CLCs from NP185 mAb immunoaffinity column. This discovery aided his thesis research on the biochemical characterization of NP185 protein. He found that NP185, stimulates clathrin cage formation, binds to CLCs in a phosphorylated manner, also binds to decoated CCVs, synaptic vesicles (SVs) and synaptosomal membranes (Ph.D., 1990; Su et al., 1991).

Douglas G. Perry, now an Associate Professor at Indiana University, was pursuing developmental expression of the NP185 protein in chick and mouse brain. Although "initially, this involved a great deal of preliminary work, to determine antibody cross-reactivities, embryonic expression, and proper conditions for immunohistochemistry" (Perry, 1991); finally he made important findings, which reveal that the expression of NP185 protein coincides with synaptogenesis, and that NP185 is synapse-specific (Ph.D., 1991; Perry et al., 1991, 1992).

When I came in Dr. Puszkin's lab in September 1990, Douglas was in the final stages of putting his data in a thesis. Being trained in biochemistry and molecular biology, I was curious to know how to make those beautiful fluorescent pictures. In my rotation in the lab, Douglas showed me how to incubate chicken eggs to lovely chicks, get embryos and brains; and how to make cryostat section; as well as how to perform immunofluorescence. It turned out that, NP185 was found to be in neuromuscular junction by using the Axiomat microscopy (\$250,000) in Physiology Department (Perry, Li,

Hanson, and Puszkin, 1992; Puszkin, Perry, Li and Hanson, 1992).

Taking together, obviously, NP185 is very important multifunctional protein, which was quite interesting to me. What was next? I was really curious and eager to know the molecular makeup of NP185, however, I didn't know how? I was not sure whether I could do it on my own, since it took a tremendous amount of courage and hard work to establish a molecular laboratory, as well as to work out all doable protocols for molecular biology techniques. Now, it is the time to summarize my line of research in Dr. Puszkin's laboratory (Li et al, 1992; 1993, 1994).

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LIST OF ABBREVIATIONS

aa:	amino acid residue in polypeptide
α -BT-F:	alpha-bungarotoxin conjugated to fluorescein
Ache:	acetylcholine receptor
Amp:	ampicillin
AP180:	assembly polypeptide 180 kDa
BCIP:	5-bromo-4-chloro-3-indolyl phosphate
BSA:	bovine serum albumin
CaM kinase II:	Ca ²⁺ /Calmodulin-dependent protein kinase II
cAMP:	cyclic adenosine monophosphate
CAPs:	clathrin-associated proteins (CLCs)
CAP ₁ :	clathrin-associated protein (36 kDa) (CLCa)
CAP ₂ :	clathrin-associated protein (33 kDa) (CLCb)
cDNA:	complementary DNA
CHCl ₃ :	chloroform
CV:	coated vesicle
CCVs:	clathrin coated vesicles
CHCs:	clathrin heavy chains
CKII:	casein kinase II
CLCs:	clathrin light chains (CAPs)
CNS:	central nervous system
DHS:	donor horse serum
DMEM:	Dulbecco's modified Eagle medium
DMSO:	dimethylsulfoxide
DTT:	dithiothreitol
EDTA:	ethylenediamine tetraacetic acid

EGTA:	ethylenebis(oxyethylenenitrilo)tetraacetic acid
ELISA:	enzyme-linked immunosorbent assay
EM:	electron microscopy
EtBr:	ethidium bromide
EtOH:	ethanol
FCS:	fetus calf serum
FITC:	fluorescein isothiocyanate
FP:	fusion protein
gDNA:	genomic DNA
GST:	glutathione S-transferase
GABA:	gamma-aminobutyric acid
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG:	isopropylthio- β -D-galactoside
kDa:	kilo-dalton
LDCVs:	large dense-cored vesicles
LN ₂ :	liquid nitrogen
mAb:	monoclonal antibody
mAbs:	monoclonal antibodies
MeOH:	methanol
MEM:	minimum non-essential amino acids
MES:	2-(N-morpholino)-ethanesulfonic acid, pH 6.5
mg:	milligram
ml:	milliliter
mM:	millimolar
NGF:	nerve growth factor
NGS:	normal goat serum
NBT:	nitro blue tetrazolium chloride
NP185:	neuron-specific protein 185 kDa

nt:	nucleotides
NTs:	neurotransmitters
OCT:	optimum cutting temperature compound: 10% polyvinyl alcohol, 5% carbowax
o.n.:	overnight
ORF:	open reading frame (encoding region)
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline: 19 mM NaH_2PO_4 , 81 mM Na_2HPO_4 , 150 mM NaCl, pH 7.4
PCR:	polymerase chain reaction
pers. comm.:	personal communication
PKA:	protein kinase A
PKC:	protein kinase C
PMSF:	phenylmethylsulfonyl fluoride
PNS:	peripheral nervous system
RT:	room temperature
RT-PCR:	reverse transcriptase polymerase chain reaction
SDS:	sodium dodecyl sulfate
SDS-PAGE:	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPM:	synaptic plasma membrane (synaptosomal plasma membrane)
SSVs:	small synaptic vesicles
SV:	synaptic vesicle
SVs:	synaptic vesicles
TBS:	tris buffered saline: 50 mM Tris, 150 mM NaCl, pH 7.4
TBS/BSA:	TBS/0.1% bovine serum albumin

TBS/Tween: TBS/0.1% Tween-20

TEMED: N,N,N',N'-tetramethylethylenediamine

Tris: Tris-(hydroxymethyl) aminomethane

ug: microgram

ul: microliter

um: micrometer

X-gal: (5-bromo-4-chloro-3-indolyl- β -D-galactoside)

INTRODUCTION

One of the most important features of mammalian nervous system is the site neuronal cells provide for the conduction and transmission of electrochemical signals, which is called synapse (Erulkar, 1989). These signals are transmitted from one cell to another at the synapses (Smith and Augustine, 1988). Most of the synapses in the mammalian cerebral cortex are components of local circuits. Not only do these local, or intrinsic, connections dominate in numerical terms, but such synapses are directly responsible for generating neuronal codes that convey sensory data and elicit behavior (Katz and Callaway, 1992).

Studies of the proteins found exclusively at synapses and of specialized function are an important premise for understanding the molecular architecture of the nerve terminal (Sudhof and Jahn, 1991; Trimble et al., 1991; Puszkin et al., 1992). So, many investigators have been driven to make tremendous efforts to isolate the nerve terminal-associated molecules, included the followings (See review, Jessell and Kandel, 1993; Kelly and Grote, 1993; Jahn and Sudhof, 1994): synapsin Ia,b/protein Ia,b (Johnson et al., 1971, 1972; De Camilli et al., 1983a,b,c; Sudhof et al., 1989b); p65/synaptotagmin (Fournier and Trifaro, 1988); tor70 (Carlson and Kelly, 1983); SV2 (Buckley and Kelly, 1985); p29 (Pfeffer and Kelly, 1985; Baumert et al., 1990); synaptophysin/p38

(Jahn et al., 1985; Wiedenmann and Franke, 1985); mediatophore (Israel et al., 1986); synapsin IIa,b/protein IIIa,b (Browning et al., 1987; Sudhof et al., 1989b); VAMP-1,2/synaptobrevin (Trimble et al., 1988, 1990; Baumert et al., 1989; Elferink et al., 1989; Sudhof et al., 1989a); synuclein (Maroteaux et al., 1988); VAT-1 (Linial et al., 1989); NT75 (Ritchie et al., 1989); synaptoporin (Knaus et al., 1990); SVAPP-120 (Bahler et al., 1991); SNAP-25 (Oyler et al., 1989; Geddes et al., 1990); and NP185/F1-20/AP180 (Kohtz and Puszkin, 1988; Su et al., 1991; Perry et al., 1991, 1992; Puszkin et al., 1992; Li et al., 1992; Ahle and Ungewickell, 1986; Morris et al., 1993; Sousa et al., 1992; Zhou et al., 1992, 1993; Li et al., 1994).

The early studies, however, have been focused only on the identification and isolation of synaptic molecules. The functional understanding of those synapse-specific molecules is still in its infancy. In particular, the molecular events involved in synaptic vesicle synthesis, transport, storage, release, and recycling, as well as in activity-dependent changes in synaptic efficacy, are less clearly understood (Sudhof and Jahn, 1991; Trimble et al., 1991; Jessell and Kandel, 1993; Unwin, 1993; Kelly, 1993; Bennett and Scheller, 1994). Some recent studies have provided insights into the mechanism of synaptic function (Thomas et al., 1988; Thomas and Betz, 1990; Han et al., 1991; Alder et al., 1992a,b; Lu et al., 1992; and Greengard et al., 1993). Still, there are more to be done for the understanding of function of synapse (Monck and Fernandez, 1994; O'Connor et al., 1994; Ferreira et al., 1994; Bennett and Scheller, 1994).

1. *Neurons and Synapses*

The human brain consists of about 10^{11} nerve cells. These nerve cells can be subdivided into about 1000 different types. Further complexity results from the way in which the component cells of the brain interconnect and function (Goodman and Shatz, 1993). Neurons are highly differentiated cells which display an elaborate regionalization of cytoskeletal and cell surface components throughout their various dendritic, axonal and nerve terminal (Goldman-Rakic, 1992). The general structural features of neurons are the cell body, dendrites, axons and the nerve terminal. The dendrites and cell body make up the input component of the neuron, the axon is the long-range signaling component and the presynaptic terminal is the output component (Kandel et al. 1991; Goldman-Rakic, 1992).

Interactions between neurons are largely restricted to specialized sites where one nerve cell comes into appropriate apposition with its target cell, e.g., neurons, gland cells or muscle cells. Axons and dendrites emerging from different neurons are intercommunicated by specialized junctional complexes known as synapses, a term proposed by Charles Sherrington in 1897 (Kandel et al., 1991).

However, it was Santiago Ramon y Cajal (1911), who demonstrated that all synapses had two conserved elements: a presynaptic terminal and a postsynaptic target site. At the time, he also referred the existence of a third element, the synaptic cleft, the space between the presynaptic and

postsynaptic elements of the synapse (Kandel et al., 1991). Today, we know that, these three elements underlie the ability of neurons to communicate with one another, the process of synaptic transmission. The intricate patterns of synaptic connections formed during the development of the brain, underlie all aspects of neural function, from sensory, perception and movement to learning and memory (Kandel et al. 1991; Goldman-Rakic, 1992; Kandel and Hawkins, 1992; Jessell and Kandel, 1993; Goodman and Shatz, 1993).

Despite the advent of molecular genetics in neurobiology, our understanding of the functional relationships of the components of the CNS remains in its infancy, particularly in the areas of the cellular interaction and synaptic modulation (Erulka, 1989), and even much less in the area of molecular events at synapses (Jessell and Kandel, 1993). Much progress has been made recently in studying on three aspects of the synaptic transmission (Bennett and Scheller, 1994), the plasticity of synapses (Patterson and Nawa, 1993), and the assembly of synapses during development (Kandel et al., 1991). It has been shown that these three processes are regulated by the bidirectional signaling of the reciprocal synapses that mediate retrograde as well as anterograde signals (Jessell and Kandel, 1993).

2. Chemical Synapses and Electrical Synapses

In terms of physiological typing, three groups of synapses are recognized: excitatory, inhibitory, and

modulatory. On the other hand, according to the types of transmissions, either chemical or electrical, they can also be divided into chemical synapse and electrical synapses (Kandel et al., 1991).

At chemical synapses, the presynaptic and postsynaptic membranes are separated by a 20- to 100-nm-wide synaptic cleft which is filled with extracellular fluid and prevents direct electrical excitation of the postsynaptic cell. Chemical synapses exhibit synapse plasticity, i.e., the efficacy of synaptic transmission can be modulated by a variety of metabolic factors and chemical agents.

In the electrical synapses, the unit membranes are closely apposed, and, indeed, the outer leaflets sometimes fuse to form a gap junction where a depolarizing current can pass from cell to cell across the regions of membrane without chemical mediation. Unlike chemical synapses, electrical synapses are designed for rapid all-or-none signal transmission, which are rare in higher vertebrates.

Sometimes, electrical synapses exist at terminals that also display typical chemical synapses; in such cases, the structure is referred to as a mixed synapse (Kandel et al., 1991).

3. *SSVs and LDCVs*

Synaptic transmission across synapses is mediated by the interaction of chemical signals released from the presynaptic vesicles with postsynaptic receptors. In presynaptic

terminals, there are at least two types of vesicles: small synaptic vesicle (SSV) and large dense-cored vesicle (LDCV) depending on their appearance in the electron-microscope (Thureson-Klein and Klein, 1990).

SSV, small synaptic vesicles with about 20 to 60 nm in diameter, contains the neurotransmitters (NTs), like acetylcholine (Ach), glutamate, GABA, and glycine (Kelly, 1993). The SSV is a specialized organelle in the terminal where it is involved in the focal release of NTs. Chemical neurotransmission occurs via the regulated exocytosis of SSVs in nerve terminals. SSVs contain a unique set of membrane proteins such as synapsin, synaptophysin, synaptobrevin, synaptotagmin, and also the small GTP-binding protein rab3A (Jessell and Kandel, 1993; Bennett and Scheller, 1994). SSVs are thought to originate from the trans-Golgi network and to be transported to the axonal terminal where they undergo repeated cycles of exo-endocytosis (Kelly, 1993; De Camilli and Jahn, 1990; Trimble et al., 1991; Sudhof and Jahn, 1991).

In addition to SSVs, there is a second class of secretory vesicles, large dense-core vesicles (LDCV), which are about 100 nm in diameter, and contain peptides and proteins (Kelly, 1993). LDCVs are similar in morphology, biogenesis, content, membrane proteins to secretory granules of non-neuronal cells (e.g., endocrine cells) and may be the neuronal equivalents of such organelles (De Camilli and Jahn, 1990). In contrast to SSVs, LDCV exocytosis is not restricted to active zones (Thureson-Klein and Klein, 1990). Nevertheless, unlike SSVs, which are now the best characterized secretory organelle,

little is known about the protein composition of LDCVs.

Sometimes, paralleling the release of neurotransmitter from SSVs is the release of neuropeptide from LDCVs. (Thureson-Klein and Klein, 1990).

4. Exocytosis and Neurotransmitter Release

When an action potential reaches the nerve terminal, only a fraction of SVs are docked at release sites, with the remaining vesicles present in reserve pools that are clustered in the vicinity of the release sites (Mulkey and Zucker, 1991). It has been proposed that NTs release involve at least four steps (Matteoli and De Camilli P, 1991; Kelly, 1993):

(1) the transport (or mobilization) of vesicles from the reserve pool to the releasable pool at active zone.

(2) the docking of vesicles to their release sites at active zones.

(3) the fusion of the synaptic vesicle membrane with the plasma membrane during exocytosis to release NT into presynaptic cleft in response to the Ca^{2+} influx (Smith and Augustine, 1988; Unwin, 1993).

(4) the retrieval and recycling of vesicle membrane following exocytosis via endocytosis.

Several proteins have been cloned and characterized, which may participate in different aspects of this release-recycling cycle (Jahn and Sudhof, 1994). As might be expected from steps described above, these proteins can be divided into

four groups as following: Synapsins may control the mobilization of vesicles; Rab3, a member of the p21 ras superfamily, may involve the intracellular trafficking and docking of synaptic vesicles; synaptotagmin, the VAMPs, neurexins, and syntaxins may involve more specifically in docking vesicles into release sites and in forming fusion pore on the presynaptic membrane; and synaptophysin may involve the formation of the vesicle fusion pore or in recycling (Bennett and Scheller, 1994).

The best-characterized of these proteins are synapsins, a family of proteins that binds to actin as well as to SVs, which is highly specific to nerve terminals (Sudhof et al., 1989; De Camilli and Jahn, 1990; Han et al., 1991; and Greengard et al., 1993). The synapsins may restrain vesicles mobilization by causing the vesicle to associate with actin cytoskeleton. The synapsins can be phosphorylated by both Ca^{2+} /Calmodulin-dependent protein kinase II (CaM kinase II) and protein kinase A (PKA) (Johnson et al., 1972). CaM kinase II also associates with synapsins (Benfenati et al., 1992). Phosphorylation of synapsin I by CaM kinase II decreases its binding to both actin and to the SVs; dephosphorylation increases its binding. Interestingly, injection into the terminal of the squid giant synapse of the dephosphorylated (or vesicle binding) form of synapsin I causes a gradual reduction of both evoked and spontaneous release. Nonetheless, injecting CaM kinase II into the presynaptic terminal enhances release (Llinas et al., 1991).

Consistent with a putative general role for small

molecular weight G proteins in membrane targeting and function, a member of this superfamily, rab3A (Zarahoni et al., 1989; Matsui et al., 1988), has been detected on SVs. Rab3A is expressed only by neuroendocrine cells and is concentrated in nerve endings where it is associated with the cytoplasmic surface of SVs. It appears to dissociate from the vesicle membrane following exocytosis (Matteoli et al., 1990; Fisher et al., 1990).

VAMP, also called synaptobrevin, may mediate docking vesicles at the active zone. There is direct evidence that cleavage of synaptobrevin by zinc-dependent endopeptidase blocks NTs release (Schiavo et al., 1992).

Syntaxins, a plasma membrane-associated protein, may mediate the docking of vesicles at release sites by interacting with both N-type Ca^{2+} channels in plasma membrane and with synaptotagmin on the SV membrane (Bennett et al., 1992, 1993).

Synaptotagmin (p65), an integral membrane protein of the SV, has several domains in its cytoplasmic carboxyl-terminal related to domains of protein kinase C (PKC) (Sudhof and Jahn, 1991; Trimble et al., 1991). These domains bind to phospholipid in the presence of elevated Ca^{2+} . In the presence of Ca^{2+} , synaptotagmin binds to the neurexins, the synaptic plasma membrane receptor for alpha-latrotoxin, a toxin that triggers the release of SVs (Ushkaryov et al., 1992). Thus, synaptotagmin is thought to induce exocytosis in response to a rise in Ca^{2+} .

Synaptophysin, another integral membrane synaptic vesicle

protein, shares structural features with gap junction channels (Buckley et al., 1987; Thomas et al., 1988; Sudhof and Jahn, 1991). It contains four putative transmembrane segments and can form a channel or fusion pore when incorporated in planar lipid bilayers (Almers and Tse, 1990; White, 1992). Consistent with this, blockade of the expression of synaptophysin by antisense oligonucleotide or by antibodies appears to block transmitter release (Alder et al., 1992a,b). It may also have other roles, e.g., in vesicle recycling.

5. "Secrets of Secretion Revealed" (Science, Nature, 1993)

As described in recent Science News (April 23, 1993, Baringa) and a Nature article and a letter (March 25, 1993; Warren G; Sollner et al., 1993; Whiteheart et al., 1993) , an extraordinary confluence of genetics, cell biology and neurobiology, has revealed that the same mechanisms govern key communications processes in cells from yeast to neurons, which has been a landmark breakthrough in these fields (O'Conner et al., 1994; Monck and Fernandez, 1994; Bennett and Scheller, 1994).

"What is really exciting...is how all these different fields of vesicular traffic are coming together" (Pietro De Camilli).

The findings "show the extraordinary universality of molecular constituents," "We are dealing with a molecular machine in the brain that about as distinctive as you could get... and yet when you find that a surprisingly large number of them are shared...not only with other parts of the cell, but with all organisms, all the way down to yeast." (Eric Kandel).

For a decade, geneticists, cell biologists, and neuroscientists have been approaching the question independently, using experimental systems ranging from yeast to the mammalian brain. It was assumed that, although the general scheme of vesicle trafficking was similar in these systems, the precise molecular mechanisms might well turn out to be quite different, i.e., it might be mediated by unrelated proteins (Bennett and Scheller, 1994). The available observations show that, NSF (for *N*-ethylmaleimide sensitive fusion protein, SEC-18 in yeast), SANPs (for soluble NSF attachment proteins) and Rab are all cytoplasmic proteins that associate transiently with membranes (Jessell and Kandel, 1993). However, it was believed, there must be more proteins related to the process. The hypothesis was that, there must be proteins permanently embedded in the membranes to act as markers and anchors for the cytoplasmic proteins to recognize and latch onto (Kelly and Grote, 1993). It was further reasoned that, these integral membrane proteins must take two forms: one for the vesicle membrane, one for the target, or "acceptor" membrane (Schekman, 1992; Kelly and Grote, 1993).

When the molecular cloning of syntaxin was reported (Bennett et al., 1992), there were no homologs in the DNA database. However, 6 months later, there were three homologous genes identified in yeast. SED5, which in yeast is essential for vesicle transport from the ER to the Golgi, was found not on the vesicle membrane, but on the acceptor membrane. SED5 is the only known integral membrane proteins on the acceptor side

of fusion events. This implies that if syntaxin resembles SED5, and resides in the acceptor membrane as SED5 does, then it probably plays a role in trafficking, as SED5 does (Bennett and Scheller, 1993).

At the same time, Jeffrey Gerst (now at Mount Sinai School of Medicine), found a pair proteins, SNC1 and 2, which are 40% identical to the mammalian vesicle-associated membrane protein known as VAMP (Gerst et al., 1992). Genetic experiments in yeast showed that the SNCs are essential for secretory vesicles to fuse with the plasma membrane. Schiavo et al.(1992), found that VAMP is the target of two lethal neurotoxins, tetanus and botulinum toxin, both of which are known to do their damage by blocking neurotransmission.

In summary, recent findings suggest a model for vesicle docking and fusion (Sollner et al., 1993; Bennett and Scheller, 1994). The model implies that, there is a set of membrane-bound proteins and a set of soluble cytoplasmic proteins, that work together as a unit to mediate vesicle transport in cell ranging from yeast to neurons. The data show that soluble proteins NSF and SNAPs (or their yeast counterparts Sec18 and Sec17) are common to fusion complexes throughout the cell, while the integral membrane proteins VAMP and syntaxin represent protein families whose members are located on different types of vesicles and take part in specific kinds of vesicle fusion (Sollner et al., 1993; Bennett and Scheller, 1993, 1994; O'Connor et al., 1994; Monck and Fernandez, 1994).

Despite the elegance of this model, however, it would be

an oversimplification to conclude that secretion in neurons is simply a replica of the process in yeast (Jahn and Sudhof, 1994). Neurons still have key distinctions, since NT releasing is highly regulated (Barinaga, 1993; Brose et al., 1992; Bennett and Scheller, 1994). Therefore, it would be reasonable that, further growth in the number of proteins which are found to be in, or associated with, the complex. In addition, little is known about the formation and dissociation of the complex, as well how it is regulated.

6. Endocytosis and Vesicle Recycling

After exocytosis, SVs may be regenerated by a neural-specific mechanisms of endocytosis at the nerve terminal and refilled locally with NT by an active transport process (Linstedt and Kelly, 1991; Puszkin et al., 1992; Bennett and Scheller, 1994). Although endocytic processes occur in all cells (Brodsky, 1988), the extremely high levels of membrane expansion occurring during neuronal activity might necessitate the establishment of specialized processes for synaptic membrane recycling (Puszkin et al., 1992). Hence, vesicle membrane is probably recycled directly at the synaptic plasma membrane and is simply refilled with NT to undergo further rounds of exocytosis (Puszkin et al., 1992).

Evidence comes from studies using conventional electron microscopy, freeze fracture analysis of the plane of the presynaptic plasma membrane and immunochemical analyses. However, these studies show that nerve terminals are not

different from neurosecretory or other endocrine systems (Heuser and Reese, 1973; Heuser, 1989).

Recently, direct evidence has emerged from biochemical and immunogold-EM analysis using specific markers (Maycox et al., 1992). It was shown that the membrane composition of nerve terminal-derived CCVs was very similar to that of SVs. Furthermore, immunogold EM revealed that virtually all CVs carried SV proteins, demonstrating that the contribution by CVs derived from other membrane traffic pathway is negligible. In addition, nerve terminal derived CVs contained AP-2 adaptor complexes, which is in agreement with their plasmalemmal origin. Also, SVs and CCVs contain the neuronal-specific proteins, NP185/AP180 and auxillin (Maycox et al., 1992). Taken collectively, together with previous data, indeed, membrane recycling through exo-endocytosis processes has been established (Linstedt and Kelly, 1991).

In general, it is now well known that CCVs contain clathrin, adaptors, and receptors in the membrane of the vesicle, and their ligands in the interior (Pearse and Crowther, 1987; Pearse and Robinson 1990). After a ligand binds to a receptor on the plasma membrane, the receptor-ligand complex is internalized in clathrin-coated pits that pinch off to become a CCV, which mediates intracellular transport processes (Goldstein et al., 1985; Brodsky, 1988).

Clathrin triskelion is composed of three identical heavy chains (CHCs) and three light chains (CLCs) in a forms of $CLCa_2b$ or $CLCab_2$ (Kirchhausen and Harrison, 1981). The CHC, forming the backbone of the triskelion, imparts the polyhedral

organization to coats and cages, which are likely to have a role that is more structural than dynamic (Jackson et al., 1987; Kirchhausen et al., 1987a). Its sequence has been shown to be well conserved (Kirchhausen et al., 1987a). On the other hand, the CLCs are very diverse in terms of sequences both in cDNA and in protein level (Jackson et al., 1987; Kirchhausen et al., 1987b).

Adaptors, as termed by Pearse and Bretscher (1981) for a family of molecular units that would sort out those molecules which are to travel in clathrin cages from those that are to remain behind. Adaptors are also defined as assembly polypeptides (APs) (Keen, 1990) or clathrin associated protein (Zaremba and Keen, 1983). They were assumed: (a) to interact with clathrin on the cytoplasmic side of the membrane, (b) to recognize specifically an address ticket carried by a receptor, and (c) perhaps to have some signal indicating to which organelle they should go (Pearse and Robinson, 1990).

Two distinct adaptors can be separated from each other by hydroxyl-apatite chromatography and hence have been termed the HA-1 and HA-2 adaptors (Pearse and Robinson 1984; Keen, 1987), which also refer to AP1 and AP2 (Keen, 1990). The HA-2/AP2, which is the more abundant form in brain preparations, is a heterotetramer with an apparent molecular weight of 270 kDa. It consists of an alpha- and beta-adaptin (subunits of 100-110 kDa), and two polypeptides of 50 and 17 kDa (Pearse and Robinson, 1984). It mediates the interaction between clathrin and the cytoplasmic tails of receptors of plasma membrane-associated, so-called plasma membrane adaptor, i.e., PM-

adaptor (Pearse and Crowther, 1987). On the other hand, the HA-1/AP1, is also a heterotetramer, but composed of gamma-, a beta'-adaptin, a 47-kDa protein, and a 20-kDa polypeptide. The beta-adaptin of HA-2/AP2 has high degree of homology (85% identical) with beta'-adaptin of HA-1/AP1, while alpha-adaptin and gamma-adaptin are least homologous (25% identical) (Robinson, 1989, 1990, 1992). HA-1/AP1 mediates the interaction of vesicle proteins in trans Golgi network (TGN), so-called Golgi adaptor, i.e., TGN-adaptor (Robinson, 1992). Both adaptors have a beta-adaptin, which appears to provide the clathrin binding site of the adaptor complex (Ahle et al., 1989). There is little relative homology between the alpha-, beta- and gamma-adaptin. Also, 47-kDa protein (HA-1) is 40% identical to the 50-kDa (HA-2), while the 20-kDa (HA-1) is 45% identical to the 17-kDa (HA-2) (Robinson, 1992).

As expected, the coat protein composition of brain-derived CCVs contains several neuronal-specific proteins. The CHC and two CLCs (CLCa and CLCb) are present in all tissues (Brodsky et al., 1991). In neurons, two additional isoforms of CLCa and one additional form of CLCb exist. They are generated by alternative splicing events resulting in the insertion of short stretches of amino acids (Jackson et al., 1987). Similarly, neuron-specific variants of alpha- and beta-adaptin were identified (Robinson, 1989, 1990; Ponnambalam et al., 1990; Kirchhausen et al., 1989). Furthermore, two new coat components have been identified that are specific for neurons: NP185/AP180 (Kohtz and Puszkin 1988; Ahle and Ungewickell, 1986) and auxillin (Ahle and Ungewickell, 1990). These neuron-

specific proteins in CCVs may function in the recycling of SVs in nerve terminals, which represents a highly specialized and greatly amplified membrane trafficking pathway (Puszkin et al., 1992).

7. Unknown Areas of Interest and Perspectives

Still largely unknown are the molecular events involved in the mobilization, targeting, docking, fusion, release, and recycling of NT vesicles at synapses, even though many proteins described above are thought to link to these processes (Jessell and Kandel, 1993; O'Connor et al., 1994). The functional significance of neuron-specific proteins and their variants is still an enigma, especially it is very difficult to do *in vivo* functional analysis. Although it is possible to block or stimulate these processes by using mAbs or drugs in order to access the function of individual molecules, the blockade or facilitation of exo-endocytosis with a particular molecule, could result from interference with any one of a number of quite different steps in the process (Jessell and Kandel, 1993).

In addition, the number of proteins involved in exo-endocytosis machinery is likely to be larger than the repertoire current available (Jessell and Kandel, 1993; O'Connor et al., 1994; Bennett and Scheller, 1994). The identification and characterization of proteins involved is progressing at a rapid pace (e.g., Kohtz and Puszkin, 1988, 1989; Bahler et al., 1991; Zhou et al., 1992; Monck and

Fernandez, 1994). Furthermore, neurons possess an unusually extensive Golgi apparatus and exhibit a variety of active endocytic-like processes. The Golgi apparatus and the endocytic phenomena both contribute, probably in multiply overlapping ways, to the epigenesis and fate of the membrane systems in axons and terminals. Comparable progress is badly needed in detailing the functional relations among the ER, Golgi apparatus, and poorly understood sets of smooth surfaced sacs, tubules and vesicles that mediate much of axonal transport (Holtzman, 1992).

Nonetheless, to relate these proteins to function will require the development of more stringent criteria, and more sophisticated systems for establishing whether, a given molecule has a role in one or another of the stages of the process. Testable models of molecular mechanisms responsible for exo-endocytosis at synapses are now emerging; e.g., synaptosomes, as well as cell lines (neuronal and endocrine), and simple organisms that are suitable for genetic manipulation (Jessell and Kandel, 1993). SV exo-endocytosis may provide an optimal system not only for learning how NT release and membrane retrieval take place, but also for learning how they can be regulated. Such studies may eventually provide some overall insight into the mechanism of learning and memory (Kandel and Hawkins, 1992; Hawkins et al. 1993).

As a novel synapse-specific protein, NP18 has important properties for the function of nerve endings in the movement of vesicles and the cargo molecules. Therefore, if it can be

fully characterized in the structure and function, it will result in understanding further how the nerve ending plays a role in nerve transmission.

8. NP185, a Novel Neuron-specific, Synapse-enriched and Multifunctional Phosphoprotein

One molecule with a potential to play an important role in synaptic function has been identified in our laboratory (Kohtz and Puszkin, 1988, 1989; Su et al., 1991; Perry et al., 1991, 1992; Li, Hanson, Gelman, Brosius and Puszkin 1992; Puszkin, Perry, Li, and Hanson, 1992; Li, Puszkin and Hanson, 1993, 1994). This molecule, NP185, has been partially characterized biochemically and immunologically, and found to display the following properties:

(1) It is a neuron-specific protein with an apparent molecular mass on SDS-PAGE of 185 kDa. It is only poorly stained by Coomassie blue and very sensitive to proteolytic attack (Kohtz and Puszkin, 1988; Su, Hanson, Perry and Puszkin, 1991).

(2) It is present in the cell bodies, cytoplasmic projections, and especially enriched in nerve endings (Kohtz and Puszkin, 1988; Perry, Hanson and Puszkin, 1991).

(3) It demonstrates binding affinity for brain CCVs, SVs and decorated CCVs, and nerve ending plasma membranes (Su, Hanson, Perry and Puszkin, 1991).

(4) It has binding affinity for tubulin (Kohtz and Puszkin, 1989). Tubulin is a microtubule subunit of the

dominant cytoskeletal protein in brain tissue (Kandel et al., 1991). In addition, tubulin is also a component of the membranes of SVs, CCVs and plasma membrane (Kelly et al., 1983). Its binding affinity for tubulin is significantly enhanced when tubulin is phosphorylated by CCV-associated casein kinase II (CKII). In contrast, phosphorylation of tubulin by a kinase activity associated with purified brain tubulin decreases its affinity for NP185. Recently, evidence has suggested that tubulin is phosphorylated by CKII during neurite development (Serrano et al., 1987; Ulloa et al., 1993). The enhanced affinity of NP185 for tubulin phosphorylated by CKII could serve to organize the transport of NP185 into the developing neurite.

(5) It appears capable of inducing clathrin polymerization to form cages, and was shown to be a monomer that interact with clathrin triskelia with a stoichiometry of one and thereby induces clathrin assembly into a homogeneous population of 60-70 nm coats (Su, Hanson, Perry and Puszkin, 1991).

(6) It binds unphosphorylated CLCs, but not to phosphorylated CLCs by the CCV-associated CKII (Su, Hanson, Perry and Puszkin, 1991).

(7) It is specific to nerve endings, and corresponds temporally and spatially to avian cerebellar synaptogenesis and synaptic maturation (Puszkin, Perry, Li and Hanson, 1992; Perry, Hancock, Hanson and Puszkin, 1991).

(8) It can be induced by NGF in PC12 cells, a NGF-responsive rat pheochromocytoma cell line (Kohtz and Puszkin,

1988).

(9) It is located at the presynaptic sites of neuromuscular synapses in PNS (Perry, Li, Hanson, and Puszkin, 1992).

(10) It is a phosphoprotein, associated with CKII activity (Su, Hanson, Perry, and Puszkin, 1991) .

There are still interesting aspects about the structure and function of NP185 in need of elucidation. Structurally, what is the gene and cDNA sequence of NP185? What other known proteins are related to NP185? Functionally, what is the real role that NP185 plays in neuronal development? What is the overall physiological function of NP185 in the exo-endocytosis cycle of SVs at synapses? How are the expression of NP185 gene regulated during early development? Since NP185 only exists at synapses, what is the mechanism governing synapse-specific expression? Does NP185 have positive regulatory role on CNS and PNS development? What are the structural and functional relationships of the NP185 to nerve terminals? **The working hypothesis for this thesis was that NP185 is a multifunctional protein in synaptic terminals.**

To prove the hypothesis, the following experiments have been initially proposed to study the structure of and expression of NP185: (a) biochemical approaches to purify NP185 protein, to obtain amino acid sequences from internal peptides of the purified protein; (b) molecular biological approaches to clone and characterize the gene; (c) physiological approaches to express the gene in neuroblastoma, neurocrine PC12 and fibroblast cell lines, as well as in

Xenopus oocyte; and (d) genetic approaches to express the gene in transgenic mice for studying function of the protein and development regulation.

Apart from the findings in our laboratory, it has been only very recently established that NP185 is probably identical or at least very closely related to AP180 (Ahle and Ungewickell, 1986, 1989) and AP3 (Keen and Black, 1986; Keen, 1987), by using 2-dimensional gel analysis, tryptic peptide mapping and mAb cross-reactivity (Murphy, Pleasure, Puszkin, Prasad, and Keen, 1991). However, progress in further characterization of this protein has been limited by the difficulty of purifying single species to homogeneity (Ahle and Ungewickell, 1986; Kohtz and Puszkin, 1988; Su et al., 1991). A major obstacle to the identification of NP185 seems to be the fact that, unlike enzymes or ion channels, functional assays for NP185 protein that can serve as a criterion for purification or expression cloning are difficult to develop. In addition, NP185 is extremely sensitive to proteolytic attack (Kohtz and Puszkin, 1988; Su, Hanson, Perry, Puszkin, 1991).

In this thesis a series of experiments have been performed for further characterization of NP185 via protein purification, cDNA cloning (Li et al., 1992) and ectopic expression (Li et al., 1993, 1994). Results from these experiments will shed light not only on the gene structure and function of NP185, but also on the function of NP185 in neuronal development, particularly in synaptic function.

MATERIALS AND METHODS

MATERIALS AND GENERAL METHODS

Source of Materials and Reagents: obtained from: ampicillin, bovine serum albumin, isopropanol, 5-bromo-4-chloro-3-indolylphosphate (BCIP), phenol, proteinase inhibitors, nitro blue tetrazolium (NBT), Sigma Chemical Co; Tween-20, J.T. Baker; non-fat powdered milk, Carnation Company; goat anti-mouse IgG-phosphatase, Sigma Chemical Co or Boehringer Mannheim Biochemicals; horse serum and fetal bovine serum, Hyclone; ammonium persulphate, acrylamide, bis-acrylamide, TEMED, X-gal, IPTG, Boehringer Mannheim Biochemicals; nitrocellulose membrane, 3 MM filter, Trans.Blott Cell, Bio-Rad; SeaKem and SeaPlaque agarose, FMC; Geneclean II Kit, Bio 101; Sepharyl S-1000, CL6B, MonoQ, glutathione sepharose 4B column, Pharmacia; DNA sequenase T4 DNA polymerase kit, United States Biochemicals; mRNA purification kit, the first-strand DNA cDNA synthesis kit, Pharmacia; GeneAmp PCR reagent kit with AmpliTaq DNA polymerase, Perkin Elmer Cetus; Nick translation system, GIBCO BRL; α -³²P-dCTP, γ -³²P-dATP, α -³⁵S-dCTP, NEN DuPont or Amersham; MacConkey agar, Difco; restriction enzymes, T4 DNA ligase, polynucleotide kinase, calf intestine phosphatase, klenow, T4 DNA polymerase, New England Biolab; etc.

1. *Bacterial Strains and Plasmid Vectors.*

Bacterial strains were used for different purposes. Three

recombination-deficient strains, *E.coli* TG1 (gift from Dr. Boris Scraybin in Professor/Dr. Brosius's laboratory), *E.coli* XL1-blue (Stratagene, gift from Dr. Wei Chen in Dr. Brosius's lab), and *E.coli* DH5 α (gift from Dr. Chia Hsiang Chen in Professor/Dr. Robert L. Desnick's laboratory), were used for transformation, propagation and selection of recombinant plasmid constructs. These three strains carry the F' episome encoding for pili that allows infection by filamentous M13 phage, or transformation by pBluscript II KS(+) plasmid vector (Stratagene, gift from Dr. Mark W. Miller) as well as its derivative vectors like pGEX series (Pharmacia), pBKCMV (Stratagene). In addition, the F' episome contains the lacI^{qZ} M15 mutation carrying α -complementation of β -galactosidase gene, which allows blue/white color selection of recombinant colonies or plaques on plates supplemented with X-gal and IPTG. As well, red/white color selection of recombinant constructs was used when the strains with recombinant plasmids grown in MacConkey agar (Difco) plate. Other strains were used such as *E.coli* JM101, JM105, JM109 for pSEM 1, 2, 3 vector expression (Knapp et al., 1990), gifts from Dr. Jianying Luo in Professor/Dr. Joseph S. Krakow's laboratory at Hunter College (Luo and Crack, 1992).

E.coli BL21LysS, a gift from Dr. Andrew D. Bergemann in Professor/Dr. Edward M. Johnson's lab, was used for expression of fusion protein, since it is a proteinase-deficient strain that allows high-level expression of genes (Das, 1990).

E.coli Y1090*hsdR*, gift from Dr. Brosius, is a strain used for immunological screening of expression libraries and

propagation of λ gt11. In this strain expression of the foreign protein is controlled by the high levels of *lac* repressor made by pMC9 carrying *lacI*^q. *E.coli* Y1089 (Stratagene) was used to generate lysogen for expressing β -galactosidase fusion protein of recombinant bacteriophages. *E.coli* LE392, gift from John Sullivan in Dr. Brosius's laboratory, is a suppressing strain commonly used to propagate bacteriophage λ vectors and their recombinants, e.g., λ gt10 cDNA library.

Plasmid Vectors (Brosius, 1989): pBluscript II KS(+) and SK(+) was a gift from Dr. Mark W. Miller in Dr. Brosius's laboratory; pGEM3Zf(+) was a gift from Professor/Dr. Hiroshi Uehara (DNA Core at Mount Sinai); pGEM5Zf(+) was purchased from Promega; pSEM 1,2,3 (Knapp et al., 1990), gifts from Dr. Jianying Luo in Professor/Dr. Joseph S. Crack laboratory at Hunter College; pGEX 1 λ T, 2T and pGEX3X (Pharmacia); pMAMneo (Clontech, gift from Ernest Sakavrasky in Dr. Edward M. Johnson's laboratory); pBKCMV (Stratagene); pCGN (gift from Dr. Jhumuku Kohtz and Dr. Stave Kohtz; Tanaka and Herr, 1990); pMV7 (Kieschmeier et al., 1988), gift from Dr. Yan Dai in Professor/Dr. Arthur Cederbaum's laboratory.

2. Bacterial Growth Conditions and Competent Cells

E.coli XL1-blue and BL21 grew in LB agar plates or LB medium. Y1090 and Y1089 grew in NZCYM agar plates (Sambrook et al., 1989). *E.coli* cells harboring plasmids vector grew in LB agar or LB liquid medium with appropriate amount of antibiotics, e.g., 50-175 ug/ml of ampicillin for pBluscript II KS(+); pGEM3Zf(+); pGEM5Zf(+); pSEM1,2,3; pGEX 1 λ T or 2T,

pGEX3X; pMAMneo; pMV7; 50 ug/ml of kanamycin for pBKCMV.

There were two ways usually used for making *E.coli* competent cells: PEG one step (Chung et al., 1989) and Hanahan's Method (Hanahan, 1983). Occasionally, CaCl_2 method was used as described in Sambrook et al., in 1989.

For PEG-mediated competent cells (Chung et al., 1989): Briefly, a fresh overnight culture from a single *E.coli* colony was diluted in 1:100 into prewarmed LB broth. The culture was incubated with shaking 250 rpm at 37 °C, until OD_{600} reached 0.5 (log phase) for about 2-3 hours at 37 °C. After cooling on ice, the cells were sedimented at 1,000xg, 3,000 rpm for 10 min at 4 °C. The pelleted cells were added with 1/10 volume of original culture 2X TSS (2X LB broth, 10% PEG-MW8000, 5% DMSO, 20-50 mM MgSO_4 or MgCl_2 , pH 6.55) precolded on ice. Pipet was used to mix up for about 0.5 hour on ice. The cells were frozen immediately in a dry-ice/EtOH bath or LN_2 in aliquot 100 ul per e-tube, and stored at -80 °C.

For Hanahan's procedure (Hanahan, 1983): Briefly, a fresh overnight culture from a single colony was diluted in 1/100 into prewarmed SOB medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl_2 , 10 mM MgSO_4) in a large flask with good aeration. The mix was incubated at 37 °C with shaking 300 rpm until OD_{600} reached 0.5, for 2-3 hours. The cells were chilled on ice for 15 min, pelleted by centrifugation at 750-1,000xg (2,000-3,000 rpm) for 10 min at 4 °C. The pelleted cells were resuspended by pipetting in a volume of RF1 (RbCl 12 g, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, Potassium acetate 35 ml of a 1 M stock pH 7.5, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1 g, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 1 g, LiCl 20

mg, Sucrose-ultrapure 150 g, adjust pH to 5.8 with 0.2 M acetic acid, water to 1 liter, and then sterilized it with 0.22 μ m Millipore filter unit), that was 1/3 of the volume collected. The cells were incubated on ice for 15 min to 2 hours. The cells were sedimented by centrifuge at 1,000xg at 4 °C. The pelleted cells were resuspended in RF2 (following) to 1/12.5 of the original volume, and incubated on ice for 15 min. The suspension was aliquoted, and immediately frozen in a dry-ice/alcohol bath or liquid nitrogen (LN₂), then stored at -80 °C. RF2 was made from 20 ml of a 0.5 M MOPS pH 6.8, RbCl 1.2 g, 11 g CaCl₂·2H₂O, 150 g Glycerol, adjust pH to final 6.8 with NaOH, water to 1 liter, and then sterilized by filtration through Millipore 0.22 μ m filter unit.

For CaCl₂ method (Dr. Boris Scryabin in Professor/Dr. Brosius's laboratory, personal communication): a single colony was incubated in 3 ml LB overnight at 37 °C with shaking 250 rpm. All 3 ml was transferred to 100 ml in large Erlenmeyer flask; grew to 0.4-0.5 of OD₆₀₀ (about 2 hours). The culture was transferred to 50 ml Falcon tube, put on ice for 10 min. 50 mM CaCl₂, 10 mM Tris.HCl pH 7.8 was kept frozen, thaw but kept on ice, i.e., with some ice floating in the tube! The cells were spun with 3,000 rpm at 4 °C for 10 min. The pelleted cells were mixed with 25 ml 50 mM CaCl₂ solution on ice for 15 min. The mixture was spun 3,000 rpm at 4 °C for 10 min. The pellet was resuspended on 7 ml CaCl₂, kept on ice, good for 24 hours.

3. Plasmid DNA Preparations

Mini-Prep: alkaline lysis method (Sambrook et al., 1989).

Briefly, 6 ml of overnight culture from a single colony was pelleted and washed with washing buffer (25 mM Tris.Cl pH 8.0, 10 mM EDTA and 86 mM NaCl). The cell pellet was resuspended on 200 ul of suspension buffer (solution I) on ice for 5 min. 400 ul lysis buffer(solution II) was added and placed on ice for 5 min until clear, and neutralized with 300 ul solution III of 3 M potassium acetate, on ice for 5 min. The majority of *E.coli* macromolecules was pelleted after microcentrifugation at 13 K rpm for 5-10 min at 4 °C. The supernatant was extracted with phenol once, phenol:chloroform (1:1) once and chloroform once. The DNA/RNA were precipitated with 2.5 volume (v/v) of 100% ethanol (-20 °C) and 1/10 of 3 M sodium acetate (NaAc), pH 5.2, or 0.9 volume (v/v) of isopropanol at RT. After spinning for 15 min at 13 K rpm (4 °C), the pellet was washed with 70% ethanol and resuspended onto TE buffer of 20 ug/ml RAase A. Alternatively, QIAGEN mini-column was used, or Wizard magic plasmid DNA purification system as well (Promega).

Maxi-Prep: Chemical method (Dr. Boris Scryabin, 1991; personal communication). Briefly, single colony was inoculated into 2 ml LB broth plus 100 ug/ml ampicillin, and grew 6-7 hours at 37 °C with 275 rpm shaking. The entire 2 ml was transferred to large Erlenmeyer flask containing approximately 200 ml of LB-Ampicillin with antibiotics, cultured in shaking incubator, overnight, at 37 °C. While streaking a plate for I.D., the rest of cells was pelleted with 4,000 rpm for 40 min at 4 °C. The pellet was washed with washing buffer (25 mM Tris.Cl pH 8.0, 10 mM EDTA and 86 mM NaCl). The cells were

drained off completely from the wash buffer after pelleting. The pellet was resuspended in 3 ml of washing buffer containing freshly made lysozyme for 5 min at RT and mixed with 2 volume of freshly made alkaline solution of 0.2 N NaOH and 1% SDS on ice for 10 min. The mix was neutralized with 1.5 volume of 5 M KAc (potassium acetate) on ice for 10 min, then transferred to polypropylene Oakridge tube, and spun 17 k rpm to get rid of high molecular weight DNA and cellular debris as pellet. The supernatant was extracted with equal amount of chloroform once. After extraction, the solution was spun 13 k rpm for 20 min at 4 °C after mixing with 0.6-0.7 volume of isopropanol at RT. The pellet was resuspended in 1 ml of dH₂O, and mixed up with 3 volumes of NaOAc (sodium acetate) saturated with dH₂O, pelleted by spinning in 3,000 rpm at 10 °C. The supernatant was transferred to plastic tube, and mixed with 0.6-0.7 volumes of isopropanol at RT. After centrifugation, the pellet was washed with 75% ethanol, and finally dissolved in 10 ug/ml RNase A dH₂O.

Alternatively, QIAGEN MAXI column was used. A large amount of high quality supercoiled plasmid DNA was obtained using alkaline lysis CsCl-ethidium bromide density gradient equilibrium centrifugation (Sambrook et al., 1989). Briefly, for 300-500 ml culture, the cell pellet was added with 20 ml of suspension solution I, 40 ml lysis buffer, and 30 ml of neutralized buffer. Centrifuged at 10,000 rpm with SS34 rotor, the supernatant was extracted once with CHCl₃. After ethanol or isopropanol precipitation, the crude DNA pellet was resuspended into ultracentrifuge tubes with 10 ml of 51% CsCl-

TE solution (10 ml TE and 11 g CsCl in Professor/Dr. Terry Ann Krulwich's laboratory; or 102 g CsCl, 100 ml H₂O, 1.25 ml 100X TE, filtered at RT, in Professor/Dr. Jay Unkeless's laboratory at Mount Sinai), and 300 ul of 10 mg/ml ethidium bromide. The tubes were sealed and centrifuged at 40,000 rpm in a Beckman Ti75 rotor at 20 °C for 24-48 hours (with Dr. Zhenhai Shen in Biochemistry Department at Mount Sinai). The Plasmid DNA band was collected, extracted with 1-butanol saturated with water or isoamyl alcohol to remove ethidium bromide, and precipitated with 2 volumes of 100% ethanol at RT. After pelleting, the concentration of plasmid DNA was determined by OD₂₆₀ and 1 at OD₂₆₀ equals to 50 mg/ml of dsDNA. The purity of the DNA was measured by ratio of OD₂₆₀/OD₂₈₀ (1.8-2.0) or by running on agarose gel with ethidium bromide.

4. Restriction Digestion, DNA Fragment Purification

The plasmid DNAs were digested with the appropriate amount of restriction enzymes and buffers, at appropriate temperature, for a sufficient period of time, by following the instruction manuals from Boehringer and New England Biolabs. The DNA fragment of interest was purified with GeneClean II kit (Bio 101) (Vogelstein et al., 1979).

5. DNA Ligation and Plasmid Transformation

Using selected restriction enzyme, the vector was linearized to produce compatible ends with the DNA fragment. Ligation reaction cocktail (Dr. Boris Scryabin and Dr. Mark Miller 1991; personal communication) was as follows: 10% of 10X ligation reaction buffer, 5% of 100X BSA (New England Biolabs), 12.5% of 40% PEG, 10% of rATP (optional), vector (v)

DNA and target (t) DNA fragment (molar ratio of v:t is about 1:2 or 1:3). After adding appropriate amount of T4 DNA ligase (New England Biolabs), the reaction was carried out at 9-16 °C for 15 min to overnight (16 hours). The transformation of recombinant plasmid DNA began by mixing diluted (1:5) DNA ligation solution with 30-100 ul of competent cells. The mixture was placed on ice for 30 min; heat-shocked at 42 °C for 90 seconds; and immediately placed on ice for 15 min. After adding 5X of S.O.C. medium (GIBCO, BRL), the mix was incubated at 37 °C for 30 min, spreaded onto LB medium agar plate with appropriate antibiotics. Those resistant colonies were selected to isolate plasmid DNA for further analysis in order to find a recombinant plasmid. If pBluscript or its derivative (pBKCMV) were transformed into *E.coli* TG1 and XL1-blue, there was blue/white color selection when on X-gal and IPTG plates, or red/white on MacConkey's agar plates.

6. DNA Sequencing

Double-strand DNA (dsDNA) template preparation (Li and Zhang JQ, personal e-mail communication) was following: Mombo preparation of DNA: From: IN%jz19+@andrew.cmu.edu "Jian-Qing Zhang" 4-JUL-1991 18:57:00.55 To: IN%SHENGWEN@msvax.mssm.edu (Shengwen Li) Subj: dsDNA TEMPLATE PREPARATION AND SEQUENCING. "I have rechecked the protocol sent to you yesterday, and I am pretty sure the problem is from the electron transferring process. The lysis buffer is 2.1 ml, NaOH is 4.2 ml, and NaOAc is 2.6 ml. The protocol is resent to you as following: Mombo preparation of DNA protocol: A single fresh *E. coli* colony (with transformed plasmid) was inoculated in 25 ml 1x Mombo

medium (1 lite 5x: 11.5 g KH_2PO_4 , 62.5 g K_2HPO_4 , 20 ml Glycerol, 60 g Trypton, 120 g Yeast Extract) for more than 12 hours. The pelleted cells were washed once with cold saline solution (100 mM NaCl, 10 mM EDTA, 50 mM Tris pH 8.0). The washed cell pellet was resuspended thoroughly in 2.1 ml lysis solution (25 mM Tris pH 7.5, 10 mM EDTA, 15% Sucrose, 2 mg/ml Lysozyme [make it fresh every time for 10 ml, 0.25 ml 1 M Tris pH 7.5, 0.4 ml 250 mM EDTA, 1.5 g sucrose, and 20 mg of lysosome] by pipetting up and down on ice for 20 min. 4.2 ml alkaline solution {0.2 M NaOH, 1% SDS[0.4 g NaOH, add 2.5 ml 20% SDS up to 50 ml with H_2O]} was added and mixed by inversion, and the tube was placed on ice for 10 min. 2.6 ml acetate solution (3 M NaOAc pH 4.7 [24.6 g NaOAc upto 100 ml, and 17 ml glacial acetic acid into 83 ml H_2O , then mix two solution pH should be 4.7]) was added, mixed by inversion and the mixture was left on ice for 20 min. 50 ul RNAase (1 mg/ml) was added to supernatant after spinning down the pellet at 15 K for 15 minutes. After 10 minutes at 37 °C, an additional 50 ul of RNAase was added for 10 min. The supernatant was extracted 2x with Phenol/Chloroform (1:1) and followed by addition of 2x volume of EtOH , and precipitation on ice for 20 min. The precipitates were spun down and the pellet was dissolved in 1.6 ul H_2O . 0.4 ml 4 M NaCl were added, mixed; 2 ml 13% PEG (MW 6000-8000) were added, and mixed. The mixture was left on ice overnight (or 4 hr on ice). The supernatant was completely removed after spinning down the pellet at 10 K x g for 10 min. The pellet was washed with 70% EtOH (just add 100-200 ul EtOH and tap with finger, then

removed the EtOH with fine tip pipeteman), dried, and resuspended in 300 μ l H_2O . Before the sequencing, 15.2 μ l fresh 2 N NaOH, 2 mM EDTA was added in 10 μ g DNA in 20 μ l volume in e-tubes, mixed by vortexing, and incubated at RT for 5 minutes. 8 μ l 1 M Tris-HCl pH 4.5 was added to the DNA solution, mixed by finger tapping, and left on ice. 3 μ l 3M NaOAc was added, mixed by finger tapping, and left on ice. 75 μ l 100% EtOH was added, and the e-tubes were incubated on dry ice for 20 min. After a 5 minutes spin, pellet was washed with ice cold 70% EtOH (100 μ l is enough), spun for 5 minutes, and dried in speed vacuum. The DNA was ready to serve as the template for sequencing. Into a e-tube containing DNA template, were added 7 μ l H_2O , 2 μ l 5X reaction buffer, 1 μ l primer (1-2 pmol) 65 $^{\circ}C$ 2 min, being slowly under 30 $^{\circ}C$ over 30 min then followed the sequenase protocol for sequencing."

Sequence reaction was carried by following USB manual of DNA sequenase sequencing kit (USB) with following modifications (Dr. Mark W. Miller, personal communication). Briefly, 1). Template-primer annealing: 10 μ l cDNA, 2 μ l primer (1-2 pmol), 4 μ l of 1 N NaOH, and 4 μ l of 1 mM of EDTA were mixed up in a e-tube, set at 37 $^{\circ}C$ for 15-30 min, and heated at 65-85 $^{\circ}C$ for 5 min. The mixture was cooled down at RT for 15-30 min. 150 μ l of ddH₂O, 20 μ l of 3 M NaAc pH 5.2, 2-4 volume of 100% EtOH were added, and kept at -80 $^{\circ}C$ for 15 min. The mixture was spun at 13 k rpm for 15 min at 4 $^{\circ}C$. The pellet was washed with 80%, and dried at speed-vac for 2 min. The pellet was resuspended in 6 μ l of ddH₂O, and added 1.5 μ l of 5X sequenase reaction buffer (Rx buffer), incubated at 37

°C for 10 min. 2). "Cocktail" for labeling reaction: in e-tube #1 for "sequenase dilution", 7 ul of enzyme dilution buffer and 1 ul of sequenase were added; e-tube #2 for "labeling mix", 3 ul of dGTP labeling mix was diluted in 6 ul of ddH₂O; in e-tube #3 for "big mix", 4 ul of DTT were mixed up with 8 ul of the e-tube #2, 4 ul of α -³⁵S-dATP, and 8 ul of the e-tube #1. 3) Labelling reaction: 6 ul of "cocktail big mix" were transferred to the template-primer sequenase reaction buffer, incubated at RT for 2-5 min. 4). Termination reaction: 2.5 ul of ddATP, ddCTP, ddGTP, and ddTTP, respectively, were added into each well of an HLA plate low profile Cat # 476511 (Nunc; Dr. Wei Chen, personal communication). 3.3 ul of labeling reaction mix were added into each well of above ddA, ddC, ddG, ddT, respectively, incubated at 37 °C for 15 min. 5). Stop the reaction: 4 ul of stop solution was added to each well. 6). Denature: The DNA was incubated at 75 °C for 5 min, then placed on ice, and immediately loaded into DNA sequencing gel. In the cases where compressions rendered the interpretation of the nucleotide sequence ambiguous, deaza-7-guanosine (Mizusawa et al., 1986) was substituted into the normal nucleotide mixes and the reactions were run on non-gradient 8% acrylamide gels (Dr. John A. Martignetti, personal communication).

Sequence plates were siliconized by Sigmacote (Sigma), and buffer gradient gels were made (Hong, 1982; Biggin et al., 1983; Sambrook et al., 1989, pp13.47-13.53) (Dr. John A. Martignetti, Dr. William Probst, personal communication), in IBI sequencing apparatus, with Bio-Rad Model 3000Xi Computer Controlled Electrophoresis Power Supply. The gel was dried

with vacuum and exposed on Kodak XAR film overnight at RT.

7. Computer analysis of cDNA Sequences

DNA sequence was manually entered from autoradiographies into a VAX program in computer and then entered into Staden format using the program BATIN (Staden, 1984a). The sequences from 373A DNA Sequencer (ABI; DNA Core at Mount Sinai) were changed into GCG format using adapter program. The nucleic acid sequences were then aligned and overlapped by the programs DBAUTO and DBUTIL (Staden, 1980, 1982). The sequences were analyzed using ANALYSEQ (Staden, 1984b) and the GCG suite of programs running at Mount Sinai Computer network (Biomathematics Department).

The nucleotide sequences were aligned and analyzed in EMBL/GenBank database to search for homologous information using FASTA algorithm program and BLAST electronic mail of National Institutes of Health (NIH) (Pearson and Lipman, 1988). The reading frame and predicted amino acid sequences were analyzed using DNA striders program (Macintosh software), or GCG (Genetic Computer Group) package of University of Wiscosin, which is established in The Mount Sinai computer network (Professor/Dr. David F. Bishop).

8. Northern Blot Analysis

a) *Extraction of RNA with guanidinium thiocyanate followed by centrifugation in cesium chloride solutions* (Sambrook et al, 1989): Briefly, to pieces of fresh tissues (e.g., brain, liver, lung, kidney, heart, spleen) 5X of guanidinium thiocyanate homogenization buffer (GTHB), e.g., 4.5 ml of 5.0 M guanidinium thiocyanate ($M_r = 118.1$), 0.1 Tris.Cl (pH 7.5),

2% β -mercaptoethanol were added and placed on ice or dry ice. The tissues were homogenized with a Polytron homogenizer (Brinkmann, RNAase-free) at high speed for 1-2 min. Sodium lauryl sarcosinate was added to a final concentration of 0.5%, mixed, centrifuged at 5,000xg for 10 min. The supernatant was layered onto a cushion of 5.7 M CsCl, 0.01 M EDTA (pH 7.5) in a clear ultracentrifuge tube (Beckman), centrifuged at 20 °C for 24 hrs in 32,000 rpm using SW40 rotor. The RNA pellet was washed with 70% ethanol at RT to get rid of CsCl, dried at RT, and dissolved in TE (pH 7.6). 0.1 volume of 3 M sodium acetate, pH 5.2, and 3 volumes of 100% ice-cold ethanol were added, mixed well, placed for 30 min at 0 °C, and then centrifuged at 12,000xg for 10 min at 4 °C in a microfuge. The pellet was washed with 70% ethanol, and resuspended in a small volume of water and 3 volumes of ethanol, and stored at -70 °C until needed.

b) Electrophoresis of RNA through Gels Containing Formaldehyde (Sambrook et al., 1989, pp 7.43).

c) Transfer of Denatured RNA to Nitrocellulose Filters

Standard capillary transfer of RNA to nitrocellulose filter (GeneScreen Hybridization membrane, NEN, Dupont) was performed as described by Sambrook et al., 1989, pp. 7.46-48.

d) Hybridization and Autoradiography

Briefly, the filter was prehybridized for 30 min to 2 hrs at 42 °C in 50% formamide, 5X SSPE, 2X Denhardt's reagent, 0.1% SDS, total yeast RNA. The denatured radiolabeled probe was directly added to the prehybridization buffer, and incubated at 42 °C for 12-18 hrs. The filter was washed twice

at 50 °C in 2X SSC, 0.5% SDS; twice in 0.5X SSC and 0.5% SDS; twice at 60 °C in 0.1X SSC and 0.5% SDS for 20 min at RT, followed by three washes of 20 min, each at 65 °C in 0.2X SSC, 0.1% SDS. An autoradiography was obtained by exposing the filter for 28-48 hrs to X-ray film (Kodak XAR-2) at -70 °C with an intensifying screen.

9. Southern Blot Analysis of Genomic DNA

For Southern blots, high-molecular-weight rat liver DNA (genomic DNA, gDNA) was prepared (Sambrook et al., 1989). The DNA was digested to completion with restriction enzymes, BamHI, EcoRI, HindIII, XhoI; subjected to electrophoresis on a 0.8% agarose gel, and electroblotted onto GeneScreen hybridization transfer membrane (NEN, DuPont). The blots were probed with nick-translated ³²P-labeled cDNA clone. After washes, the blots were autoradiographed as described as Northern blot above.

Alternatively, genomic DNA (gDNA) was isolated from cultured cells (Ma et al., 1994). Briefly, cultured cells were lysed in 10 mM EDTA, 50 mM Tris pH 8.0, and 0.5% SDS. Pancreatic ribonuclease (Boehringer) was added to a final concentration of 20 ug/ml. Samples were incubated in 37 °C for 2 hours. Then proteinase K was added to a final concentration of 0.1 mg/ml. The samples were incubated in 50 °C for 5-12 hours. DNA was extracted 3 times with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), once with chloroform, and once with ether. DNA was precipitated with 0.1 volume of 3.0 M sodium acetate, pH 5.2, 3 volumes of 100% ethanol (-20 °C). High molecular weight genomic DNA was spooled out with

glass rods, gently rinsed with 70% ethanol, and dissolved in 1 mM EDTA, 10 mM Tris, pH 8.0. gDNA concentration was determined by spectrophotometry.

PART I. IMMUNOFLUORESCENCE HISTOCHEMISTRY FOR THE LOCALIZATION OF NP185 IN THE SYNAPTIC SITES

1. *Western Blot and SDS-PAGE Analysis*

Gel electrophoresis was adapted for the brain and muscle tissue preparations following the procedure of Laemmli (1970) as described for chick brain embryo NP185 (Perry, et al., 1991). Sample preparations were loaded onto one-dimensional gels of a gradient of 5-15% acrylamide. The gradient was made from 15% and 5% pools of acrylamide, i.e., 15% tank was from 1.9 ml of 3 M Tris.Cl pH 8.8, 7.5 ml of 30% acrylamide and 0.8% bis-acrylamide, 150 ul of 10% SDS, 40 ul of 10% ammonium persulphate, 10 ul of TEMED, water to 15 ml; and 5% tank was from 1.9 ml of 3 M Tris.Cl pH 8.8, 2.5 ml of 30% acrylamide and 0.8% bis-acrylamide, 150 ul of 10% SDS, 40 ul of ammonium persulphate, 10 ul of TEMED, water to 15 ml. Both 15% and 5% mixes were blended in by using Buchler Multistaltic Pump (Searle), casted into glass plates assembler (Hoefer Scientific Instruments). For stacking gel, mixed were 2 ml 0.5 M Tris.Cl pH 6.8, 1 ml of 30% acrylamide and 0.8% bis, 100 ul of 10% SDS and 100 ul of ammonium persulphate, 10 ul of TEMED, and watered to 8 ml; immediately casted into the plates.

For immunoblotting, the polyacrylamide gels with migrated proteins were electrophoresed onto nitrocellulose sheets using

a Trans-Blot Cell apparatus (Bio-Rad). Sheets were fixed and non-specific reactivity was neutralized with 0.1% bovine serum albumin (BSA) or 1% horse serum or 1% fetal bovine serum and 0.05% Tween-20 in Tris-buffered saline (TBS), pH 7.5. The filter strips were incubated at 4 °C overnight with the primary antibody in 0.1% BSA, 0.05% Tween-20, and TBS. The strips were washed three times with 0.05% Tween-20 and TBS, then incubated for 1 hour at 4 °C with phosphatase-conjugated goat anti-mouse IgG (Boehringer).

2. NP185 Enriched Preparations from Muscle

To establish that mAb 8G8 reacted with NP185 polypeptides of brain and muscle tissues from chick, NP185-enriched preparations were made immediately after excising the brain tissue and striated muscle fibers. The soft tissues were quickly homogenized with buffers containing proteolytic inhibitors to preserve the NP185 molecule intact. Otherwise, the NP185 polypeptide is highly susceptible to proteolytic degradation (Su et al., 1991). Obtaining strong mAb reactivity with NP185 bands from each tissue source indicated the possibility of determining immunologically the presence and distribution of NP185 in muscle fibers. Recent published data (Perry et al., 1991) have shown that the mAb 8G8 cross-reacted with NP185 in neurons of chick embryo CNS. Initial determinations using whole muscle homogenate failed to detect a signal for NP185 (unpublished results). To overcome previous attempts that yielded negative NP185 detection, used were two new modified preparations, an homogenate supernatant devoid of insoluble muscle fiber debris and an immunoprecipitate from

this supernatant mixture. This approach could yield a detectable amount of a polypeptides having epitopes recognized by the NP185 mAb. In addition to use chick muscle, fertilized chicken eggs with embryos of late-stage growth was also included as the source of fresh muscle and brain tissue (Puszkun et al., 1992). Tissue from the same embryo were used fresh and served equally well to obtain results used for comparison purposes and the remainder stored frozen at -80°C for future uses in developmental studies (Puszkun et al., 1992).

Certified pathogen-free fertilized chicken eggs were obtained from a commercial supplier (SPAFAS) and placed inside a rocking egg incubator (Lyon Electric). Embryos were harvested, weighed and staged following established method (Foelix and Oppenheim, 1974). Innervation in chicken striated muscle begins between day 15-16. Therefore, for the purpose of this study, embryos were used after day 17 to assure the presence of developed neuromuscular junctions where most structural elements and other components were in place and functional.

Pectoral and neck muscle fibers from embryos were excised, gently dissociated with fine tweezers and forceps, rinsed in PBS (pH 7.4), pooled, and weighed. An equal volume of a 0.2 M MES buffer solution pH 6.5 was added, the muscle was chopped with scissors into small cubes, and the content dounced in a glass grinder at 4°C . The extract was centrifuged at $15,000\times g$ for 20 min and the pellet saved. The pellet was resuspended with an equal volume 0.5 M Tris buffer

pH 8.0 containing 1.8 M NaCl and homogenized as above. The homogenate was centrifuged, the supernatant dialyzed against the same buffer without NaCl, and concentrated. Aliquots were separated, the remainder dounced in a small grinder, centrifuged at 75,000xg for 20 min and the supernatant saved. For comparison purposes, the brains from these embryos were subjected to protein extraction with an identical procedure to that described for muscle. Aliquots from the brain and muscle second homogenate and their supernatants were diluted 1:1 with sample buffer and used for SDS-PAGE and Western blotting.

3. Immunolocalization

Thin muscle fibers were excised and fixed in 3.8% formaldehyde/PBS for 0.5-2 hours at 4 °C. In most cases the fixed tissue was then soaked in 15% sucrose and PBS 0.5-3 hours or overnight at 4 °C. Fibers were immersed in O.C.T. compound embedding medium for frozen tissue specimens (1988 Miles Inc), frozen in liquid nitrogen, and immediately sectioned in a Harris cryostat. The sections were mounted on slides and rinsed in PBS. Tissue sections were permeabilized with 0.5% Triton in PBS for 5 min, rinsed, and blocked with goat antimouse IgG 1:100 for 30 min at 4 °C. After rinsing, the sections were incubated overnight at 4 °C with primary antibody diluted 1:100 with 1% BSA in PBS pH 7.4, washed, and then incubated with goat antimouse IgG-FITC for 45 min at RT. Control tissue fibers were incubated with: (1) the first antibody absorbed with excess antigen, (2) the second antibody only, and (3) mouse control ascites and second antibody. Sections were given a last rinse in PBS. To minimize bleaching

of the fluorescent label, the final sections were covered with a mixture of 1% p-phenylenediamine in 90% v/v glycerol buffered with PBS. Slides were sealed with coverslips and nail polish.

4. Monoclonal Antibodies

Monoclonal antibody 8G8, was generated according to procedure described by Kohtz and Puszkin (1988). Hybridoma cell lines were carried in RPMI-1640 medium supplemented with 15% fetal calf serum, 1 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM minimum essential medium (MEM) with nonessential amino acids, MEM vitamins, 15 ug/ml hypoxanthine, and 7.6 ug/ml thymidine. Ascites fluid was generated in pristine-primed Balb/c mice.

5. Double-Immunolabelling (Co-Labeling) Procedures

As shown in Table 1, avian pectoral or neck muscle fibers were fixed for 30 min in picric acid/formaldehyde (PAF) solution: 0.2% picric acid, 4% paraformaldehyde, and phosphate-buffered saline (PBS) pH 7.4, or 4% paraformaldehyde in PBS pH 7.4. The fixed tissue was then soaked in 15% sucrose/PBS for 0.5-2 hours or overnight at 4 °C. The tissue was immersed in rapid-freeze medium, frozen in liquid nitrogen, and sectioned at 8 um in a Harris cryostat. The sections were mounted on slides, rinsed in PBS, and blocked with goat antimouse IgG 1:100 for 30 min at 4 °C. Following another rinse, the sections were incubated overnight at 4 °C with mAb 8G8 diluted 1:100 with 1% bovine serum albumin in PBS, pH 7.4, washed, and then incubated with α -bungarotoxin conjugated to fluorescein (α BT-F) under the same conditions

but for 1-2 hrs at RT. Sections were washed and then incubated with goat anti-mouse IgG conjugated to rhodamine (IgG-R) for 45 min at RT. The sections were given a last rinse in PBS. To minimize fluorescence photobleaching, the sections were covered with a mixture of 1% p-phenylenediamine in 90% glycerol buffered with PBS. The sections were then sealed with coverslips and nail polish. Epifluorescence was observed with a Zeiss Axioplan microscope through a 63X objective and with a Zeiss Confocal microscope.

6. Scanning Electron Microscopy

Single muscle fiber samples separated from the pool of fibers prelabeled with either or both, antibodies and the AchR markers were dehydrated through 100% ethanol and critical-point dried in liquid CO₂ in a critical-point drying apparatus (Model 814; Tousinmis Scientific Instruments, Rockville, MD), mounted onto stubs, and sputter-coated with gold in an evaporator (MED 010; Balzers, Hudson, NH). Fibers were observed using the upper stage of a scanning EM (model DS 130; International Scientific Instruments, Milpitas, CA) equipped with an LaB6 emitter operated at 10 kV with a spot size of 3-10 nm.

PART II. PURIFICATION OF NP185 BY IMMUNO-AFFINITY CHROMATOGRAPHY AND PROTEIN MICROSEQUENCING

1. Purification of Clathrin Coated Vesicles (CCVs) (Table 2)

(1) Differential centrifugation: Bovine brains were obtained from a slaughterhouse, immediately submersed into ice (Schook

and Puszkin, 1985). All following procedures were performed at 4 °C or on ice in presence of several proteolytic inhibitors. The meninges were removed, and the gray matter was separated from the white by suction. The grey matter was resuspended in 0.1 M MES, pH 6.5, 1 mM EGTA, 0.5 mM MgCl₂, 0.02% sodium azide plus 0.5 mM phenylmethylsulfonylfluoride, 15 uM DTT, 1 mM benzamidine, 5 uM leupeptin, 5 uM apotinin, 5 uM pepstatin, 2 ug/ml soybean trypsin inhibitor and 0.3% 2-mercaptoethanol. Tissue was homogenized in a Waring Blender, and the homogenate was spun down at 17,000 rpm for 15 min in a Sorvall RC2B centrifuge with a SS34 rotor. This pellet (unbroken cells and nucleus) was discarded and the supernatant (microsomes, mitochondria, synaptosomes) was spun down at 35,000 rpm in a Beckman ultracentrifuge for 60 min with a Ti45 rotor (Beckman). The supernatant (cytosol) was discarded, and the pellet was resuspended in 0.1 MES buffer (1:5) and mixed with an equal volume of 12.5% Ficoll and 12.5% sucrose in 0.1 M MES buffer, and spun at 40,000xg for 60 min. The pellet (mitochondria) was discarded and supernatant (microsome fractions, CCVs, etc.) was diluted five times in 0.1 M MES buffer (1:5). The vesicle fraction was recovered from this solution as a pellet after centrifugation in a Ti45 rotor at 35,000 rpm for 75 min. The supernatant containing soluble proteins, vesicle fraction was mixed up with fractions of membranes.

(2) Sepharyl S-1000 column (gel filtration, Pharmacia): The pellet above was suspended in 0.1 M MES buffer and applied twice on a 100 cm x 2.5 cm Sepharyl S-1000 column (gel

filtration, Pharmacia) connected with FPLC machine (Pharmacia). 5 ml fractions were collected, and protein peaks were monitored by absorbance at 280 nm. This procedure yields approximately 10 mg of highly purified CCVs per 500 grams starting material.

2. Purification of NP185 Protein with Chromatography

(1) Sepharose CL6B (gel filtration, Pharmacia) was used to further separate clathrins, NP185, and assembly peptides. Briefly, CCVs from above were concentrated using 40% ammonium sulfate. The pellet (CCVs) was decoated by resuspending in running buffer of 0.5 M Tris pH 7.0, and 0.5 M NaBr; spun 35,000 rpm for 1 hour with Ti45 rotor (Beckman); the pellet (vesicle membranes) was discarded and the supernatant (disassembled clathrins) was concentrated by 40% ammonium sulfate. The pellet was resuspended in running buffer and dialyzed against running buffer (above); the retained dialysate was applied on a 100 cm x 2.5 cm Sepharose CL6B column connected with FPLC machine; elute the protein with running buffer. The fractions were detected by either enzyme-linked immunosorbent assay (ELISA) or immunoblotting using anti-NP185 mAbs, anti-clathrin mAbs or other mAbs or polyclonal antibodies (pAbs) for cross-detection.

CLCs were prepared as described by Lisanti et al., (1981, 1982), and phosphorylation of CLCs was performed as shown in Kohtz et al. (1987).

(2) MonoQ (ion exchange column, Pharmacia) was used because it is a strong anion exchanger based on MonoBeads, which binds

negatively charge biomolecules through quaternary amine groups that remain equally charged over the entire useful pH range of 2-12. From previous experiment, NP185 is shown to be acidic (Kohtz and Puszkun 1988; Su et al., 1991). Briefly, (a), the NP185 positive (detected by ELISA or immunoblotting using mAbs 8G8 and 6G7) fractions from the last column, were pooled and dialyzed against MonoQ buffer, 20 mM ethanolamine, pH 9.0; (b), the retained dialysate was loaded on the column coupled to FPLC machine; (c), elute the protein with 0.5 M NaCl gradient in MonoQ buffer; and (d), collect the fractions, detected with NP185 antibodies by ELISA or immunoblotting.

(3) Affinity column was made by purified anti-NP185 mAbs, 8G8 and 6G7.

a) Purification of IgG by Avid All column from ascites fluids

Purification IgG from ascites fluids by using a new product, Avid All gel column from BioProbe Internationale Inc., Unison Technology, 14272 Franklin Ave, Tustin, CA 92680-7017, Fax 714-544-0322. Briefly, 10 ml of Avid All beads were resuspended in 40 ml of Buffer C (20% MeOH and 80% of 1% acetic acid), packed in a Pharmacia FPLC chromatography column (diameter: 1.5 cm; height: 5.5 cm; volume: approx. 10 ml) at a speed of 1.5 ml/min, and equilibrated in Buffer A (0.01 M sodium phosphate pH 7.4, 0.15 M sodium chloride, 0.02% sodium azide). The ascites was clarified by spinning 33 k rpm for 30 min at 4 °C, diluted 1:4 with Buffer A, and loaded on the column at 0.5 ml/min. The column was washed with approximately 250 ml Buffer A at 1.0 ml/min. The IgG1 was eluted with approximately 60 ml of Buffer B (0.05 M sodium acetate pH 2.8,

20% glycerol. The purified IgG1 solution was concentrated by using the MonoQ column (Pharmacia). 10 mg of IgG1 was recovered from 30 ml of the ascites, while 13 mg of IgG1 was purified from another 30 ml of the ascites. Together, 23 mg of IgG1 was in 6 ml of volume.

b) Purified IgG1 was coupled to Hydrazide Ax gel

The purified IgG1 was coupled to Hydrazide Ax gel (BioProbe). Briefly, 23 mg IgG1 in 6 ml (above) was dialyzed for 17 hours against 2 liters of the Coupling Buffer (0.05 M Sodium Acetate pH 6.0); 500 ml of the Coupling Buffer were replaced with the fresh one for the dialysis each over a period of 6 hours, and repeated for 4 times. 600 μ l of 0.1 M Sodium Periodate (21.4 mg in 1 ml ddH₂O) was added dropwise to the sample in coupling buffer; the sample was wrapped in foil and shaken gently and placed on rotator 50 min at RT. The sample was dialyzed against 1 liter of the Coupling Buffer for 40 mins at 4 °C, repeated once; then the buffer was changed and dialyzed 17 hours against 2 liters of the coupling buffer. The concentration of the dialysate was determined by Lowry's Assay (Lowry et al., 1951), 22 mg of IgG1 was recovered. The sample was redialyzed for 1 hour against 500 ml of the Coupling Buffer. 4 ml suspension of Hydrazide Ax beads were placed in a 15 ml polystyrene tube at 4 °C, the beads was settled to a volume of 2 ml, and the liquid was aspirated off. 12 ml of the coupling buffer were added, settled to 2 ml, and the liquid was aspired off; repeated 3 times. The 2 ml of the washed beads were mixed with the 6 ml of oxidized IgG1 (above), the mixture was wrapped in foil and placed on a

rotator for 19 hours at 4 °C. The 6 ml of liquid were removed from the beads, the beads were washed 3 times with 6 ml of the coupling buffer, three times with 10 ml wash buffer (Phosphate Buffered Saline pH 7.4 with 0.5 M Sodium Chloride), and three times with 10 ml storage buffer (Phosphate Buffered Saline, pH 7.4, with 0.02% Sodium Azide). Then the beads were stored in storage buffer at 4 °C for about 48 hours. The beads were packed in a 4 ml FPLC chromatography column at a flow rate of 1 ml/min. The column was topped with 2 ml Sephadex G25. The dimensions of the column were diameter 0.5 cm and height 19 cm.

c) Running the affinity column

For running the affinity column, briefly, a), the NP185 positive (detected by either ELISA or immunoblotting using anti-NP185 mAbs, 8G8 and 6G7) fractions from the last column were pooled and dialyzed against 20X volumes of 10 mM PBS pH 7.4 and 100 mM NaCl, 0.1% 2-mercaptoethanol, 0.02% Na₃, and 0.02 mM PMSF; b) at 4 °C, the retained dialysate was then loaded on the above immuno-affinity column coupled to FPLC machine in a flow rate 0.1 ml/min, for 4 ml of sample, total time on column was 2 hrs and 40 min; c), the column was washed with 10 column volumes of the wash buffer (10 mM Sodium Phosphate pH 7.4, 150 mM Sodium Chloride) at flow rate 1 ml/min, to get rid of unbound proteins; d), the column was washed (double washes) with 5 column volumes of the pre-elution buffer (10 mM sodium phosphate pH 6.8, 150 mM NaCl), to get rid of non-specific binding proteins; e), NP185 was eluted with 30 ml of 100 mM glycine pH 2.5 at flow rate 1

ml/min; f), the sample was immediately neutralized with 1/10 the volume of the neutralization buffer (1 M Tris), dialyzed, and concentrated by Amicon filter apparatus (Amicon).

d) *Sample preparation for protein microsequencing*

The Purified NP185 was subjected to electrophoresis and transferred to polyvinylidene difluoride filters (PVDF) or Immobilon membrane (Millipore) for protein microsequencing (Matsudaira, 1987). Alternatively, the NP185 protein in gels was excised and sent out by following the instruction of "Sample preparation for in situ digestion procedure in SDS-PAGE gels" (Protein Chemistry Group, Biotechnology Resource Laboratory, W.M. Keck Foundation at Yale University). Briefly, the gel slot of NP185 band was excised from SDS-PAGE gel after Coomassie blue staining, and the gel slot without protein but with the same thickness was included for a control. The gel slots were kept on dry-ice and sent out for microsequencing, or kept at -80 °C.

3. Amino Acid Composition and Protein Microsequence Analysis

The purified NP185 protein was analyzed by Dr. Kathy Stone and Dr. Kenneth Williams in Protein Chemistry Laboratory, W.M. Keck Foundation at Yale University. Briefly, The amino acid composition from purified NP185 was obtained by complete digestion with 6 N HCl at 110 °C for 24 hours, and analysis in amino acid analyzer by spectrophotometry.

Protein microsequencings were performed by Dr. Kathy Stone and Dr. Kenneth Williams in Protein Chemistry Laboratory of W. M. Keck Foundation at Yale University. Since the N-

terminus of the NP185 protein was found to be blocked in our previous experiments (Li et al., 1991), internal peptides were made by trypsin digestion of the NP185 protein. The enzymatic cleavage fragments were separated on a narrow-bore (2.1-mm i.d.) reverse-phase high-performance liquid chromatography (HPLC), Vydac C18 column in 0.1% trifluoroacetic acid in H₂O and acetonitrile as the organic modifier, using a dual-syringe Brownlee micropump (Aebersold et al., 1987). The peptide fractions with clear peak pattern were chosen for peptide sequencing by automated Edman degradation on an Applied Biosystem 470A sequencer equipped with an on-line 120A PTH analyzer. The amino acid sequences of those peptides were aligned to the GenBank and the EMBL database with FASTA (Swissprot) program by the University of Wisconsin Genetics Computer Group (GCG) package (Pearson and Lipman, 1988) running at the Mount Sinai School of Medicine, and BLAST network service by the National Center for Biotechnology Information (NCBI) of the National Institutes of Health (NIH) (Altschul et al., 1990).

PART III. cDNA CLONING, SUBCLONING, EXPRESSION; and *in vitro* BIOCHEMICAL STUDIES

1. Phage dot hybridization was used for searching the full-length ORF of NP185/F1-20 cDNA

The source of rat cDNA libraries was as the following: A rat brain lambda gt11 cDNA library was a gift from Professor/Dr. David R. Colman at Columbia University (Bernier

LA, Alvarez F, Norgard EM, Raible DW, Mentaberry A, Schembri JG, Sabatini DD and Colman DR, 1987). A p15 rat brain ZAP-II cDNA expression library was a gift from Professor/Dr. Steve R.J. Salton of Mount Sinai School of Medicine; a rat adult brain ZAP-II cDNA expression library was purchased from Stratagene. A rat brain lambda gt10 cDNA library was a gift from Professor/Dr. Jurgen Brosius.

The positive clones from immunoscreening of NP185 (Li et al., 1992) were used to probe the libraries as described in Sambrook et al., 1989. The positive clones from above were rescreened with two primers flanking the ORF of F1-20 cDNA sequence (Zhou et al., 1992), separately, by **PHAGE DOT HYBRIDIZATION** (Probst et al., 1990; Salton et al., 1991). Briefly, 300 μ l of plating bacteria (Sambrook et al., 1989) and 7.5 ml of molten top agarose were mixed, and immediately poured onto an LB agar plate (150-mm). The plate was incubated for 1-2 hrs at 37 °C until the bacterial lawn was formed. By using a 20 μ l Pipettman tip, 1 μ l (containing approximately 10^5 pfu) of each of putative phage stocks was applied to its surface in a pattern based upon a paper template placed below the plate on the light box. A copy of this template was retained with a legend relating each clone to this position number. In order to facilitate subsequent orientation of the filter to the plate, the phage should be spotted in an asymmetric pattern. Following absorption of the phage, the plates were incubated for 6-12 hrs at 37 °C. The plates were cooled to 4 °C prior to transfer of the phages to dry and detergent-free nitrocellulose filters (Millipore HATG). Using

blunt-ended forceps, a sterile filter, numbered side down, was overlaid on the above plate. When the filter was thoroughly wet for 1 min, peeled from the plate, a replica filter was overlaid for 2 min. The filters were treated in 0.5 N NaOH and 1.5 M NaCl, 2.5 min at RT, and neutralized with 1.5 M NaCl and 0.5 M Tris.Cl, pH 7.4 for 5 min. The filters were rinsed in 2X SSPE (Sambrook et al., 1989), air-dried for 5 min at RT, sandwiched between Whitman 3 MM paper, baked for 2 hrs at 80 °C under the vacuum, and stored at -20 °C in a sealed plastic bag until needed. Hybridization was performed as described above in the Northern blotting analysis using ³²P-labeled cDNA as probe.

2. Reverse Transcriptase Polymerase Chain Reactions (RT-PCR)

For preparation of total RNA, mouse brain was isolated and immediately frozen in liquid nitrogen following decapitation and grounding in a Bio-Pulvinizer (Bergemann et al., 1992). Total RNA was extracted from brains as described above (Chirgwin et al., 1979). Poly (A⁺) RNA was obtained by passing through oligo (dT)-cellulose columns from mRNA purification kit (Pharmacia) (Aviv and Leder, 1972).

Total cellular RNA (20-30 ug) or poly (A⁺) RNA (1 ug) was used for reverse transcription with the first-strand cDNA synthesis kit (Pharmacia) by following the manufacturer's instructional manual using the antisense primer (reverse primer as described as follow). The entire first-strand DNA sample (15 ul) was heated to 90 °C for 5 min to denature the RNA-cDNA duplex and inactivate the reverse transcriptase, then chilled on ice. The entire first-strand DNA sample was then

amplified in PCR (Ausubel et al., 1990; Innis et al., 1990) using primers flanking the open reading frame of F1-20 cDNA, forward: USX130 5'-CTCACTCGAGGGCCGGTGAAGATGTC-3', and reverse: DSX2922 5'-CTCAACTCGAGAATCTTATCTGAAGTTTCC-3', modified from Zhou et al. (1992, 1993). RT-PCR reactions (Ausubel et al., 1990; Kolls et al., 1993) were carried out by mixing 80 ul of PCR mix (60 ul of 10X PCR buffer--150 mM Tris.Cl, pH 8.8, 600 mM KCl; 6 ul of 25 mM MgCl₂; 4 ul of dNTPs mix--1.25 mM final from the solution of 4.5 ul of each 10 mM dNTP plus 18 ul ddH₂O; 0.5 ul of AMpli Taq DNA polymerase--Cetus 5 unit/ul; 60 ul ddH₂O), 2.5 ul of each primer (total 200 ng, 10 pmol/ul), 15 ul template cDNA (50 ng/ul) and 0 ul ddH₂O. Topped was 1-2 drops of mineral oil per tube. DNA samples were amplified with cycling program as follows: denaturation at 94 °C for 1 min; annealing reaction at 50 °C for 2 min; extension at 72 °C for 3 min, extend 30 seconds; for 25 cycles, which was ended by incubating a final 15 min at 72 °C, then soak at 4 °C. The PCR product was electrophoresed on 1% agarose gels and purified by the GeneClean II procedure (Bio 101).

Alternatively, four rat brain cDNA libraries were used to screen for the full-length encoding region of F1-20 using the forward or reverse primer, separately. The putative plaques were rescreened with both primers by phage dot hybridization method as described above. In addition, all the four rat brain cDNA libraries were used for PCR amplification with the pairs of primers flanking the open reading frame (ORF) as shown above (Dr. Min Li, Johns Hopkins University, personal

communication).

3. Subcloning and Overexpression

The PCR product was electrophoresed on agarose gels and purified by the GeneClean II procedure (Bio 101), end-repaired and ligated with SmaI cut pGEX3X to generate the recombinant plasmid pSL185. After transforming into *E.coli* XL1-blue, the recombinant plasmids were screened by in situ lysis of bacterial colonies on nitrocellulose filters, followed by DNA hybridization with both forward and reverse primers as probes (Sambrook, et al., 1989; pp 1.90-1.110). The recombinant plasmids with correct orientation and reading frame were determined by restriction enzyme mapping and DNA sequencing. In respect to the subcloning, a blunt-ending procedure and a novel method was used (Bankier et al., 1988).

For standard Kinasing/end-repair and blunt-end subcloning (Dr. John A. Martignetti, personal communication), 20 ul of the PCRed DNA, 3 ul 10X Kinase buffer, 1 ul of 10 mM γ ATP, 5 units of polynucleotide kinase, 5 ul H₂O, incubated at 37 °C for 45 min. Into the 30 ul kinasing reaction above, were added 2 ul Fill-in buffer, 4 ul of 0.5 mM mixture of deoxynucleotides, 5 units of Klenow, 5 units of T4 DNA polymerase. The mixture was incubated for 45 min at RT. After phenol extract, the DNA was precipitated to remove proteins and free nucleotides (Bankier et al., 1988). Or, the end-repaired fragment was run out onto a 0.8% agarose gel and the fragment purified with GeneClean II kit (Bio 101). For this novel method of end repair, it was reasoned that, if Taq polymerase leaves homopolymeric overhanging ends, mild

treatment with S1 nuclease following the kinasing reaction should also generate a product which can subsequently be cloned. When this was attempted, cloning efficiency was 10-fold greater than that achieved with the blunt-ending protocol. However, products generated were not all full-length (Dr. John A. Martignetti, personal communication).

The pSL185 construct was transformed into *E.coli* BL21 (gift from Dr. Andrew D. Bergemann in Professor/Dr. Edward M. Johnson's laboratory), and grown in LB broth with Ampicillin (Das 1990; Gould, 1990; Studier et al., 1990). The optimal time for IPTG induction was determined by time course experiment as follows: overnight cultures of the freshly transformed BL21 cells was diluted 1:100 in LB-ampicillin. Cells were grown by shaking 250 rpm at 37 °C. A series of 1 ml aliquots of cultures were taken per 0.5 hour until an O.D.₆₀₀ of 0.3-0.5, about 2 hours. 0.5 M IPTG was added to a final 1 mM to overexpress GST-F1-20/NP185 fusion protein. 1 ml aliquotes were taken each half hour after IPTG induction. The cells were pelleted by centrifugation, solubilized in SDS sample buffer containing proteinase inhibitors, and cooked for 5 min. The insoluble materials were removed by centrifugation. The SDS-PAGEs were performed and immunoblotted as described above with the Sturdier vertical slab gel unit, model SE400 (Hoefer Scientific Int.) and a Trans-Blot apparatus (Bio-Rad).

4. Purification of GST-NP185/F1-20 Fusion Protein

Purification of GST-NP185/F1-20 protein was performed using a Glutathione Sepharose 4B column (Pharmacia) according to manufacturer's instruction with following modifications

(Marston, 1986). The induced cells (500 ml to 2 liters) were harvested by low speed centrifugation. The pellet was washed by 25 mM Tris.Cl, pH 7.5, 10 mM EDTA, 86 mM NaCl and 50 mM glucose. The cells were immediately added with proteinase inhibitors and frozen in liquid nitrogen (Li and Shue G, personal communication). After thawing, the cells were resuspended in TE buffer (Sambrook et al., 1989), lysozyme was added into the cell suspension (final concentration of 2 mg/ml) for 15 min on ice. After the cells were sonicated, DNase II or DNase I was added to digest DNAs and RNAs, then centrifuged by 15,000 rpm for 10 min with SS34 rotor (Dr. Chavdar P. Krachmarov in Professor/Dr. Johnson's laboratory, Personal communication). The supernatant was diluted in PBS (150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄, pH 7.3), plus 1% Triton X-100, and applied to the column as described in the manufacturer's manual.

Alternatively, the GST-F1-20/NP185 fusion protein was purified by Centrilon Micro-elutor Microelectroelutor (Amicon)(Dr. Ronald Kohansky, Protein Core at Mount Sinai). Briefly: after staining of SDS-PAGE and checking with immunoblot, the specific band of the fusion protein was excised from the gel, pieced and submersed into e-tube. The fusion protein was electro-eluted out of the gel slab into the buffer, and concentrated by Centricon concentrator system (Amicon).

5. Clathrin Assembly Assay

Separation of heavy and light chains was performed by following the procedure of Winkler and Stanley (1983). The

effect of NP185 on the polymerization of clathrin was determined by layering purified clathrin incubated with either purified bovine NP185 or the purified GST-NP185/F1-20 fusion protein, on top of a 10% sucrose solution. The tubes were centrifuged at 100,000g to permit polymerized structures to sediment at the bottom of the sucrose layer. Aliquots from the top solution and the resuspended pellet were assayed by ELISA to detect clathrin and NP185. The pellets were resuspended for negative staining electron microscopy to visualize the type of structure formed.

Approximately 10 ug of the pellet from the sucrose bed containing NP185-induced clathrin cage polymers was suspended in 0.1 M Tris buffer pH 7.0 and used for negative staining. Samples were placed on Formvar-coated grids, negatively stained with 1% uranyl acetate, air dried according to the procedure of Valentine et al., (1968), and examined in a Joel 100-B electron microscope at an acceleration voltage of 80 kV (Su et al., 1991). Where indicated, pellets of vesicles were processed for transmission electron microscopy as described elsewhere (Lisanti et al., 1982).

6. Far-Western Blotting

Far-western blotting (Ruppert et al., 1993; Hisatake et al., 1993; Lee et al., 1991) or sandwich blotting (Kohtz and Puszkin, 1989), was used for GST-NP185 tubulin binding analysis. GST-NP185 protein (20 ug), purified with the column (above), 20 ug of partially purified NP185 from bovine brain and 20 ug of purified CHCs were resolved on a 5-15% SDS-PAGE. Proteins were electrotransferred to nitrocellulose (Bio-Rad)

at 300 amps for 3.5 hours. The protein on blot was denatured and then renatured by treating the blot with sequential washings in solution CZ 0.1 M (20 mM HEPES pH 7.9, 0-17% glycerol, 0.1 M KCl, 5 mM MgCl₂, 0.1 mM EDTA, and 2 mM dithiothreitol) plus 0.5 mg/ml BSA, 0.02% polyvinylpyrrolidone, and 6 M guanidine-HCl for 30 min, one time; then solution CZ 0.1 M plus 0.5 mg/ml BSA, 0.02% polyvinylpyrrolidone, and 0.1 M guanidine-HCl for 1 hour, two times; then solution CZ 0.1 M plus 0.5 mg/ml BSA, 0.02% polyvinylpyrrolidone for 2 hours, three times; and 10 mM HEPES (pH 7.9) plus 5% BSA for 1 hour. All washings were performed at 4 °C. The blot was then incubated with 10 ml of 1:1 dilution of CZ 0.1 M mixed with brain tubulin (kindly provided by Dr. David R. Colman) for 12 hours at 4 °C. The blot was then washed with 20 ml of 1:1 dilution of CZ 0.1 M for 10 min two times, incubated with mouse anti-tubulin mAbs IgG (from Dr. Gianni Piperno) for 2-8 hrs at 4 °C. The wet protein blot was washed with 1:1 of CZ 0.1 M for 10 min two times, and incubated with goat anti-mouse IgG conjugated with alkaline phosphatase. After washings, the signals were detected by BCIP/NBT in developing buffer.

7. Identification of NP185-Associated Proteins from Mouse Brain Cytosol

A pull-down assay was modified and used for this experiment (Hagemeier et al., 1994). Briefly, mouse brain cytosol was prepared as described in Table 2 and used for isolation of NP185-associated proteins. After dialysis against PBS pH 7.4 buffer, the cytosol was mixed with Glutathione

Sepharose 4B beads which bound to GST-NP185/F1-20 fusion protein, and incubated for 0.5-8 hrs at 4 °C at rotatory mixer. The mixture was packed into a FPLC coupled column. The column was washed with PBS pH 7.4, 0.1% BSA buffer. The complex of NP185/F1-20 and its associated proteins was eluted using 50 mM glutathione solution of 50 mM Tris pH 7.5 as described above. After dialysis, phosphorylation of proteins was conducted in 10 mM Tris, 10 mM MgCl₂, 100 mM MES buffer pH 7.5. Reaction mixture (0.1 ml) contained 0.1 mg of the elute from above, 20 uM ATP, 1 uCi γ -³²P-ATP. Incubation was performed for 10-20 min at RT. Reaction was stopped by adding of SDS-PAGE sample buffer (0.1% SDS, 15% 1-mercaptoethanol, 20% glycerol, and 0.2% phenol red). The sample was loaded and run through 5-15% gradient SDS-PAGE. Gels were stained and dried. An autoradiography was obtained by overlaying Kodak X-omat R film onto the dry gel sandwiched with intensifying screen for 2-15 hrs at -80 °C.

Alternatively, a Pull-down assay was directly used as described in Hagemeier et al., (1994). Briefly, proteins were expressed in and purified from E.coli as the same as above. Pull-down assays were performed by pre-incubating 50 ng of GST or GST fusion protein with BSA (final concentration, 1 mg/ml) at RT. After 5 min, the mixture of GST or GST fusion protein and the cytosol was incubated on a rotating wheel for 45 min or 2 hrs at 4 °C. The glutathione sepharose 4B beads were added to the mixture and incubated for 0.5-1 hr. The beads were then washed three times in appropriate buffer, and finally taken up in SDS-PAGE sample buffer. Copurified

proteins were resolved on 5%-15% SDS-polyacrylamide gradient gels. The gels were fixed, stained, soaked in 5% glycerol, dried, and subjected to autoradiography at -70 °C as described above.

PART IV. MAPPINGS OF ANTIGENIC DETERMINANTS, TUBULIN BINDING DOMAIN, CLATHRIN LIGHT CHAIN ASSOCIATED DOMAIN

The pSL185 (pGEX-NP185/F1-20) was cut by EcoRI, the 2 Kb insert was purified with GeneClean II (Bio 101) from agarose gel, and subcloned into pGEX 1 λ T vector (Pharmacia), this construct was named as pSLEC. The left-over of pSL185 was also gel-purified with GeneClean II (Bio 101), and self-ligated, formed pSLEN construct. These two constructs were transformed into *E.coli* XL1-blue, the plasmid DNAs were isolated, and analyzed by restriction mapping to figure out the right orientation. After transformed into *E.coli* BL21, the fusion proteins from pSLEN and pSLEC were induced to express by adding final concentration of 1 ml IPTG. The immunoblotting was performed with anti-NP185 antibodies.

Construction of NP185 truncation mutants (Luo and Krakow, 1992; Luo et al., 1993): 5 ug of pSLEC was partially digested in 20 ul solution (the buffer recommended by Boehringer Mannheim Biochemicals or New England Biolabs) by a restriction enzyme PstI. After the digestion with different amount of the restriction enzyme in different time at 37 °C, the volume of the reaction mixture was increased by addition of 0.3 M sodium acetate (pH 5.2) to 200 ul. After phenol-chloroform extraction

and ethanol precipitation, the pellet was dissolved in 20 μ l of TE buffer (10 mM Tris-HCl pH 8.0, and 1 mM EDTA). For this, 4 μ l was used for analysis on 0.8% agarose gel, 12 μ l for ligation at 14 $^{\circ}$ C overnight in the presence of 1 unit of T4 DNA ligase and 4 μ l was saved as a non-ligated DNA control for transformation. The ligation mixture as well as the non-ligated DNA control were transformed into competent *E.coli* XL1-blue cells. Positive transformants were selected on LB-ampicillin plates and grown in liquid culture (LB-ampicillin) overnight. Plasmid minipreps were used for restriction enzyme analysis.

The pGEX-NP185/F1-20 derived plasmids identified as containing a truncated NP185 were transformed into *E.coli* BL21. Synthesis of the truncated NP185 protein were induced by temperature shift from 30 $^{\circ}$ C to 37 $^{\circ}$ C with 0.5 mM IPTG at an A_{600nm} of 0.6 to 1.5 for three hours. Total cell protein was prepared by lysing the cell pellet collected from a 1 ml culture in 100 μ l of SDS-PAGE sample buffer (0.1% SDS, 5% glycerol, 0.05% bromophenol blue plus 1% of 2-mercaptoethanol). After heating in a boiling water bath for 5 min, 50 μ l were used for analysis by SDS-PAGE.

Western blotting was used to map the antigen determinants, while Far-Western blotting or GST-column was used to map the tubulin and CLC binding site. For all the experiment, the protein from the pGEX vector were used as a negative control, the NP185 protein from bovine brain were included as a positive control.

PCR fragments (Ausubel et al., 1990; Kaufman and Evans,

1990; Innis et al., 1990): a series of cDNA fragments from Fl-20/NP185 gene were generated by PCR and subcloned into the expression vector pGEX 1λT using EcoRI site for overexpression in *E.coli*. as followings (Underlined are EcoRI sites incorporated into both primers):

pPCR224 (224 residues) contains the fragment generated by primers USE869 5'-CTAGAATGACGAGGGTGT-3' and DSE1557 5'-CTCGAATTCTGAGCATGCATCAAGAGC-3'.

pPCR56 (56 residues) was made from the fragment generated by primers USP2680 5'-CTCGAATTCAGTTCTCAGCAGCCAGTC-3' and DSA2922 5'-CTCGAATTCTCTTATCTGAAGTTTCC-3'.

pPCR124 (124 residues) was from the fragment generated by primers USE869 5'-CTAGAATGACGAGGGTGT-3' and DSE1259 5'-CTCGAATTCCCAGAAGGAGGC-3' (tubulin binding domain).

pPCR28 (28 residues) was from the fragment, generated by primers USE1174 5'-CTCGAATTCAAGCCATCAAGCGATCTCC-3' and DSE1259 5'-CTCGAATTCCCAGAAGGAGGCACTAC-3'.

pPCR129 (129 residues) contains the fragment generated by primers USE1174 5'-CTCGAATTCAAGCCATCAAGCGATCTCC-3' and DSE1557 5'-CTCGAATTCTGAGCATGCATCAAGAGC-3' (epitopes for 8G8 and 6G7 mAbs).

pPCR66 (66 residues) contains the fragment generated by primers USE1174 5'-CTCGAATTCAAGCCATCAAGCGATCTCC-3' and DSE1374 5'-CTCGAATTCGTAGTAGGAGGGGAAGGC-3'.

pPCR60 (60 residues) contains the fragment generated by primers USE1378 5'-CTCGAATTCACCACTGAGCCTGCTTC-3' and DSE1557 5'-CTCGAATTCTGAGCATGCATCAAGAGC-3' (epitopes for 8G8 and 6G7 mAbs).

RESULTS

1. Immunolocalization of NP185 in the Neuromuscular Junction of Peripheral Nerve System

As demonstrated in Fig. 1, the results showing the dotted immunofluorescence patterns with the NP185 mAb, the typical morphological appearance, and the preservation of neuromuscular junctions were photographed through phase of contrast optics, DIC Nomarski optics, confocal microscopy and scanning electron microscopy. Also in Fig. 1, the patterns of immunofluorescence indicate that NP185 is located in the neuromuscular junction of chick muscle fibers. The fluorescent labellings are depicted in Fig. 1 (c, e, f). Dots and patches of fluorescence were associated with structures present on the fiber surface, which had the features avian neuromuscular junctions. Fig. 1a shows two muscle fibers with their transverse striations. At low magnification, the neuromuscular junction were not visible, and control determinations with the primary antibody omitted showed the muscle fibers devoid of labelling (Fig. 1b).

The NP185 immunofluorescence shown in Fig. 1c was confined to small, clearly marked dots. Patches of these dots or lanes of dots were seen on the muscle fiber surface. The types of structures marked by the antibody was visualized by scanning electron microscopy of the labeled fibers and shown in Fig 1g-i. The pattern of immunofluorescence was consistent with the morphology of the neuromuscular junction described by

other investigators (reviewed in Purves and Litchman, 1985). The motor neuron axonal terminal contacting the muscle membrane is often referred to as a single synapse, but in fact it is a collection of many linked synaptic boutons. The boutons are polymorphic connected sacs extending into the complementary furrows of the postsynaptic endplate which are clearly visible in the scanning electron micrographs (Fig 1g-i). These figures illustrate views of endplates on muscle fibers after immunolabelling. They also render clear views of the fiber surface, their preservation after antibody treatment, and after their processing for scanning electron microscopy.

The arrowheads in Fig. 1g mark the contour of a neuromuscular endplate, whereas the solid ovals are placed on the remnants of presynaptic boutons of axonal terminals showing open and resealed saclike formations. The bulky, polymorphic, smooth, and elongated structures shown in Fig. 1h-i represent the presynaptic boutons positioned on top of the partially exposed postsynaptic folds packing the acetylcholine receptors. These partly constricted, open or resealed, sausage-like structures of the axonal terminal bouton are responsible for intense fluorescent dots. They in turn suggest that the NP185 molecules are located on the presynaptic side of the neuromuscular junction (Silver, 1963; Torri-Tarelli et al., 1990).

In the immunohistochemical tagging of a presynaptic protein, the effect obtained is manifested as an archipelago of irregular size dots showing intense immunolabelling when

the neuromuscular junction is viewed from above as seen in the figures. In Fig. 1d, a muscle fiber viewed by phase contrast optics reveals the contour of an elongated neuromuscular junction delineated by arrowheads., The equivalent structure with NP185 fluorescence is illustrated in Fig 1e. Likewise, Fig 1f displays an isolated area of the fiber in Fig. 1c at higher magnification. The speckled labelling is loosely organized, occasionally having its highest concentration in a centralized bright region. The fluorescent punctate labelling is irregularly distributed in the muscle surface region and varies from area to area. The dots frequently appear as small constellations of curves and loops. This looping is reminiscent of the endplate visualized previously (Anderson et al., 1977; Gunther and Letinsky, 1982). When skeletal muscle was viewed in cross-section, labelling for NP185 occurred only along the muscle fiber perimeter (not shown).

The incidence of dual focal labelling was high. Examined was total of 150 muscle tissue sections mounted individually on coverslips representing five separate labelling experiments. The incidence of positive muscle surface area labelling for NP185 ranged from 0.05-0.085% of the total muscle surface scanned. The dimensions of the labelled areas agreed with the findings of others who visualized the avian neuromuscular junction by histochemical methods (Scott et al., 1988).

2. Immunoprecipitation of NP185 Extracted from Muscle Tissue

The above results indicate the presence of NP185 in

skeletal muscle and suggest NP185 sequestration to particular regions of the muscle fiber. However, these results alone do not prove that NP185 immunofluorescence is in the neuromuscular junctions. Also, immunofluorescent patterns alone do not prove that the monoclonal antibodies reacted with a NP185 molecule similar to that of brain. The mAbs could have recognized epitopes similar to those present in NP185 of brain tissue but present in other unrelated muscle proteins. For these reasons, we adapted a procedure to purify NP185 from brain, and carried out a microscale purification and enrichment of a putative NP185 molecule from muscle fibers. Chick muscle fibers were extracted with a high molar Tris buffer pH 8.0 that contained 0.9 M KCl. Then immunoabsorbed NP185/8G8 antibody with protein-G, eluted the retained molecules, and concentrated the supernatant with the putative NP185 molecules by acid precipitation. The precipitate was dissolved in SDS sample buffer, the protein bands were recovered, transferred, reacted with the mAb, and the results shown in the immunoblot lanes of Fig. 2.

Fig. 2A is the protein stained with Coomassie Brilliant Blue. The brain tissue clathrin coated vesicles are in lane 1, the extracted and concentrated proteins by immunoprecipitation are on lane 2. A clear narrow polypeptide band of 185 kDa is seen accompanied by a pair of polypeptides of 33 and 36 kDa possibly representing the clathrin light chains (Su et al., 1991). Lane 3 shows NP185 from muscle. It was obtained from a muscle fiber protein extract from which NP185-like molecules were immunoprecipitated by NP185 mAb coated beads; lane 4 has

the eluted protein bands concentrated by acid salt precipitation. Fig. 2B is the immunoblot of the gel in Fig. 2A. The antibody bound to similar 185 kDa polypeptides derived from muscle and brain. These results suggest that the NP185 molecules extracted from muscle fibers share epitopes identical to the neuronal polypeptide and that their site of origin is most likely the neuromuscular junction. It was noticed that NP185 isolated from the neuromuscular junction emerged with lower molecular weight proteins, which are different from those accompanying NP185 from chick embryo CNS. These bands could be the light chains from muscle clathrin whose lattices were seen beneath the sarcolemma (Pumplin and Bloch, 1987).

3. NP185 is in Neuromuscular Junction Indicated by Double Labelling of NP185 and AchR

The results indicated that similar NP185 polypeptides were present in muscle and brain tissue extracts. It remained to demonstrate that the muscle structures displaying NP185 fluorescence were indeed neuromuscular junctions. The experimental approach chosen was to carry out double-labelling determinations and confocal microscopy (Fig. 3). To label AchRs at the neuromuscular junction, α -bungarotoxin was used as conjugation with fluorescein (α BT-F). The snake venom binds selectively and irreversibly to AchRs in the postsynaptic plate of the neuromuscular junction. Also, it binds to both intracellular and cell surface receptors (Bursztajn, et al., 1985). Concomitantly, rhodamine-conjugated

IgG (IgG-R) was used to probe for mAb 8G8 immunoreacted with NP185 (Fig. 3).

Fig. 3 illustrates the results obtained from double labelling of AchRs and NP185. At low magnification the fluorescent dotted patterns appeared to correlate and to overlap with each other. The phase contrast optics image of the muscle area with neuromuscular junctions positive for both markers is shown in Fig. 3a. Marked by arrowheads are ribbons of the neuromuscular terminals. Control determinations using mAb absorbed with brain NP185 and unlabeled bungarotoxin were negative (Fig. 3b). Fig. 3c, d present the immunofluorescence patterns obtained after labelling with fluorescein- α -bungarotoxin and rhodamine-second mAb/8G8., respectively. Close examination of the dots in these patterns revealed that, although an overall correlation existed among the structures labelled by both markers, they were not overlapping. Indeed, comparison of many fluorescent dots at varying depths from the tissue's surface revealed subtle differences. A number of dots seen sharply with one marker were out of focus or at another plane with the other marker. These photographs were taken with different filters appropriate for each fluorescent marker but keeping the same plane of focus in the tissue.

The different planar location of dots was clearly resolved at higher magnification and on smaller nerve terminals visualized by Nomarski (DIC) optics after double labelling (Fig. 4). The dots for NP185 labelling illustrated in Fig. 4a appear markedly sharp and are clearly distinguished from those labelling the AchRs shown in Fig. 4b. To illustrate

these details in the figures, the plane of focus was adjusted until a sharp edge coinciding with high fluorescence intensity was obtained for a group dots. The neuromuscular junction illustrated in this figure displays similar morphology to that shown by scanning electron microscopy (Fig. 1g-i). The speckled label for AchR (arrowheads, Fig. 4b) is at a slightly different position than that displayed by the NP185 mAb (arrowheads, Fig. 4a). Thus these results must represent antigen labelling at pre- and postsynaptic locations. In chick myofibers, AchRs are commonly aggregated as lanes of patches in the sarcolemma (Cohen and Pumplin, 1979). This pattern is readily visible under epifluorescent light microscopy.

4. NP185 Protein was Successfully Purified to be Homogeneity by Anti-NP185 mAb Immuno-Affinity Chromatography

Bovine brains were homogenized and subjected to differential centrifugation, and followed by several chromatographies in order to purify NP185 protein (Table 2). CCV was separated from plasma membrane using Sephacryl S-1000 gel filtration column equilibrated with 0.1 M MES buffer, pH 7.8 (Fig. 5).

Proteins from CCVs were further separated by the Sepharose CL6B gel filtration column (Fig. 6). Western blotting revealed NP185 eluting in fractions within the clathrin peak (Fig. 6, 7). When these fractions was subjected to hydroxylapatite chromatography (MonoQ anion exchange column), NP185 protein was separated from clathrin, but not from assembly polypeptides (Fig. 8).

NP185 protein was finally resolved from assembly polypeptides by the immuno-affinity chromatography (Fig. 9, 10). NP185 protein was eluted in two peaks: a minor peak (Peak I) and a major peak (Peak II)(Fig. 9). These two peaks may represent two isoforms of NP185 protein. The fractions of the minor peak (Peak I) may represent an isoform of NP185 protein which has a low binding affinity to antibody so that it was firstly eluted from the column. However, the fractions from the major peak (Peak II) was used for further analysis.

NP185 protein was successfully purified to be homogeneity by using immuno-affinity chromatography with our anti-NP185 mAb, 8G8 (Fig. 10). The SDS-PAGE of the purified protein shows a single band (Fig. 10A), and anti-NP185 mAbs recognized the protein (Fig. 10B); while none of the other mAbs, e.g., anti-clathrin mAb, anti-assembly polypeptide mAb was bound to the band (data not shown). As a control, albumin was not retained on the mAb 8G8-conjugated Sepharose 4B column, and emerged with the washing buffer (data not shown).

5. The Amino Acid Sequences of Internal Peptides from NP185 Protein Purified by Anti-NP185 mAb Affinity Chromatography Were Found to Contain Matches in Deduced Amino Acid Sequence from F1-20 cDNA, While the Amino Acid Composition of the Purified NP185 Protein Showed the Similar pattern to the Amino Acid Content Predicted from F1-20 cDNA

Analyses of the amino acid composition of the purified NP185 protein showed the similar pattern to the amino acid content predicted from F1-20 cDNA. Two batches of the

purification of NP185 protein showed the same amino acid composition (Table 3, 4), therefore, the NP185 protein was purified to be homogeneity. The amino acid composition of the purified NP185 protein indicates very high content of alanine, serine, proline, threonine, and leucine; which is consistent with that predicted from F1-20 cDNA. While the number of tyrosine from F1-20 cDNA is 9, 11 tyrosines were found for the purified NP185.

The N-terminal of NP185 protein was found to be blocked in our previous experiment (Li et al., 1991), therefore, NP185 protein was digested by trypsin and the internal peptides were isolated by HPLC. As shown in Fig. 11, the tryptic fragments of purified NP185 protein were separated by reverse-phase HPLC Vydac C-18 column. Briefly, about 100 pmoles of NP185 protein (15.5 ug, 94 pmoles) was digested with trypsin; while, as a control, 200 pmoles or 15 ug of transferrin was digested with trypsin, blank was SDS-PAGE gel slot with the same thickness but without proteins (not shown). The profiles of HPLC shows that the digestion of NP185 protein (the lower panel, Fig. 11) was reasonably successful by the comparison plots with transferrin (the upper panel, Fig. 11). Some well separated peaks of peptides were used for laser desorption mass spectrophotometry (LDMS) on 1-3% of the peaks prior to amino acid sequencing. LDMS is usually used to determine followings: (1) it shows whether the peak is an artifact peak or is in fact a peptide; (2) it shows whether the peak contains one peptide or more than one peptide; and (3) it reveals the mass of the peptide which can then be compared to the predicted

mass that would be obtained from the sequence itself. After analyses of LDMS, five internal peptides from peak #43, #48, #65, and #72 (the lower panel, Fig. 19) were chosen for amino acid sequencing (Table 5).

The amino acid sequences from the internal peptides were analyzed with FASTA (Swissprot) program by the University of Wisconsin Genetics computer Group (GCG) package running at the Mount Sinai School of Medicine, and with BLAST network service by National Center for Biotechnology Information (NCBI) of National Institutes of Health (NIH). As shown in Fig. 12 by underlines, there were 5 peptide sequences: AFSYR (peak #43), QMAFDGAR (peak #65), VSEFLK (peak #72), KGDLQWNAGEK (peak #48), and LTGGANWQPK (peak #48), which contains a match with residues 135-139, 140-147, 247-252, 770-780, and 782-791, respectively, predicted from mouse F1-20 phosphoprotein cDNA (Zhou et al., 1992). These five peptides can be found in both alternative splicing mRNA forms of the F1-20 as underlined (Fig. 12). It was shown that each of these peptide sequence matches contains tryptic site right before the amino terminus of the peptide, i.e., a tryptic digest would generate these fragments from the protein (Fig. 12).

6. Anti-NP185 mAbs, 8G8 and 6G7, Were Found to Cross-react with both Mouse and Bovine Brain Homogenate

Having found these matches as described above in Database computer searching, we set to determine to whether NP185 protein is identical or related to mouse F1-20 protein by doing immunological analyses. NP185 mAbs were generated

against crude extracts from bovine CCVs (Kohtz and Puszkin, 1988), while F1-20 mAb was generated against mouse brain homogenate (Sousa et al., 1990). The antibody cross-reactivity between bovine and mouse has not been determined previously. Both anti-NP185 and anti-F1-20 antibodies can recognize bovine and mouse brain homogenate proteins with similar migrated band of 185-190 kDa on SDS-PAGE (data not shown). However, we did not know whether both antibodies bind to the same protein based on the above experiment since there may be several proteins that migrate with 185 kDa or 190 kDa on SDS-PAGE gel. Therefore, the following experiments were performed.

7. Both Anti-NP185 mAbs and anti-F1-20 mAb Specifically Recognize the NP185 Protein Purified by Anti-NP185 mAb Affinity Column

With CCV protein as a control, the NP185 protein purified from bovine brain by immuno-affinity column can be specifically recognized by both anti-NP185 and anti-F1-20 monoclonal antibodies (Fig. 13B,C). Therefore, NP185 and F1-20 protein contain, at least, an identical domain, sharing the epitope region for both mAbs, anti-NP185 and anti-F1-20, if not the same.

8. The Full-length Open Reading Frame (Encoding Region) of F1-20 cDNA Was Generated by Reverse Transcriptase Polymerase Chain Reaction

Taken together, it was quite convincing that NP185 is most likely similar or identical to F1-20, called NP185/F1-20

as the following. While the full-length cDNA of rat NP185/F1-20 was continuing to carry out by screening those cDNA libraries as described previously (Li et al., 1992), a procedure was performed to generate the full-length encoding region for both rat and mouse NP185/F1-20 by using the published composite F1-20 cDNA sequence. All four rat brain cDNA libraries were screened using two primers flanking the ORF of F1-20 cDNA as oligonucleotide probes, separately; none of the clones was reacted with both ³²P-labelled probes by phage dot hybridization. Therefore, mouse brain mRNA was isolated, and the entire ORF was generated by reverse transcriptase polymerase chain reaction (RT-PCR). As predicted, 2.8 kb (2792 nt as designed) of cDNA was found on 0.8% agarose gel stained with ethidium bromide (Fig. 14). The PCR products were extracted with phenol and chroloform, and precipitated with salt-ethanol. The sequence of PCR product was confirmed by Northern blot analysis, restriction mapping, DNA sequencing, and Southern blot analysis.

Northern blot analyses show that the mRNA probed by the 2.8 kb cDNA probe is brain-specific with about 6 kb in size, not shown in other tissues: liver, lung, kidney, spleen (Fig. 15). The broadness of the band may reflect either the heterogeneity of the original mRNA (Fig. 15A) as shown elsewhere (Zhou et al., 1992; Morris et al., 1993), or degradation in this case (Fig. 15B). Southern blot analyses of mouse genomic DNA are similar to a single-copy mouse F1-20 gene (Zhou et al., 1992). Digestion of genomic DNA with four different restriction enzymes (BamHI, EcoRI, HindIII, and

XhoI), followed by Southern blotting and hybridization with the 2.8 kb cDNA as probe. The results showed the similar pattern to that from F1-20, although with the sizes of fragments different from those of mouse F1-20, which might result from different length of probes used.

9. GST-NP185/F1-20 Fusion Protein Expressed in *E.coli* Can be Specifically Recognized by both Anti-NP185 and Anti-F1-20 mAbs

One of goals of the expression of NP185/F1-20 protein in *E.coli* is to eventually obtain large amounts of a homogeneous NP185/F1-20 protein for functional studies in vitro. Therefore, the cDNA fragments from PCR above were isolated by GeneClean II kit, and subcloned into pGEX3X expression vector, resulting in construct pSL185 (Fig. 16). The orientation, reading frame, and sequence were confirmed by restriction mapping and DNA sequencing as well as Western blot analysis.

To further study, the recombinant plasmid pSL185 was transformed into *E.coli* BL21LysS, and overexpressed in *E.coli*. by an inducer, IPTG. In order to determine the optimal time for IPTG induction, the time course of induction was performed (Fig. 17). It was shown that, without IPTG induction, there was no or little amount of GST or GST-NP185/F1-20 expression as shown in lanes 1,2,1',2'. However, when IPTG added, GST-NP185/F1-20 was produced after 0.5 hour, migrated with molecular weight 200 kDa in SDS-PAGE as indicated in lanes 3',4',5',6',7'; while GST was produced at the same time, migrated with molecular weight 26 kDa in lanes 3,4,5,6,7. When the protein accumulated, it was aggregated and insoluble in

SDS sample buffer (lane 6', 7', Fig. 17). In this case, degradation was observed (lane 6' and 7', Fig. 17). The amount of, either GST-NP185/F1-20 or GST proteins, respectively, was accumulated through the time up to 6 hours, and then decreased due to cell death (data not shown). Moreover, the best time for the purpose of purified GST-NP185/F1-20 proteins was 1.5-2 hours after induction in order to avoid the degradation of the protein by accumulated proteinases.

In immunoblotting analyses (Fig. 18), the GST-NP185/F1-20 protein is specifically recognized by both anti-NP185 mAb, 8G8 (Fig. 18C); and anti-F1-20 mAb (Fig. 18B), in the predicted location as indicated. There is no signal when probed with anti-clathrin mAb, 2D9 (Fig. 18D). If stained longer with the mAbs and developed longer with substrates, the minor band of GST-NP185/F1-20 could be detected even without IPTG induction in GST-NP185/F1-20 recombinant plasmid transformed cells, which reveals the low levels of expression (leaking); while there was no comparable staining of protein from the cells transformed with the expression vector alone (Fig. 18A). It was noted that GST-NP185/F1-20 was extremely unstable and quickly degraded, and proteolytic fragments were shown in both Coomassie blue stained gel (lane 4, Fig. 18A), and mAb stained blots) (Lane 4, Fig. 18B, C).

10. GST-NP185/F1-20 Fusion Protein Was Purified with Glutathione Sepharose 4B Column

GST-NP185/F1-20 fusion protein was purified to be homogeneity by Glutathione Sepharose 4B column (Fig. 19A). The

column is very easy to contaminate with proteinases, therefore, degraded product usually emerges from the regenerated column. Alternatively, the large amount of the GST-NP185/F1-20 fusion protein was purified by electroelution apparatus (Amicon) from preparatory SDS-PAGE gels (Protein Core, Mount Sinai Medical Center). The fusion protein was specifically recognized by anti-NP185 mAb as shown in immunoblotting analysis (lane 2, Fig. 19B).

11. GST-NP185/F1-20 Fusion Protein Promotes Clathrin Assembly

Clathrin assembly assay using the purified GST-NP185/F1-20 protein showed that, indeed, the fusion protein promoted the cage formation of purified clathrins as demonstrated by scanning electron microscopy (Fig. 20). Assembly of cages mediated by NP185 protein purified from bovine brain showed the much more uniform or homogeneous in cage formation, while buffer control did not show any polymerized structures under the described conditions (data not shown).

12. Truncation Mutants of NP185/F1-20 by Partial Restriction Enzyme Digestion

As shown in Fig. 21, the truncation mutants was generated by complete and partial digestion with restriction enzymes. The 2 kb fragment was cut out of pSL185 plasmid and subcloned into pGEX1 λ T vector, named pSLEC construct (Fig. 22). The left-over of the vector plus 0.7 kb insert was religated into pSLEN construct (Fig. 21). These two constructs were transformed to E.coli, and the fusion proteins were induced,

resolved on SDS-PAGE, and blotted with mAb probe; the result shows that both NP185 mAbs, 8G8 and 6G7, recognize the truncated protein in the C-terminal portion of NP185/F1-20 (Fig. 23). Using PstI restriction enzyme, the pSLEC was partially digested, and religated; and the constructs were classified by restriction mapping and DNA sequencing analyses (Fig. 21).

Selected truncated mutants were retransformed into competent E.coli BL21LysS cells, and overexpressed by inducer IPTG. Variety of truncated fusion proteins expressed in E.coli, were resolved on SDS-PAGE, stained with Coomassie blue (Fig. 24), and probed with mAb 8G8 (Fig. 25). The shortest fragment, pP990 (Fig. 21), contains the epitopes for both anti-NP185 mAbs 8G8 and 6G7, as shown by the immunoblot (lane 1, Fig. 25).

13. Deletion Mutants of NP185/F1-20 Were Made by Polymerase Chain Reaction (PCR)

When the NP185/F1-20 cDNA becomes shorter, it is unlikely to find a restriction enzyme which can be used to do further truncation fitting both the insert and the vector. Therefore, a series of PCR were performed to amplify particular fragments of the cDNA. As shown in diagram (Fig. 26), seven PCRs were carried out to generate 7 fragments with 7 pairs of primers according to the cDNA sequence (Zhou et al., 1992).

The PCR products were resolved onto 0.8-1.0% agarose gel. The bands of PCR product were isolated, cut with EcoRI as incorporated into the both primers, and subcloned into EcoRI-

cut pGEX1 λ T vector, and the orientations were confirmed by restriction enzymes and partial DNA sequencing. Those construct plasmids were retransformed into E.coli BL21LysS cells, and overexpressed by inducer IPTG. The proteins were shown on SDS-PAGE gel, stained with Coomassie blue (Fig. 27). The blots were probed with anti-NP185 mAbs, 8G8 and 6G7, showing that the shortest fragment of cDNA is 60 aa between residue 411 and residue 471 of the F1-20, which is the epitope for both 8G8 and 6G7 (Fig. 33B, C). Interestingly, the 60 aa show that there are two CKII motifs. This fragment was fused to GST, expressed as GST-fusion protein, which was purified by Glutathione Sepharose 4B column (Fig. 35), and the separation of two mAb epitopes by antibody-proteinase-protection assay, HPLC and Edman degradation sequencing is underway.

14. GST-NP185/F1-20 Fusion Protein Bound to Brain Tubulin, and the Binding Domain Was Mapped to 124 Amino Acids by Far-Western Blotting

In order to study relation between NP185 and other proteins, a newly revised method was adapted to investigate the protein-protein interaction (Rupper et al., 1993; Hisatake et al., 1993), named Far-Western blotting or Sandwich blotting (Kohtz and Puszkin, 1989). Forward (Fig. 28A) and reverse Far-Western blotting (Fig. 28B) showed that GST-NP185/F1-20 binds to tubulin, not purified clathrins. In forward Far-Western blotting (Fig. 28A), tubulin was resolved in SDS-PAGE gel and transferred to a nitrocellulose membrane, GST-NP185/F1-20 fusion protein was overlayed on the membrane, and then

detected with anti-NP185 mAb (Fig. 28A).

In reverse Far-Western blotting (Fig. 28B), GST-NP185/F1-20 fusion protein was resolved in SDS-PAGE gel and transferred to a nitrocellulose membrane, tubulin was overlayed on the membrane, and then detected with anti-tubulin mAbs. It shows that tubulin specifically binds to GST-NP185/F1-20 protein on the membrane (Fig. 28B).

When the truncated NP185/F1-20 protein was used, the binding was much stronger on the same Far-Western blotting condition (lane 4, Fig. 29). It is reasonable that the truncated protein would more easily fold in a proper configuration that leaves the tubulin binding domain exposed. The binding domain was mapped to 124 amino acid between residue 249 and residue 372 of F1-20 cDNA predicted aa sequence (lane 4, Fig. 29; Fig. 30), a tubulin band from clathrin preparation was probed out by anti-tubulin mAb (lane 5, Fig. 30B). The amino acid composition of the tubulin binding domain indicates that it is quite hydrophobic, showing high content in alanine, proline, valine, phenylalanine. In addition, there are two CKII motifs in the region, indicating that the tubulin binding may be regulated by phosphorylation (Fig. 34).

15. Non-Phosphorylated Clathrin Light Chain Binds to NP185 Protein Purified from Bovine Brain, but Failed to Bind to Bacterially Expressed NP185; Phosphorylate CLCs Failed to Bind to CHCs

As shown in Fig. 31, non-phosphorylate CLCs bound to the

bovine NP185 protein (lane 1, Fig. 31C), but not NP185/F1-20 expressed in *E.coli* (lane 3, Fig. 31C); while non-phosphorylated CLCs bound to CHCs (lane 4, Fig. 31C). On the other hand, phosphorylated CLCs did not bind to CHC (lane 4, Fig. 31B). It is possible that CLC binding to NP185 is phosphorylation-dependent, since *E.coli* system does not have posttranslational modification, i.e., phosphorylation system. The results are consistent with biochemical data of that NP185 binds to unphosphorylated light chain (Su et al., 1991).

16. One Possible Kinase Associated with NP185/F1-20 Protein Was Isolated from Mouse Brain Cytosol by Glutathione Sepharose 4B bound GST-NP185/F1-20 Fusion Protein

Preliminary data showed that NP185 protein may bind to an unidentified kinase from mouse brain cytosol. This method was successfully used to find many ligand binding proteins as described elsewhere (Whiteheart et al., 1993; Sollner et al., 1993; Hagemeier et al., 1994). In this experiment, GST-NP185/F1-20 fusion protein was bound to Glutathione Sepharose 4B beads; while mouse cytosol was used for $\gamma^{32}\text{P}$ -ATP reaction, and mixed with the beads above. The column was packed with the beads, the sample was eluted, mixed with SDS-PAGE sample buffer, resolved on SDS-PAGE gel. The autoradiography shows that a possible kinase or kinase subunit is associated with GST-NP185/F1-20 protein (data not shown).

DISCUSSIONS

1. Neuron-specific protein, NP185, may be adapted to specialized neuronal functions, in particular in the synapse

Clathrin coated vesicles (CCVs) are phylogenetically distributed in most plant and animal cells, and are responsible for intracellular transport processes (Fine and Ockleford, 1984; Brodsky, 1985; Keen, 1990). CCVs have been purified from numerous tissues, including: bovine (Schook et al., 1979) and porcine brain (Pearse, 1975), human placenta (Ockleford and Whyte, 1977), rat liver (Campbell et al., 1983), bovine adrenal medulla (Crowther et al., 1976), oocytes (Woods et al., 1978), yeast (Mueller and Branton, 1982; Payne and Schekman, 1985; Payne et al., 1987; Lemmon et al., 1991), and cultured lymphoma (Pearse, 1976) and Chinese hamster ovary cells (Rothman et al., 1980). How do they adapt to serve such varied functions?

There are, at least, three ways used for CCVs specificity in evolution (Kohtz and Puszkin, 1988).

(1) Clathrin is adapted to specialized function through genetic variability or posttranslation modification. Predicted from Clathrin heavy chain (CHC) cDNA sequences, the deduced amino acid sequence is unrelated to known proteins, however, CHCs from human, rat, mouse (Kirchhausen et al., 1987a), yeast, and bovine brain showed that, it is structurally conserved between different tissues and species (Keen, 1990).

(2) On the other hand, modification of CCV accessory proteins (e.g., CLCs, assembly polypeptides or adaptors) may

serve to CCV specificity. Four CLC isoforms have so far been characterized: brain and nonbrain LC α and LC β (Jackson et al., 1987; Hill et al., 1988). Brain LC α and LC β have brain-specific hydrophobic insert of 30 aa and 18 aa, respectively (Jackson et al., 1987; Kirchhausen et al., 1987b). Similarly, adaptins, subunits of adaptors, have been divided into three types: α -, β - γ -. The β -adaptins are well conserved between species. The α - and the γ -adaptins, however, have brain-specific isoforms, which reinforce the supposition that there is a specialized pathway of receptor-mediated intracellular transport in brain (Ponnambalam et al., 1990).

(3) Moreover, CCVs may be adapted to specialized cellular functions through the association of novel proteins with the vesicle structure. So far, two such proteins, NP185 (Kohtz and Puszkin, 1988) and auxilin (Ahle and Ungewickell, 1990), have been described.

Previously, NP185 was shown in CNS neurons (Kohtz and Puszkin, 1988; Su et al., 1991). More recently during developmental study, the expression of NP185 was found to coincides with the onset of synaptogenesis (Perry et al., 1991). Thus, it is reasonable to speculate that, NP185 is present in the neuromuscular junctions formed from axonal branching of PNS neurons to support its neuronal specificity (Perry et al., 1992). The results presented above clearly demonstrate that the molecules visualized by two specific fluorescent markers, using light and confocal microscopy, are located at different planes and sites of the neuromuscular junctions. The NP185, due to its known neuronal specificity,

is located more externally and above the embryonic or chick muscle fiber surface (in presynaptic buttons). The AchRs are at their known postsynaptic location slightly deeper and beneath the muscle fiber outer surface layer (packed at the postsynaptic furrows of the sarcolemma). Also, it was shown that the labelling of NP185 was co-extensive with the labelling of AchR, on structures that are teetered together on the same muscle fiber. The Nomarski optics photographs revealed that all the visualized structures possessed the characteristics of avian neuromuscular junctions, namely, broad distribution and discoid-like morphology (Silver, 1963). Finally, the confirming evidence was obtained by applying the NP185 mAb in the Western blots to molecules resolved from respective tissues, embryonic or chick brain and striated muscle. The blots showed strong positive immunoreactivity with the NP185 mAb, showing NP185 protein bands of 185 kDa, which had been coimmunoprecipitated and comigrated on SDS-PAGE.

2. NP185 is present in the presynaptic site of neuromuscular junction of PNS, and may be involved in the recycling of synaptic vesicles

Theoretically, endocytosis processes may occur at both sites of the neuromuscular junction, regulated by different sets of molecules. Ultrastructural observation, however, indicated that CCVs are present only in presynaptic site of the neuromuscular junction (Heuser and Reese, 1973). The proposed function was to retrieve by endocytosis excess synaptic vesicle membrane fused with the synaptosomal plasma

membrane following neurotransmitter release. Nonetheless, refined electron microscopy permitted in recent years to establish the presence of morphologically typical clathrin lattices (0.5-1 μm) and clathrin-coated pits (0.1-0.15 μm) beneath the postsynaptic membrane of embryonic neuromuscular junctions (Bloch and Pumplin, 1988). Several reports showed that CVs and coated pits in muscle participate in the insertion and removal of AchRs at the postsynaptic membrane (Bursztajn and Fischbach, 1984), and AchR transport (Bursztajn et al., 1987).

Clathrin lattices, CCVs, and the complementary number of clathrin-associated proteins in CNS presynaptic boutons were positively identified with polyclonal antibodies (Puszkin et al., 1983). In view of NP185's association with CCVs (Kohtz and Puszkin, 1988; Puszkin et al., 1989; Perry et al., 1991, 1992; Su et al., 1991), similarities with other clathrin assembly proteins (Murphy et al., 1991; Su et al., 1991; Puszkin et al., 1992), and its binding for SVs and SPM, it would appear that the NP185 molecules are present only in the presynaptic site of neuromuscular junction.

NP185 may play a role in recycling of SV membrane through endocytosis. NP185 molecules are probably marking membrane areas with embedded components and "adapt" them for endocytosis and transport them to other locations. These processes are specialized for neurons of both the CNS and PNS. The synaptic boutons are sites where the rapid release and re-uptake of active neurotransmitter molecules occur mediated by complex flow of ions and chemical compounds. Such flow is

sustained from a compartment provided by a pool of synaptic terminal vesicles (Betz and Bewick, 1992). A likely role for NP185 would be to retrieve rapidly excess synaptic plasma membrane with other bound molecules after vesicle fusion and neurotransmitter release. This idea is based on the ability of NP185 molecules to induce *in vitro* rapid and effective assembly and closure of clathrin molecules in lattices and into close cages, as shown previously (Su et al., 1991) and later with bacterially expressed NP185 (Li et al., 1994). There are several molecules in the group of "adaptors", which perform similar roles, interact as complexes of several molecules, and are visualized at other locations in cells (Robinson and Kreis, 1992).

3. Molecular analyses showed that NP185 is identical to F1-20 and AP180 phosphoprotein

The relation of NP185 to F1-20 had not been determined before, while NP185 has only recently been refereed to be identical to AP180 (Alhe and Ungewickell, 1986) and AP3 (155 kDa) (Keen and Black, 1986), based on peptide mapping and immunoblot analyses (Murphy et al., 1991). NP185 has been characterized biochemically (Kohtz and Puszkin, 1988, 1989; Su et al., 1991), and immunohistochemically (Perry et al., 1991, 1992; Puszkin et al., 1992), as well as by partial cDNA cloning (Li et al., 1992); while F1-20 has been cloned by immunoscreening without any evidence showing relationship with other proteins (Sousa et al., 1992; Zhou et al., 1992). In view of the previous studies, NP185 and F1-20 have a number of

similarities as the following: they are (1) neuron-specific (Kohtz and Puszkin, 1988; Sousa et al., 1992); (2) synapse-specific and enriched (Perry et al., 1991 and 1992; Sousa et al., 1992); (3) phosphoprotein (Keen and Black, 1986; Morris et al., 1990; Zhou et al., 1992); (4) acidic with anomalous migration on SDS-PAGE (Ahle and Ungewickell, 1986; Keen and Black, 1986; Kohtz and Puszkin, 1988; Murphy et al., 1991; Sousa et al., 1992) (5) developmentally regulated (Perry et al., 1991; Sousa et al., 1992).

In the current studies, NP185 was found to be identical to F1-20 protein, or otherwise, at least contains many identical domains of F1-20 protein. Firstly, both NP185 mAbs (raised from bovine protein) and F1-20 mAb (raised from mouse brain protein) can be cross-reactive to a band with 185-190 kDa on the Western blots and SDS-PAGE from both bovine and mouse brain homogenate. Secondly, both NP185 and F1-20 mAb can recognize the purified NP185 protein. Thirdly, the partial cDNA clone sequence of NP185 by immunoscreening was found to overlap with F1-20 cDNA sequence (Zhou et al., 1992). Most importantly, five amino acid sequences of internal peptides from the purified NP185 protein can be found to completely match the internal amino acid sequences predicted from F1-20 cDNA. In addition, both anti-NP185 mAb and anti-F1-20 mAb can specifically recognize the GST-NP185/F1-20 protein expressed in *E.coli* with molecular weight 200-211 kDa migrated on SDS-PAGE, although the molecular mass of F1-20 as calculated from translated cDNA is only 91 kDa (Zhou et al., 1992, 1993). Relevantly, both acidic proteins and proline-rich protein were

showed to migrate anomalously on SDS-PAGE, with differences between calculated and migrated molecular weights as great as a factor of 2 (Julien and Mushynski, 1982; Kleinschmidt et al., 1986; Myers et al., 1987; Takano et al., 1988; Chin and Liem, 1990; See and Jackowski, 1990). The reason for the molecular weight discrepancy of NP185 and F1-20 on SDS-PAGE could result from, either posttranslational modifications of the primary translation products, e.g., phosphorylation; or the highly acidic proteins, e.g., F1-20 with pI 3.85 (Zhou et al., 1992).

While we were performing the experiments to establish the relation between NP185 and F1-20 protein; a paper describing the cloning and sequencing of rat AP180 was most recently published by Ungewickell's group (Morris et al., 1993; EMBL database accession numbers X68877 and X68878). It shows that the coding sequence of rat AP180 is 98% identical to mouse F1-20 protein on the amino acid level; while rat NP185 partial cDNA sequence (Li et al., 1992), almost overlaps with part of rat AP180 cDNA sequence. This confirmed findings that AP180 is probably identical to NP185 (Murphy et al., 1991).

In summary, some of differences may be due to species variation. It is still not clear why NP185, F1-20 and AP180 show such a wide variation in pI value: pI 6.0 for NP185 by 2-dimensional gel analysis (Kohtz and Puszkin, 1988), pI 3.85 for F1-20 calculated from cDNA (Zhou et al., 1992), pI 5.5 by 2-dimensional gel analysis (Ahle and Ungewickell, 1986) or pI 4.6 calculated from predicted amino acids of cDNA for AP180 (Morris et al., 1993). Even using an identical electrophoresis

system to examine the bovine protein and mouse protein, a pI of 4.6 has been found in the bovine protein, while the mouse protein has a pI of 3.85 (Zhou et al., 1993). In addition, the numbers of potential phosphorylation sites for AP180 are different from that of F1-20 as the following: In AP180, 12 motifs for CKII, 6 for PKC, 4 for cdc kinase, and 2 for cAMP-dependent protein kinase (Morris et al., 1993); while 6 motifs for CKII, 4 for PKC, 1 for cAMP-dependent protein kinase, and 3 for Ca²⁺/Calmodulin-dependent protein kinase (Zhou et al., 1992).

Since presently definitive data on the functional analysis of NP185, F1-20 and AP180 is still not available, it might be reasonably to assume that NP185, F1-20 and AP180, are highly related structurally. In other words, it is likely that these three proteins are members of a new gene family. NP185 is structurally or functionally related to F1-20 protein and AP180 in synaptic vesicle transport and synaptogenesis.

4. NP185 has two isoforms in bovine brain, F1-20 has at least three isoforms in mouse brain, and AP180 has two isoforms in rat brain

As shown in Fig. 9, two isoforms were found in bovine brains: one with high affinity to the anti-NP185 mAb and the other with low affinity to the mAb. During the development of PC12 cells induced by NGF, two forms of NP185 were described, one with high mobility, the other with low mobility (Kohtz and Puszkin, 1988). Consistent is that two isoforms were revealed in F1-20 protein (Fig. 12) (Zhou et al., 1992), one more

isoform was found later on (Zhou et al., 1993). It is possible that both isoforms of NP185 are involved in the regulation of the function of the NP185 protein. Similarly, in the rat cDNA of AP180, two isoforms were found in the presence or absence of 19 residues (Morris et al., 1993).

Most interestingly, the removal of a potential phosphorylation site resulting from this alternative splicing and/or posttranslational modifications, as observed, point out a possible functional significance of the event. The cDNA structures of most of the well-characterized synaptic proteins have been found to be present in multiple isoforms (Elferink et al., 1989; Sudhof et al., 1989b; Archer et al., 1990; Geppert et al., 1991; Zhou et al., 1992). In addition, the isoforms have been shown to have differential distributions in brain (Geppert et al., 1991). NP185 was found to associate with CKII activity (Su, 1989; Su et al., 1991), two of the isoforms of F1-20 cDNA indeed eliminate a potential CKII motif, S-X-X-D/E, as described by Zhou et al. (1992). Coincidentally, AP180/NP185 is phosphorylated at serine residues both *in vivo* (Keen and Black, 1986) and *in vitro* (Morris et al., 1990). Therefore, the isoforms of NP185/AP180/F1-20 protein may play an important role in the regulation of synaptic function.

5. Does NP185 contain annexin-like domains?

When the internal peptide VSEFLKT from the purified NP185 protein was aligned to GenBank and EMBL database, in addition to completely match to predicted amino acid (aa) sequence from

F1-20 cDNA, it shows high homologous (100% identity in 6 aa overlap) to annexin I (lipocortin) (GenBank accession numbers: P04083, P10107, P07150, P14950). This 6 aa, VSEFLK, are conserved in human, mouse, rat, pigeon, guinea pig (Wallner et al., 1986; Smith et al., 1994; Kaplan et al., 1988; Arcone et al., 1993). Annexin I is a member of the newly defined family known as annexins (Kaplan et al., 1988; Smith et al., 1994). Since little is known for the function of NP185/F1-20/AP180, this information might shine a spotlight on understanding the function of this protein.

Annexins undergo Ca^{2+} -dependent binding to phospholipid that are preferentially located on the cytosolic face of the plasma membrane. The individual proteins in this family were discovered by investigators who had the apparently unrelated goals of identifying mediators of exocytosis, substrates for protein-tyrosine kinases, inhibitors of phospholipase A2, and components of the cytoskeleton. The physiological role has not been determined for any of these proteins, but the determination of their primary sequences has defined their relationships to one another and provided a structural basis for both their common and unique characteristics. The proteins share little sequence similarity in the amino-terminal domain but have 40-60% sequence identity in the core domain. The Ca^{2+} and phospholipid binding sites that are present in all proteins in the family are located in the conserved core domain (Gleeney, 1986; Schlaepfer and Haigler, 1987); thus, it is likely that they all exert their biological effects at the plasma membrane or endosome and their activity may be

modulated in response to changing cellular Ca^{2+} levels (Cohen and Fava, 1985).

In contrast to the conserved core domain, the nonconserved amino-acid domain is responsible for properties unique to individual members of the family. e.g., Calpactin I and lipocortin I contain a tyrosine residue that is phosphorylated, the phosphorylation is catalyzed by two different kinases, pp60^{src} (Radke and Martin, 1979), and the epidermal growth factor receptor/kinase (Pepinsky and Sinclair, 1986). All found members in the family are phosphoprotein, it was speculated to related to signal transduction or metabolic regulation. However, since annexins are present in relatively large amounts (up to 0.5% of cellular protein), it is unlikely that they play a catalytic role (Kaplan et al., 1988). Most interestingly, annexin VI was recently found to be required for budding of clathrin-coated pits (Lin et al., 1992). Activated by cytosol-dependent factors, Ca^{2+} and ATP, annexin VI was shown to be, not only an active associated protein in the detachment of coated pits from the membrane, but also a regulatory protein for the formation of CCVs (Lin et al., 1992).

NP185 was shown that it binds to synaptosomal plasma membrane, SVs, CCVs (Su et al, 1991), that may be like annexins binding to phospholipid. In addition, NP185/AP180/F1-20 is phosphorylated (Ahle and Ungewickell, 1986; Zhou et al., 1992). Furthermore, NP185 is also present in large amount, reasonably related to structural component, such as related to tubulin and anchored to ion channel (Puszkun et al., 1992), or

involving in neurite formation (Kohtz and Puszkin, 1988, 1989). Besides, the F1-20 protein is a substrate for Ca^{2+} -dependent proteolysis (Zhou et al., 1992), in other words, the expression and function of NP185/F1-20 would be regulated by Ca^{2+} .

6. NP185 may function as an adaptin-like molecule, since bacterial-expressed NP185 induces *in vitro* clathrin assemble.

It was showed that NP185 induces *in vitro* rapid and effective assembly and closure of clathrin molecules into lattices and into closed cages with purified bovine protein (Ahle and Ungewickell, 1986; Su et al., 1991). NP185 itself, however, may not function as an adaptin-like molecule, since HA2/AP2 is also present in CCVs (Maycox et al., 1992). The NP185 or AP180 used in those assays (Alhe and Ungewickell, 1986) may contain HA2/AP2 contaminants, which, as observed, bind to NP185, and could be responsible for inducing coat assemble (Kohtz and Puszkin, 1988).

To solve the ambiguous, the NP185 protein expressed in bacteria was used to do clathrin assembly assay. The results showed that, NP185 induced clathrin assembly into cages (Fig. 20), although the cage shapes were not as uniform or as homogeneous as when the purified bovine NP185 was used. The following are possible explanations.

(1) It is possible that GST fragment in the fusion protein interferes with the interaction between NP185 and clathrins by blocking the N-terminus of NP185/F1-20 protein. The GST fragment may also interfere the protein folding. The

N-terminus of NP185/AP180 protein was biochemically demonstrated to be required for interaction with clathrins (Murphy and Keen, 1992; Morris et al., 1993).

(2) NP185/AP180 is a phosphoprotein (Ahle and Ungewickell, 1986). It is well known that *E.coli* does not have any posttranslational modification system, e.g., phosphorylation (Maeda, 1989). In another words, posttranslational modifications may be required for full assembly activity of NP185/AP180 protein. Nonetheless, the bacterially expressed NP185/F1-20 proteins were able to fold into their biological conformation, thus stimulate the cage formation.

7. NP185 directly binds to brain tubulin.

Preliminary data showed that, the GST-NP185/F1-20 fusion protein, has a binding affinity to tubulin (Fig. 28). Thus tubulin binding domain was determined with *E.coli* expressed NP185 protein. The information on tubulin binding domain will be useful in addressing the functional significance of NP185-tubulin interaction *in vivo* experiment with the deletion mutants.

A large number of tubulin binding proteins have been described, but their nature appears to vary in several aspects. The tubulin binding proteins are as the following: MAP2 (Lewis et al., 1988), MAP-1B (Nobel et al., 1989), MAP5 (Noble et al., 1989), neuraxin (Kirsch, et al., 1990); tau (Lee et al., 1989); synapsin Ia and Ib (Baines, 1989; Petrucci and Morrow, 1991). The tau protein, however, was demonstrated

that three 18 residues evenly spaced imperfectly repeats appears to be tubulin binding sites *in vitro* (Lee et al., 1989). These 18 amino acids are as the following: ValLysSerLysIleGlySerThrGluAsnLeuLysHisGlnProGlyGlyGly, with a characteristic Pro-Gly-Gly-Gly motif (Lee et al., 1989; Luduena et al., 1992). The similar structure was observed in MAP2 (Lewis et al., 1988; Littauer et al., 1986) and tau isoforms (Goedert and Jakes, 1990; Goedert et al., 1991; Serrano et al., 1985). Other proteins, which do not contain similar repeat, were found to bind to tubulin (Luduena et al., 1992; Stetzkowski-Marden et al., 1991; Langosch et al., 1992). Nonetheless, a MAP2 peptide corresponding to the tubulin binding region inhibits MAP1B binding to tubulin, even though the sequences of the two proteins' tubulin-binding domains are very different (Joly and Purich, 1990). These apparently contradictory results could perhaps be explained by assuming that, the interaction between tubulin and tubulin-binding protein is, either hydrophobic, or electrostatic (Luduena et al., 1992).

The tubulin binding domain was mapped to 124 aa region, which is near N-terminal portion of predicted amino acid from the F1-20 cDNA. In this region, there are a CKII motif and a PKC motif as well as stretches of hydrophobic residues. Similarly, the actin and tubulin binding domain of synapsins Ia and Ib was mapped to 14 kDa peptide close to N-terminus, the "globular" head region of synapsin I (Petrucci and Morrow, 1991). Thus NP185 may mediate the interaction of vesicular organelles with cytoskeletal protein, e.g., by binding to

tubulin of these organelles (Kohtz and Puszkin, 1988, 1989).

8. Epitope mapping for NP185 mAbs will be a useful tool to study the function and the structure of NP185 protein

NP185 mAbs, 8G8 and 6G7, were successfully used for biochemical characterizations (Kohtz and Puszkin, 1988, 1989; Su et al., 1991), developmental expression (Perry et al., 1991), detection of NP185 in neuromuscular junction (Perry et al., 1992), as well as for making immuno-affinity chromatography to purify the protein (Li et al., 1993). Since both mAbs can recognize NP185 in immunoblot transferred from SDS-PAGE gel, it is reasonable that the epitopes are sequential, not conformational. Mapping epitopes for mAbs will provide information for detecting the structure and the function of NP185, such as probing conformational change in protein-protein interaction. Also, domain for tubulin binding will help to make specific deletion mutant in further *in vivo* experiment.

There are two potential CKII motif in the epitopes of NP185 mAbs. Therefore, the mAbs may be used for functional characterization of phosphorylation sites both *in vivo* and *in vitro*. In addition, as shown in Fig. 36, the epitope of F1-20 mAb was found in the middle of F1-20 sequence in between residue 400 and residue 760 (Zhou et al., 1992); while the epitope of AP180 mAb was defined to reside in between residue 562 and residue 744 (Morris et al., 1993). Taken together, these four mAbs could be used as a tool to probe the structure and function of the protein both *in vitro* and *in vivo*.

9. NP185 may be expected to involve neuritogenesis.

a), Is NP185 itself sufficient to trigger neuritogenesis?

As shown previously (Kohtz and Puszkin, 1988), little or no NP185 was immunologically detected in PC12 cells grown in the absence of NGF, but NP185 was detected as soon as 12 hours after the addition of NGF. The substantial increase in NP185 protein content of PC12 cells was found during neuritogenesis. Immunofluorescence microscopy of 7-day differentiated PC12 cells with mAb 8G8 revealed that NP185 is distributed in a punctate manner through the developing neurites and the growth cones. Developmental experiment shows that NP185 expression coincide with synaptogenesis (Perry et al., 1991; Sousa et al., 1992). These studies suggest that NP185 may involve neurite formation.

There are a number of evidences that expression of a single gene can induce the morphological changes in PC12 cells, e.g., N-ras gene (Guerrero et al., 1986, 1988), pp60^{v-src}, CaM kinase II (Goshima et al., 1993), tau and MAP2 (Kelly and Grote, 1993) and 42C (Masiakowski and Shooter, 1990). The 42C protein, a possible target of viral and growth factor receptor-associated protein kinase, is induced to express by NGF, however, it can induce the differentiation of PC12 cells when introduced into PC12 and expressed in the absence of NGF (Masiakowski and Shooter, 1990). Since NP185 is phosphoprotein associated with CKII activity (Su et al., 1991), CKII has been proposed to promote neuritogenesis through the phosphorylation of several cytoskeletal proteins including microtubule-associated protein MAP1B and the neuronal-specific beta-III

tubulin (Ulloa et al., 1993). Therefore, NP185 may play a role in neuritogenesis. To test this hypothesis, NP185 would be introduced into PC12 cells and induced to express the protein in the absence of NGF. Morphological change will be examined under phase contrast microscopy with the quantitation of neurite outgrowth (Greene et al., 1991).

b) NP185 may interact with other proteins to promote neurite outgrowth if it alone is not sufficient for the process.

To test this assumption, NGF will be used to induce the expression of endogenous NP185 genes in the NP185-PC12 subline cells, while exogenous NP185 molecules will be induced to express by an inducer, e.g., dexamethasone if using pMAMneo vector. Thus overexpression of exogenous NP185 may speed up the PC12 cells to form neurites, while the suppression of NP185 expression by antisense oligonucleotides may slow down the process.

As known, NP185 associates with CKII activity (Su, 1989). Since CKII is much more abundant in brain than in any other tissue (Girault et al., 1990; Krek et al., 1992), one of the proposed roles for CKII in developing neurons would be the promotion of neuritogenesis through the phosphorylation of several cytoskeletal proteins, e.g., MAP1B (Diaz-Nido et al., 1988, 1990a, 1990b, 1992) and the neuronal-specific β III tubulin (Serrano et al., 1987; Diaz-Nido et al., 1990a,b). Gatica et al., (1987) demonstrated that, the stimulation of CKII activity by a lysine-rich mediator could be important to the initiation of neurite formation. When tubulin is phosphorylated by CKII, its affinity for NP185 is increased

(Kohtz and Puszkin, 1989). More specifically, phosphorylation of tubulin by CKII could serve to organize transport of NP185 into the neurite (Kohtz and Puszkin, 1989). Ulloa et al., (1993) showed that when the expression of CKII was blocked by antisense oligonucleotides, it result in depriving the formation of neurite.

In addition, preliminary data shows that a possible unidentified kinase would be an NP185-associated protein in developing mouse brain cytosol. Taken together with that NP185 binds to tubulin and clthrins (Kohtz and Puszkin, 1989; Su et al., 1991), NP185 most likely plays an important role in neuritogenesis in developing neurons.

10. NP185 may be expected to contain a targeting signal in nerve endings.

The key targeting mechanisms associated with neurite extension appear to be location-specific, microtubule-based targeting, but may include selective retention, via interaction with the actin-based cytoskeleton of the growth cone (Ginzburg, 1991; Kelly and Grote, 1993; Craig and Banker, 1994). In order to decipher the targeting of NP185 to nerve endings, the following questions would be addressed.

a) Is there any targeting domain of NP185 to neurite and growth cone?

Synapse forms when a growth cone touches its target. Because the conversion of growth cone to synapse can be very fast, growth cones are expected to contain many of the elements needed to form a synapse. Synapsins and SVs are

present in the immature axon and quickly accumulate when synaptic contact is made (Kelly and Grote, 1993). Similarly, NP185 is present in the growth cone during neuritogenesis (Kohtz and Puszkin, 1988), and enriched in mature neurons (Su et al., 1991; Perry et al., 1991; 1992; Puszkin et al., 1992). It is likely that NP185 may contain a targeting signal.

Analysis of mutant forms of NP185 cDNA subcloned into Flu-tag vector with an internal marker (e.g., pCGN, Tanaka and Herr, 1990), which are transfected into PC12 cells (Muller et al., 1990; Troy et al., 1992), will provide information on structural components important for the localization of NP185 molecules in nerve ending.

b) NP185 may contain domains that interact with other proteins to form a complex in which there is a target signal.

Tubulin, especially membrane-bound tubulin, may provide an anchor for NP185 in nerve endings. A mutant form of NP185 that lacks tubulin-binding domain will be introduced into PC12 cells, immunofluorescence will be used to localized the mutated NP185 protein with an internal marker like flu-tag.

Currently, available information on targeting domains of vesicle protein is only found in endocytotic targeting of synaptophysin. If synaptophysin without its carboxyl-terminal cytoplasmic domain is expressed in fibroblasts, it is no longer targeted to endosome (Linstedt and Kelly, 1991c).

If NP185 is not targeting to nerve endings, it may use mechanisms such as microtubule stabilization, microtubule-dependent organelle transport, membrane protein self-association, enhanced resistance to turnover, clathrin-coated

vesicles, mRNA targeting, local phosphorylation, and cytoskeletal rearrangements at points of cell-cell contact (Kelly and Grote, 1993).

11. NP185 is a multifunctional protein, specialized in neuronal functions in mammalian synapse

As shown in Fig. 36, the organization of the NP185/AP180/AP3 gene structure showed that, it contains: (1) a clathrin binding domain in the N-terminal 300 residues (pI 8.7) with assembly promoting properties and its ability to interact with membranes (Morris et al., 1993); (2) a tubulin binding domain in the middle 124 amino acids between residue 249 and residue 372 of F1-20 sequence with its ability to organize the transport of NP185 into the neurite (Kohtz and Puszkin, 1989; Li et al., 1994); and (3) an unidentified CLC and SPM binding domains (Su et al., 1991), which would be in C-terminal of the protein (Fig. 36).

Furthermore, NP185 may involve the neuritogenesis and synaptic function by interacting either with cytoskeleton protein (Kohtz and Puszkin, 1988), or unidentified components. Alternatively, NP185 may be part of ion channel, or related to receptor molecule in endocytosis (Puszkin et al., 1992).

In summary, a model can be suggested by integrating our knowledge of NP185 to explain the function of NP185 in synapse. When NP185 is phosphorylated, its middle domain binds to membrane-bound tubulin anchored on vesicles or SPM (only 30% of the NP185/AP180 is associated with membrane in bovine brain, Lindner and Ungewickell, 1992; Ungewickell and

Oestergaard, 1989), while its C-terminal binds to non-phosphorylated CLC, and its N-terminal domain binds to CHC, resulting in the formation of CCVs to retrieve the SV membrane. On the other hand, when NP185 is dephosphorylated or CLC is phosphorylated, they dissociate from each other, CCVs decoate and release the SVs. While tubulin is phosphorylated by CCV-associated CKII, it binds to NP185; moreover, when tubulin is dephosphorylated, it dissociates from NP185, then NP185 is released into the cytosolic pool, which forms the bulk (70%) (Ungewickell and Oestergaard, 1989). Finally, NP185 isoforms may play different roles in the regulation of synaptic function.

Taking together, from observations made in several laboratories (Ahle and Ungewickell, 1986; Keen and Black, 1986; Kohtz and Puszkin, 1988, 1989; Murphy et al., 1991; Su et al., 1991; Morris et al., 1993; Li et al., 1994), it can be further postulated that NP185 fulfills multiple functions in synaptic terminals. One function is that of a plasma membrane docking or channel protein; second function may be that of a signaling molecule for neuronal vesicles to reach the synaptic terminal region; and a third function may be that of a recycling mediated molecule, binding to protein components on the lipid bilayer of the synaptic plasma membrane during the process of endocytosis (Puszkin et al., 1992).

FIGURES AND LEGENDS

Figure 1. Immunofluorescence. Muscle fibers obtained from E17 chick embryos subjected to immunofluorescent labelling (b-i), and visualized by Nomarski optics. (a) Two parallel muscle fibers. (b) Same fibers after immunolabelling omitting mAb 8G8 developed against NP185 display background, non specific fluorescence. (c) Same fibers showing typical labelling of ribbons of neuromuscular junctions using 8G8 monoclonal antibody against NP185. (d) Phase contrast optics of muscle fiber delineating at higher magnification (arrowheads) the contour of a neuromuscular junction. (e) Typical fluorescence pattern of muscle fiber shown in d after labelling with mAb 8G8 developed against NP185. (f) Neuromuscular junction shown at higher magnification from an area of the muscle fiber illustrated in (c). (g-i) Neuromuscular endplates displaying details of the myofibrilla architecture of presynaptic (white solid ovals) and post-synaptic sides (white solid arrowheads) of the neuromuscular junction plate. The furrows with the post-synaptic membrane receptors are always embedded within and below the sarcolemma membrane as illustrated.

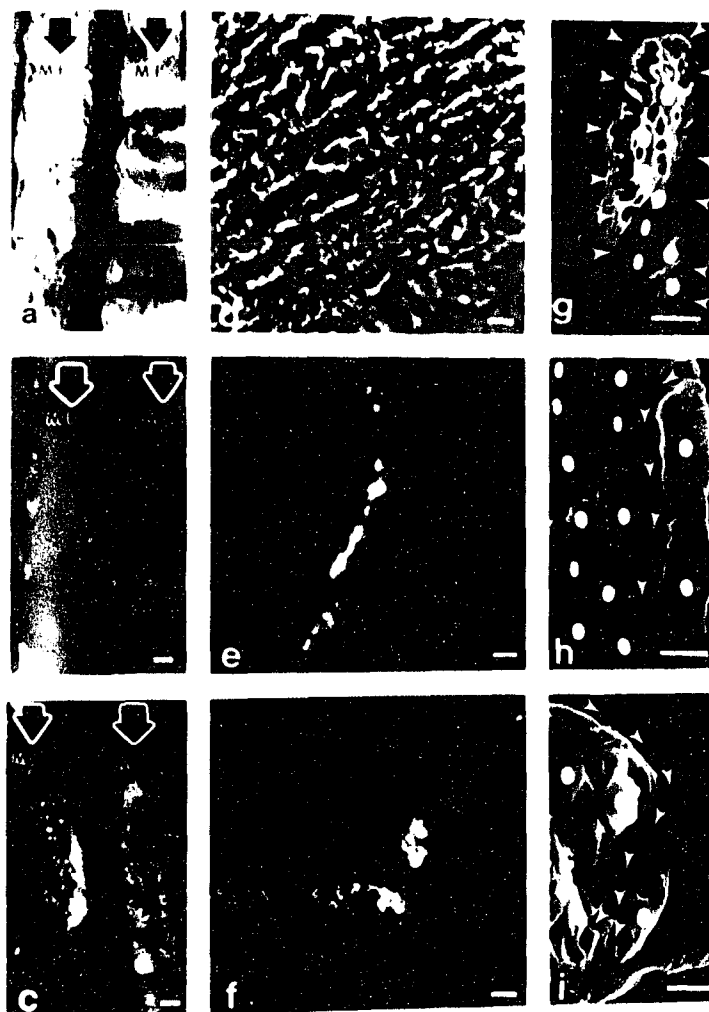


Figure 2. SDS-PAGE and immunoblot with anti-NP185 mAb. Extracts of crude pellets enriched with clathrin-coated vesicles from E17 chick embryo brain and chick embryo muscle fibers immunoprecipitated with mAb 8G8 and protein A. Panel A: Coomassie blue protein stain. Panel B: Western blot of Panel A. Lane 1: supernatant from an extract of chick brain crude pellet enriched with clathrin-coated vesicles. Lane 2: Immunoprecipitate from extract shown in lane 1. Lane 3: Chick embryo muscle extract supernatant from a pellet enriched with clathrin-coated vesicles using mAb 8G8. Lane 4: Immunoprecipitate from extract shown in lane 3 using mAb 8G8.

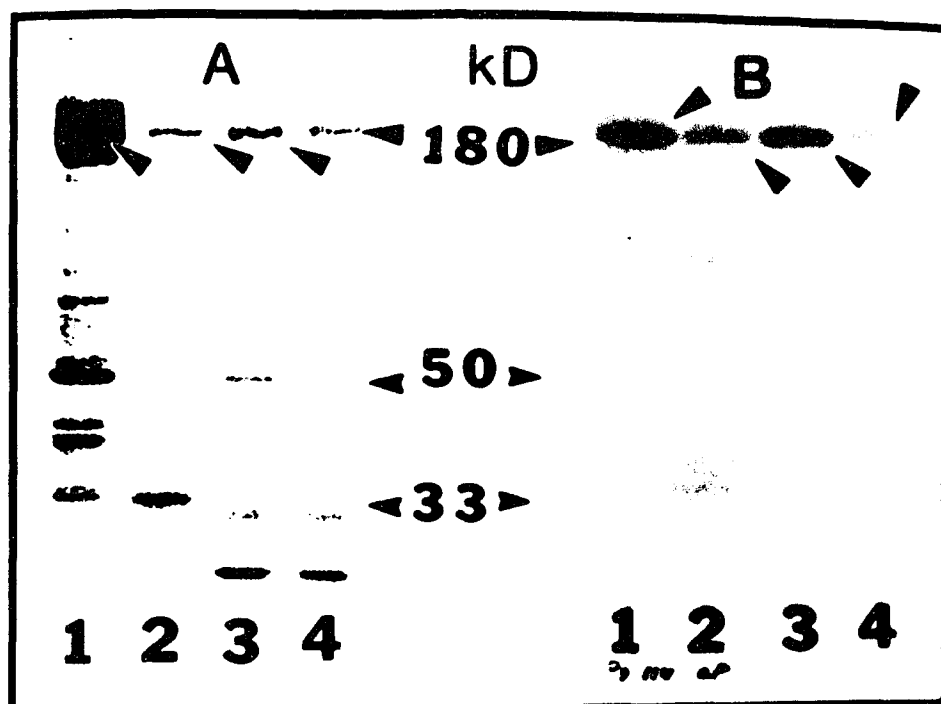


Figure 3. Double labelling with α -bungarotoxin-fluorescein conjugate (AChR) and mAb 8G8 (NP185). (a) Phase contrast optics of muscle fiber showing ribbons of neuromuscular junctions (arrowheads) after double labelling. (b) Control muscle fiber with NP185 absorbed mAb 8G8 and bungarotoxin. (c) α -bungarotoxin/fluorescein labelling showing lanes of neuromuscular junction (AChR). (d) Labelling for NP185 with mAb 8G8 (rhodamine). Examination of fluorescence at the same tissue depth reveals general correlation of patterns but the dotted fluorescence is partly overlapping.

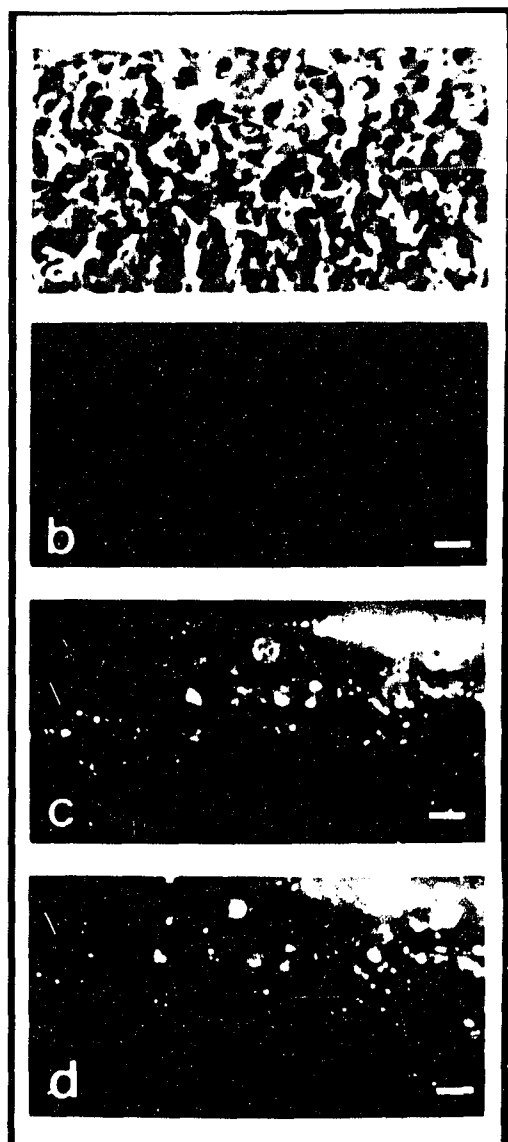


Figure 4. Confocal microscopy of a neuromuscular junction double labeled with α -bungarotoxin-fluorescein and mAb 8G8. (a) NP185 labelling with the mAb showing dotted fluorescence (rhodamine). (b) α -bungarotoxin labelling (fluorescein/AchR) with the focus on a deeper plane in the tissue to show strongest fluorescent intensity. Fluorescent dots show some general correlation but do not overlap indicating that labelling occurs at different sites of the neuromuscular junction. (c) Nomarski optics of the neuromuscular junction shown in (a) and (b).

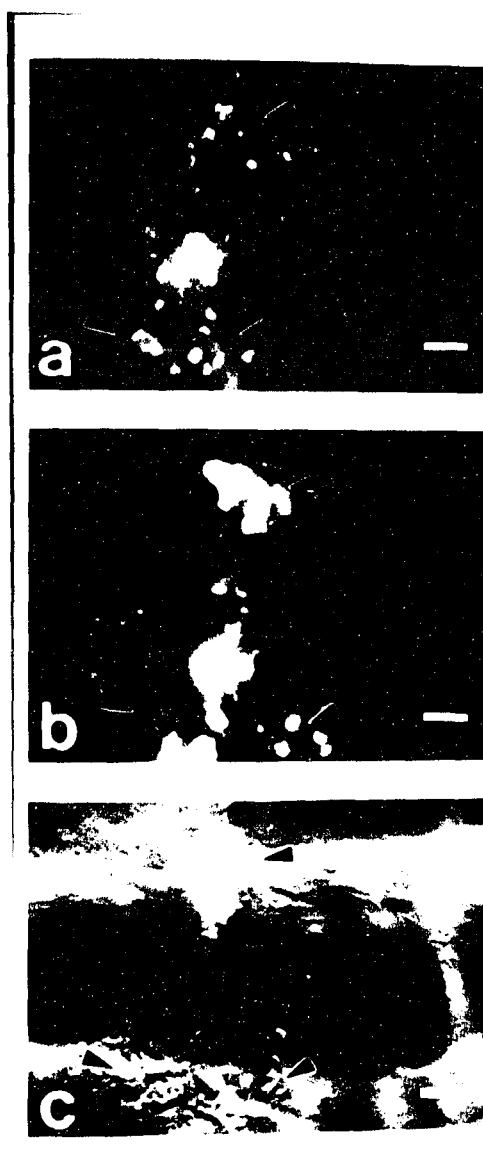


Table 1. The procedure of double-immunolabelling for NP185 protein and acetylcholine receptor in neuromuscular junction. (refer to Materials and Methods for details)

Double labelling of NP185 and AchR in neuromuscular junction

1. Striated muscle fibers.
2. Fix, 30 min, in picric acid/formaldehyde (PAF) solution: 0.2% picric acid, 4% paraformaldehyde, and phosphate-buffered saline (PBS) pH 7.4.
3. Soak in 15% sucrose/PBS 0.5 hr to o.n. at 4 °C.
4. Rapid-freeze the tissue in liquid nitrogen (LN₂), and pack in O.C.T. compound, embedding medium for frozen tissue specimens.
5. Section at 8 um in Harris Cryostat.
6. Mount on slides, rinse in PBS, block with goat anti-mouse IgG for 30 min at 4 °C.
7. Incubate o.n. at 4 °C with anti-NP185 mAb 8G8.
8. Incubate with α -bungarotoxin conjugated to fluorescein (α -BT-F), specifically label acetylcholine receptor (AchR), for 1-2 hrs.
9. Incubate with goat anti-mouse IgG conjugated to rhodamine (IgG-R) for 45 min at RT.
10. Elutriate in PBS pH 7.4.
11. Add Johnson's mountant.
12. Seal with coverslip and nail polish.
13. Immunofluorescence microscopy and confocal microscopy.

Table 2. The procedure of NP185 protein purification from bovine brain. NP185 was successfully purified by differential centrifugation, sizing columns, ion exchange column, and immuno-affinity column.

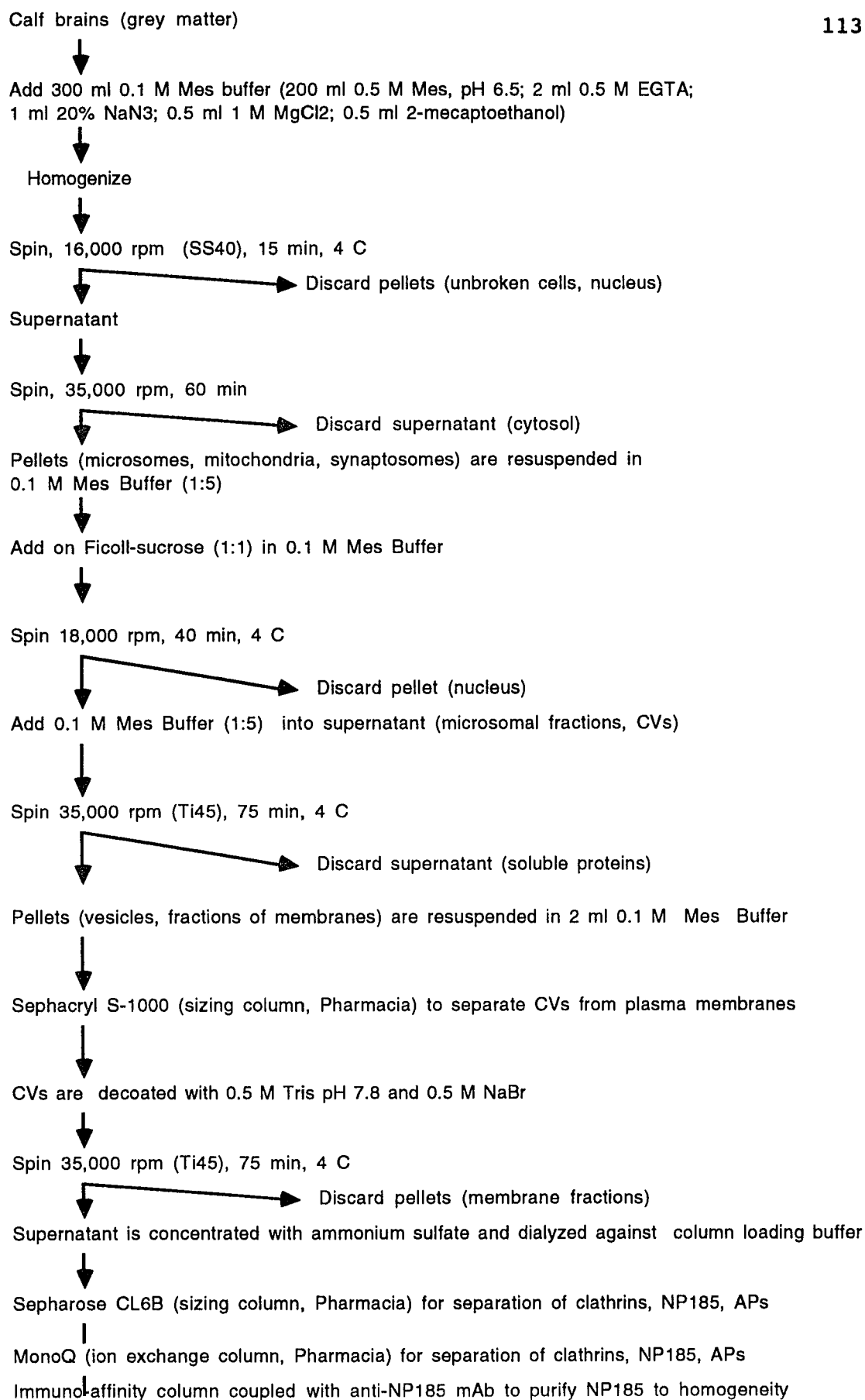


Table 3. The Quantitation of Amino Acid Contents of NP185 from the Protein Purified by Anti-NP185 Immuno-Affinity Chromatography (digested and analyzed by Dr. Kenneth Williams and Dr. Kathy Stone in Protein Chemistry Laboratory at K.M. Keck Foundation of Yale University).

<u>AMINO ACID</u>	<u>NMOLES</u>	<u>MW</u>	<u>uGRAMS</u>
A ala alanine	1.820	71.07	0.1294
C cys cysteine*			
D asp aspartic acid			
(N asn asparagine)	1.075	114.09	0.1227
E glu glutamic acid			
(Q gln glutamine)	1.007	128.12	0.1291
F phe phenylalanine	0.478	147.18	0.0703
G gly glycine*			
H his histidine	0.865	137.14	0.1186
I ile isoleucine	0.304	113.16	0.0344
K lys lysine	0.549	129.18	0.0709
L leu leucine	0.934	113.16	0.1057
M met methionine*			
P pro proline	1.355	97.12	0.1315
R arg arginine**			
S ser serine	1.380	87.08	0.1201
T thr threonine	0.973	101.11	0.0984
V val valine	0.665	99.13	0.0659
W trp tryptophan***			
Y tyr tyrosine	0.079	163.18	0.0128
Total micrograms of protein:			1.210 ug
* not detected			
** obscured			
*** not determined			

Table 4. Amino Acid Composition of NP185 from the Protein Purified by Anti-NP185 mAb Immuno-Affinity Chromatography (digested and analyzed by Dr. Kenneth Williams and Dr. Kathy Stone in Protein Chemistry Laboratory at K.M. Keck Foundation of Yale University).

<u>AMINO ACID</u>	<u># OF RESIDUES</u>	<u>MOLE PERCENT</u>
A ala alanine	248.2	15.848
C cys cysteine*		
D asp aspartic acid (N asn asparagine)	146.6	9.363
E glu glutamic acid (Q gln glutamine)	137.4	8.773
F phe phenylalanine	65.2	4.162
G gly glycine*		
H his histidine	118.0	7.532
I ile isoleucine	41.5	2.647
K lys lysine	74.9	4.780
L leu leucine	127.4	8.136
M met methionine*		
P pro proline	184.7	11.794
R arg arginine**		
S ser serine	188.1	12.013
T thr threonine	132.7	8.475
V val valine	90.7	5.792
W trp tryptophan***		
Y tyr tyrosine	10.7	0.684

* not detected

** obscured

*** not determined

Table 5. The Amino Acid Sequences of Internal peptides from Protein Microsequencing of Tryptic-NP185 Protein Purified by Anti-NP185 mAb Immuno-Affinity Chromatography from Bovine Brain. (Sequenced by Protein Chemistry Laboratory, K.M. Keck Foundation at Yale University, by Dr. Kenneth Williams and Dr. Kathy Stone)\$

1. Peak 43	AFSYR (Ala-Phe-Ser-Tyr-Arg)
2. Peak 65	QMAFD FAR (Gln-Met-Ala-Phe-Asp-Phe-Ala-Arg)
3. Peak 48*a	KGDLQWNAGEK(LysGlyAspLeuGlnTrpAsnAlaGlyGluLys)
4. Peak 48*b	LTGGANWQPK (LeuThrGlyGlyAlaAsnTrpGlnProLys)
5. Peak 72	VSEFLK (Val-Ser-Glu-Phe-Leu-Lys)

\$ More peptides could be sequenced if necessary and available for funding since there was still a lot of amount of protein sample (internal peptides isolated by HPLC) in stock in the Foundation.

1. The sequence from peak 43 corresponds to residues 135-139 of F1-20 cDNA predicted residues.
2. The sequence in peak 65 contains a match with residues 140-147 of the F-20 cDNA predicted residues.
- 3.4. Peak 48 contained a primary and a secondary sequence, both of which match predicted amino acid sequence of F1-20 cDNA. The primary sequence (*b) matches residues 782-791, while the secondary (*a) matches residues 770-780.
5. The sequence from peak 72 matches residues 247-252 of predicted amino acid sequence from F1-20 cDNA.

Table 6. Comparison of the features for NP185, AP180 and F1-20 phosphoprotein: NP185 (Kohtz and Puszkin, 1988, 1989; Su et al., 1991; Perry et al., 1991, 1992; Li et al., 1992, 1993, 1994); F1-20 (Sousa et al., 1992; Zhou et al., 1992, 1993); and AP180 (Ahle and Ungewickell, 1986; Morris et al., 1993).

Feature Comparison of NP185, F1-20 and AP180

	<u>NP185</u>	<u>F1-20</u>	<u>AP180</u>
Laboratory	Puszkyn's	Lafer's	Ungewickell's
Discover time	1988	1992	1986
Biochemical	CCV, CLC and tubulin binding cage formation bind to SVs and decoated vesicles peptide mapping	not detected	bind to CCVs, CHC, induce cage formation peptide mapping
Purification	Yes (1992)	No	Yes (1993)
Peptide sequence	Yes	No	Yes
Phosphorylation	not published	Yes	Yes
Physical features			
pI of 2 D	6.0	n.d.	5.5
SDS-PAGE	185 kDa	190 kDa	180 kDa
# of subunit	1	1	1
Localization	brain neuron-specific synapse-specific PNS, NMJ	brain neuron-specific synapse-specific n.d.	brain n.d. n.d. n.d.
Development	synaptogenesis	synaptogenesis	n.d.
PC 12 cells	NGF-inducible 12 hrs after NGF	n.d.	n.d.
cDNA cloning			
strategy	mAbs	mAb	mAb
animal	rat brain	mouse brain	rat brain
predicted	partial	91.3 kDa	91.4 kDa
calculated (pI)		4.6	3.85
# of total aa		902	915
time published		Jun., 1992	March, 1993
Journal		J. Neurosci.	EMBO. J.

Table 7. The procedure of Far-Western blotting analyses for protein-protein interaction, NP185 binds to tubulin. This method was also called as Sandwich blotting (Kohtz and Puszkin, 1989; modified by Lee et al., 1991; Ruppert et al., 1993; Hisatake et al., 1993). The principle for the procedure is that the protein on blot was denatured and then renatured by treating the blot with sequential washings in several solutions as described in "Materials and Methods". Through the procedure, the protein is folding in a proper way for protein-protein interaction.

FAR WESTERN(SANDWICH) BLOTTING

GST-NP185 fusion protein and mutated proteins are resolved on a 5-15% gradient SDS-PAGE

↓
Electransfer to nitrocellulose .

↓
CZ 0.1 M (20 mM HEPES pH 7.9, 2% glycerol, 0.1 M KCl, 5 mM MgCl₂, 0.1 mM EDTA, 2 mM dithiothreitol); 0.5 mg/ml BSA, 0.02% polyvinylpyrrolidone, 6 M guanidine-HCl, 30 min, 4 C.

↓
CZ 0.1 M, 0.5 mg/ml BSA, 0.02% polyvinylpyrrolidone, 0.1 M guanidine-HCl, 1 hr, 2X, 4 C.

↓
CZ 0.1 M, 0.5 mg/ml BSA, 0.02% polyvinylpyrrolidone, 2 hrs, 3X, 4 C.

↓
10 mM HEPES (pH 7.9), 1% horse serum, 1 hr, 4 C.

↓
30 ml of 1:1 dilution of CZ 0.1 M, brain tubulin(TEAE column purified), incubate 12 hrs, 4 C.

↓
Wash with 1:1 dilution of CZ 0.1 M, 10 min, 3X, 4 C.

↓
Mouse anti-tubulin mAbs in 1:1 dilution of CZ 0.1 M, 2 hrs, 4 C.

↓
Wash with 1:1 dilution of CZ 0.1 M, 10 min, 3X, 4 C.

↓
Goat anti-mouse IgG alkaline phosphatase conjugate(2 Ab) in 1:1 dilution of CZ 0.1 M, 2 hrs, 4 C.

↓
Wash with 1:1 dilution of CZ 0.1 M, 10 min, 3X, 4 C.

↓
Develop with BCIP and NBT in 0.1 M Tris.Cl (pH 9.0), 0.5 mM MgCl₂, 4 C.

Figure 5. Sepharyl S-1000 gel filtration chromatography. CCVs were separated from plasma membranes. Peak I was plasma membranes (liposomes). Peak II was clathrin coated vesicles (CCVs). Peak III and IV were other small vesicles (small liposomes).

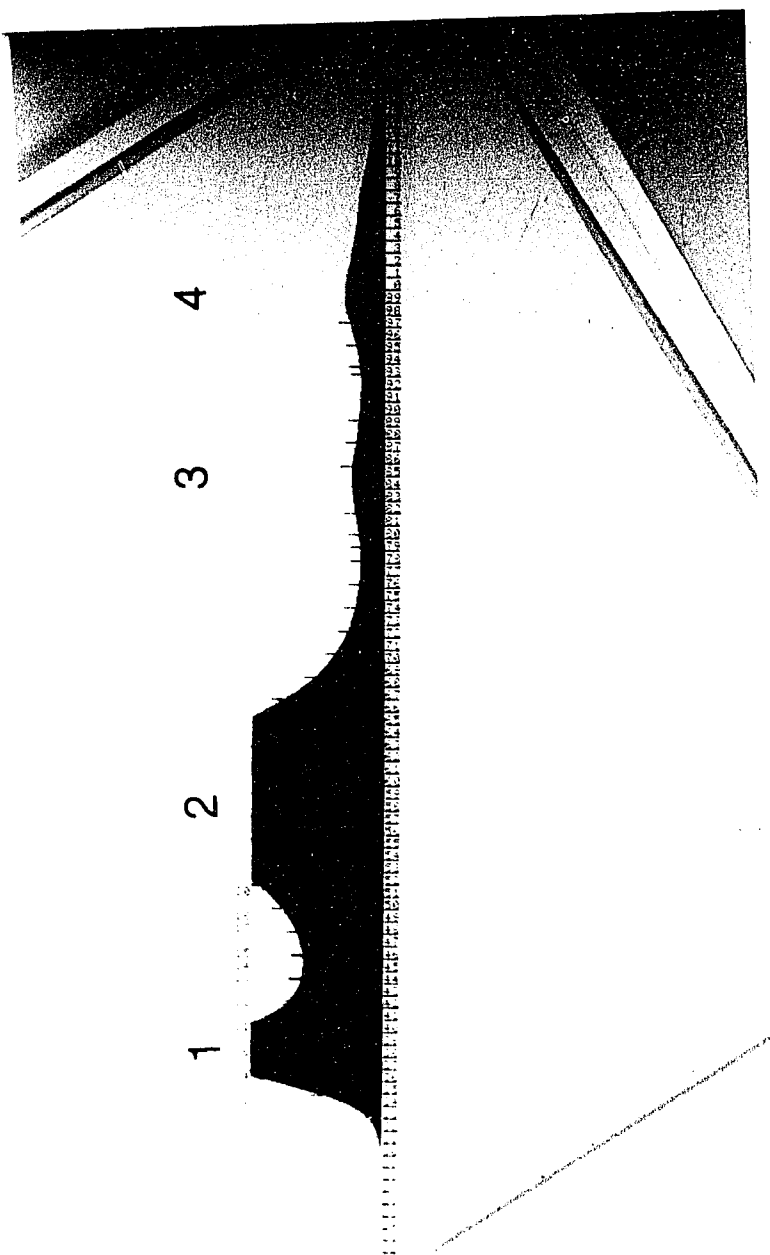


Figure 6. NP185 is present in fractions eluted from a Sepharose CL6B chromatography column within the clathrin peak. The coat proteins of CCVs were dissociated with 0.9 M NaCl in 0.5 M Tris buffer, pH 7.0, and chromatographed through a Sepharose CL6B column. Every third fraction was placed on the polyacrylamide gel starting from the top of the clathrin peak (lane 1) and was resolved by SDS-PAGE on two slabs, one stained with Coomassie blue (B) and the other immunoblotted with mAb 8G8 (A). The lane 4 indicate the peak fraction of NP185 in the CL6B column. C, D: clathrins, NP185 and assembly polypeptides were separated by Sepharose CL6B column. As detected by NP185 mAbs and SDS-PAGE analyses, the patterns are as the following, Peak I: minor NP185 or others. Peak II: CCVs only, no NP185. Peak III: clathrins (CHC and CLC), or NP185. Peak IV: major NP185, assembly polypeptides 100 kDa, 50 kDa, and 30 kDa, sometimes contaminated with clathrins. Peak V: major 40 kDa, covesins (Kohtz et al., 1990).

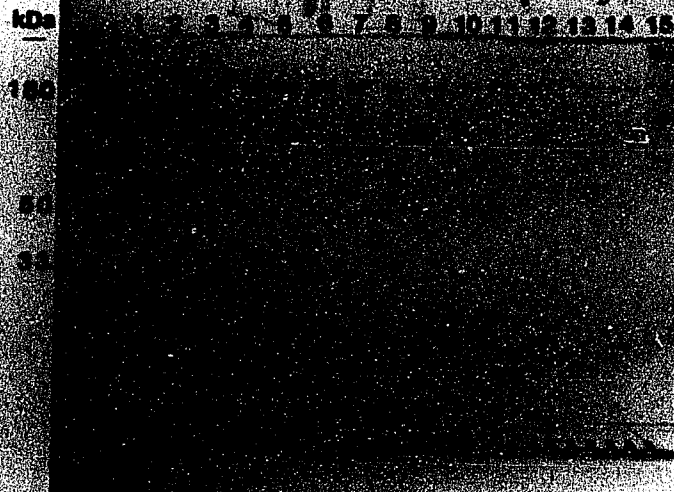
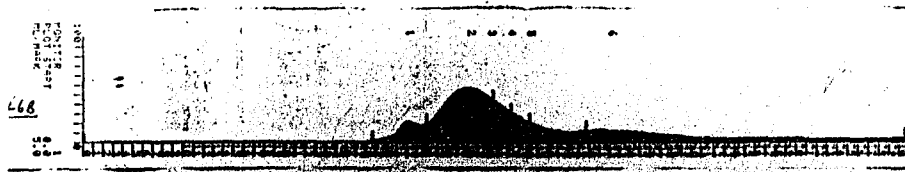
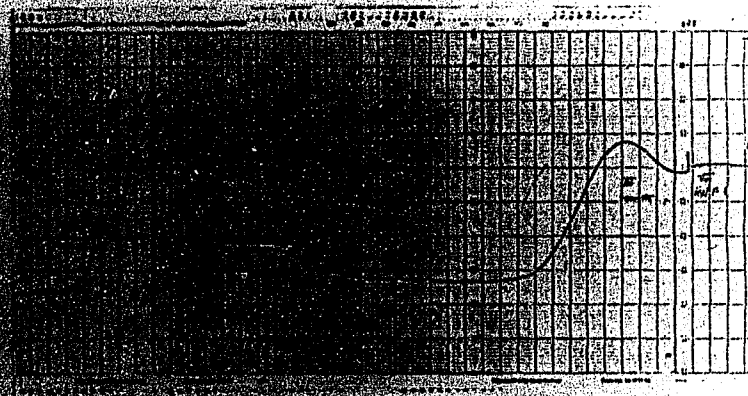
A**B****C****D**

Figure 7. The same as shown in Fig. 8., NP185 is present in fractions eluted from a Sepharose CL6B column within the clathrin peak. Lane 1: molecular markers. Lane 2: homogenate of bovine brain. Lane 3 to 7, 11 shows the pattern of CCV proteins. Lane 8-10: traces of NP185 and clathrin.

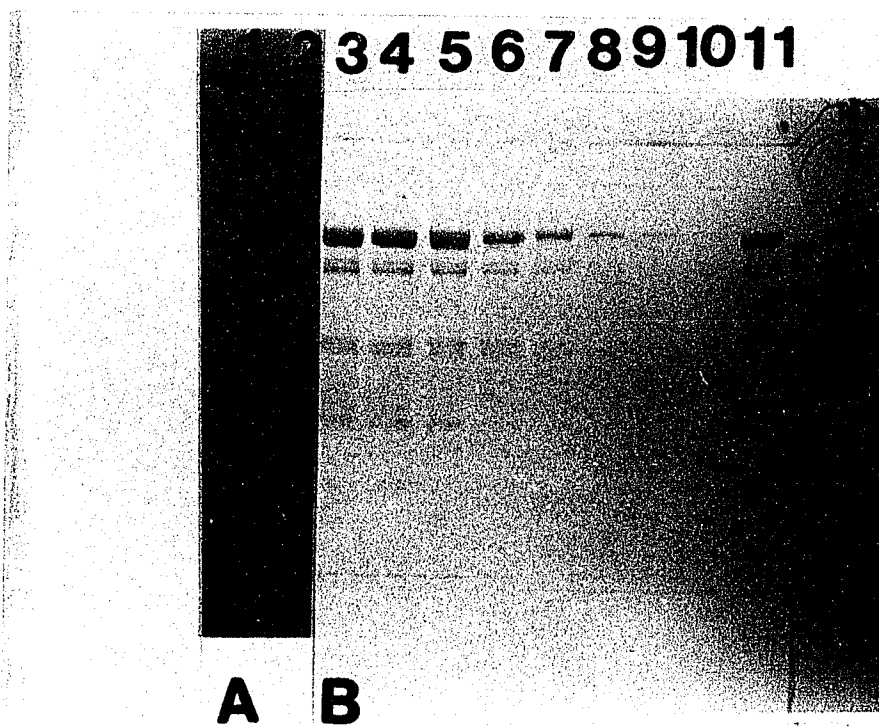


Figure 8. Mono Q anion exchange chromatography. NP185 was separated from clathrins, but not from assembly polypeptide (AP). When the column fractions were screened by immunoblotting or ELISA, it was shown as followings. Peak I: minor NP185 peak; peak II: minor NP185 peak; peak III: major NP185 peak, and assembly polypeptide, 100-110 kDa, was eluted at 0.15 M NaCl; peak IV: clathrins.

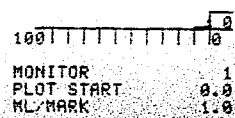
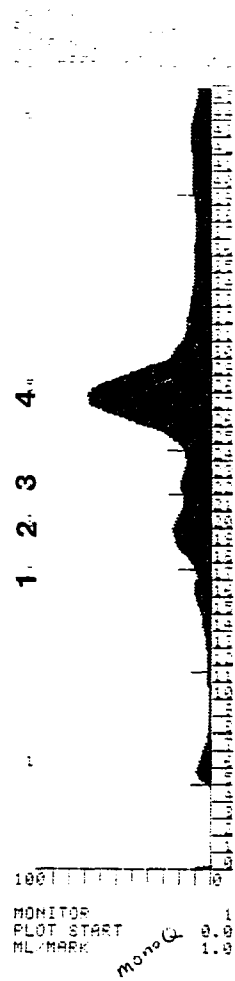


Figure 9. Two isoforms came out from the immuno-affinity chromatography with anti-NP185 mAb, 8G8. NP185 was resolved from assembly polypeptide through the column. Peak I: minor NP185 peak, in which the NP185 protein has low affinity to the mAb of anti-NP185; peak II: major NP185 peak, which was used for further analysis, in which the NP185 protein has high affinity to the NP185 mAb.

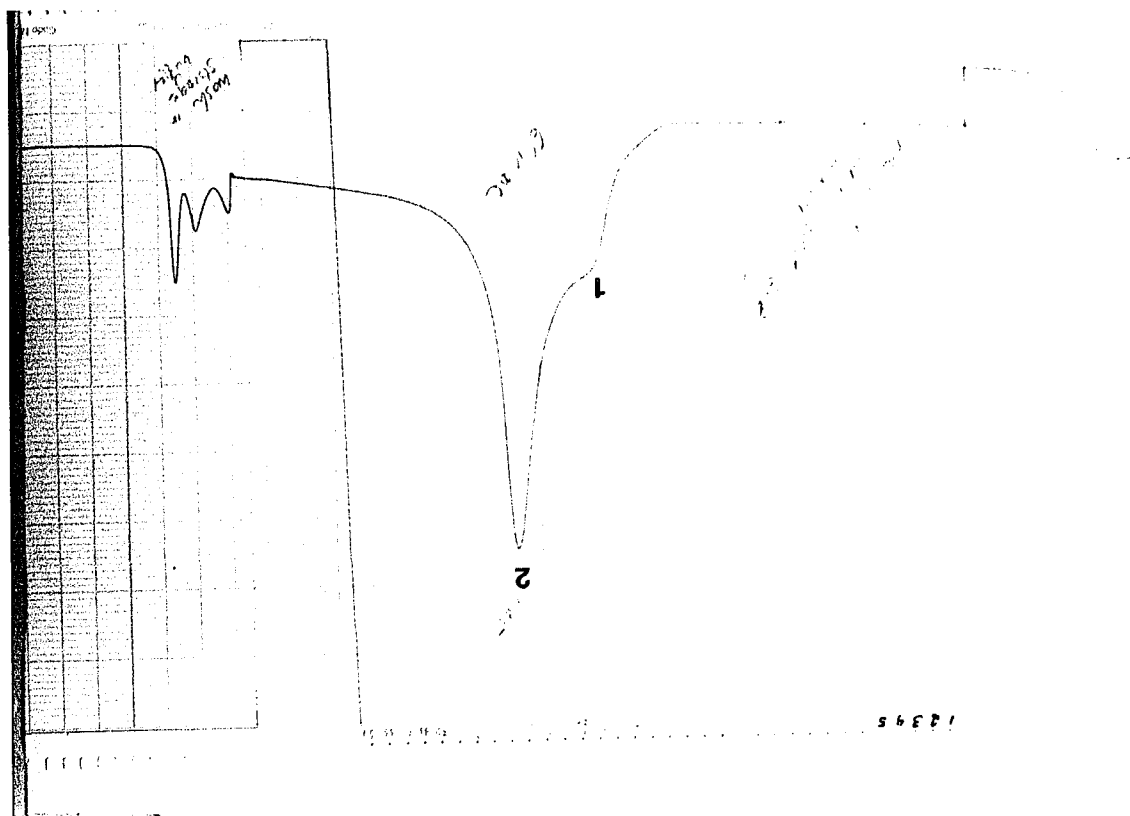


Figure 10. NP185 was purified to homogeneity by immuno-affinity chromatography of Hydrazide AvidGel Ax gel coupled anti-NP185 mAb, 8G8. Panel A: NP185 was purified to homogeneity, as shown by staining with Coomassie blue. Lane 1, homogenized bovine brain proteins; lane 2, CCVs; lane 3, high salt extract of CCVs, it was shown that NP185 is enriched by 0.9 N of NaCl (Kohtz and Puszkin, 1988); lane 4-10, fractions eluted from the immuno-affinity column.

Panel B: immunoblot was probed with anti-NP185 mAb, 8G8; the lane numbers in Panel B were corresponding to lane number of panel A. Panel C: immunoblot was probed with anti-assembly polypeptide antibody; the lane numbers were corresponding to lane number of panel A (not shown).

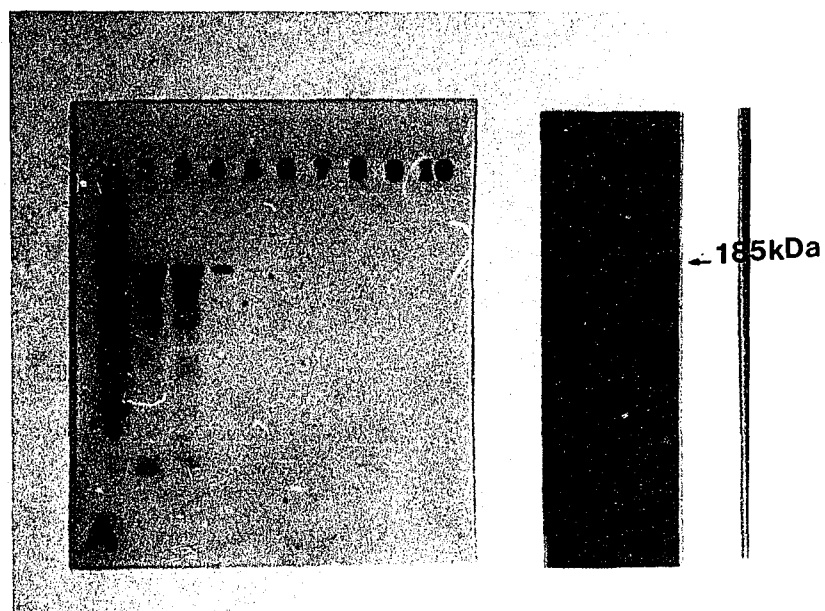


Figure 11. The tryptic fragments of NP185 were separated by reverse-phase HPLC Vydac C-18 Chromatography. NP185 was digested with trypsin with a positive control, TRANS CNTRL (for transferrin control); BLANK is a blank control without protein sample (not shown). Those clear separated peaks were chosen for laser desorption mass spectrophotometer (LDMS) on 1-3% of the peaks prior to sequencing. Peaks (peak #43, #48, #65, #72) selected by LDMS were used for peptide sequencing by automated Edman degradation on an Applied Biosystem 470A sequencer equipped with an on-line 120A PTH analyzer performed by Protein Chemistry Laboratory, Biotechnology Resource Laboratory of W.M. Keck Foundation at Yale University (Dr. Kenneth Williams and Dr. Kathy Stone).

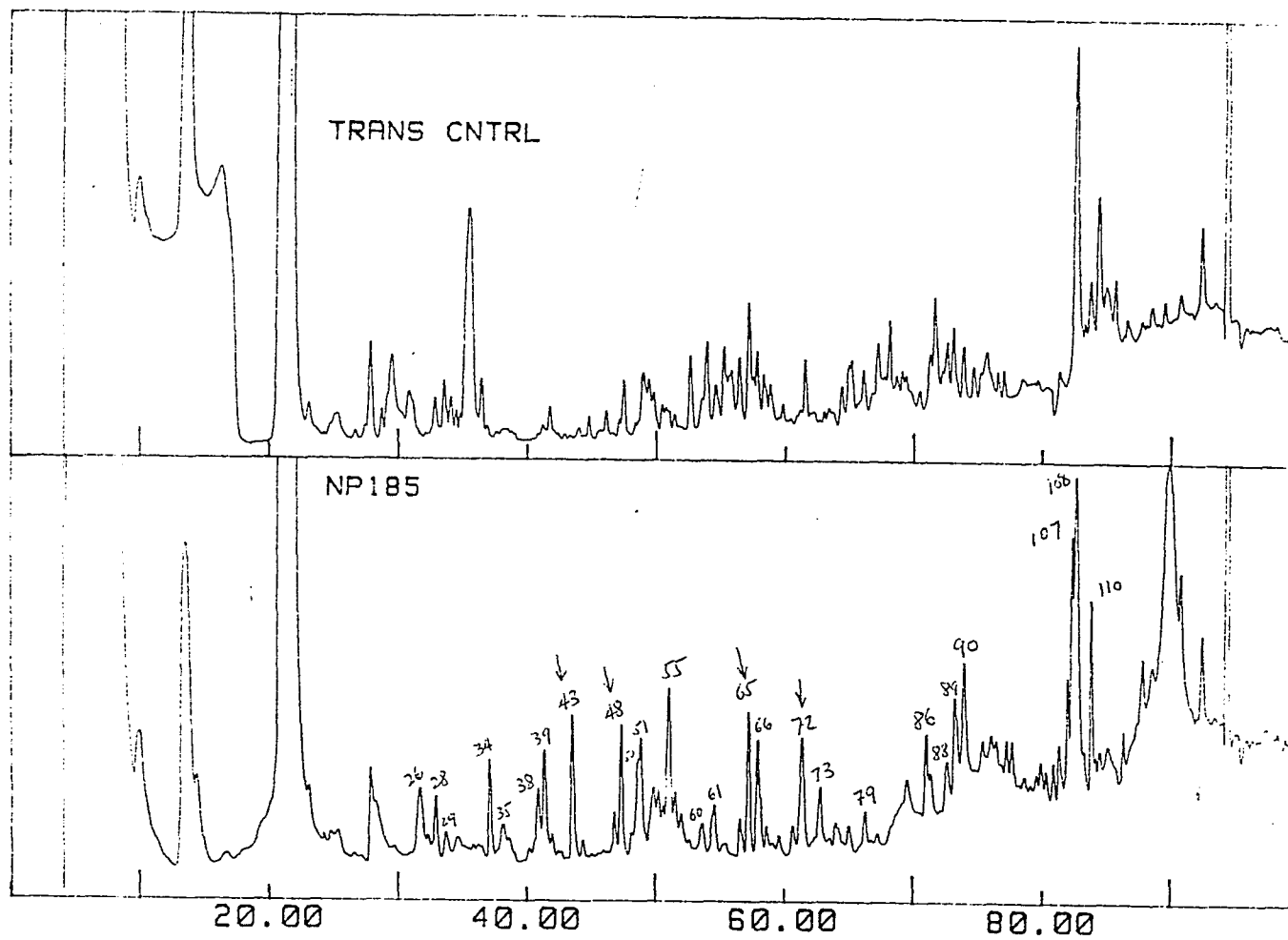


Figure 12. As double-underlined, amino acid sequence of five internal peptides from NP185 can be found in two forms of the predicted amino acid sequence of F1-20 phosphoprotein cDNA (*Mus musculus*). Note: each of these peptide sequence matches contains tryptic site right before the amino terminus of the peptide, i.e., a tryptic digest would generate these fragments from the protein.

From: IN%"retrieve@ncbi.nlm.nih.gov" "Retrieve Server"
 To: IN%"shengwen@msvax.mssm.edu" (Shengwen Li)
 CC:
 Subj: Results-RETRIEVE Server

LOCUS MUSF120A, 4260 bp ss-mRNA
 DEFINITION Mouse phosphoprotein (F1-20) mRNA, complete cds.
 ACCESSION M83985
 KEYWORDS phosphoprotein F1-20.
 SOURCE *Mus musculus* (library: cDNA) adult brain cDNA to mRNA.
 ORGANISM *Mus musculus*, Eukaryota; Animalia; Chordata; Vertebrata; Mammalia; Theria; Eutheria; Rodentia; Myomorpha; Muridae; Murinae.
 1 (bases 1 to 4260)

REFERENCE

AUTHORS Lafer,E., Zhou,S., Sousa,R. and Tannery,N.H.
 TITLE Characterization of a novel synapse-specific protein.
 II. cDNA cloning and sequence analysis of the F1-20 protein

JOURNAL J. Neurosci. 12, 2144-2155 (1992)

STANDARD full automatic

FEATURES	Location/Qualifiers
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CDS	join(145..2285,2301..2850) /gene="F1-20" /note="alternatively spliced"

/product="phosphoprotein"

/codon_start=1

/translation="MSGQTLTDRIAAQYSVTGSAVARAVCKATTHEVMGPKKKHLDY

LIQATNETNVNIPQMADTLFERATNSSWVVF~~KALVTTHHLMVHGNERFIQYL~~ASRNT

LFNLSNFLDKSGSHGYDMSTFIRRY~~SRYLNEKA~~AFSYROMAFDFARVKKGADGVMRTMV

(1) (2)

PEKLLKSMPILQGQIDALLEFDVHPNELTNGVINAAFM~~LLFKDLIKLFAC~~YNDGVINL

LEKFFEMKKGQCKDALEIYKRFLTRMTRV~~SEFLK~~VAEQVGIDKGDIPDLTQAPSSLME

(3)

TLEQHLNTLEGKKPGNNEGSGAPSPLSKSSPATTVTSPNSTPAKTIDTSPPVDIFATA

SAAAPVSSAKPSSDLLDLQPDFSGAAAGAAAPVVPPSGGATAWGDLLGEDSLAALSSV

PCEAPISDPFAPEPSPPTTTTEPASASASTTTAVTAVTTEVDLFGDAFAASPGEAPAA

SEGATAPATPAPVAAALDACSGNDPFAPSEGSAAEAPEL~~DLFAMKPPETSAPVV~~TPTA

STAPPVPATAPSPAPTAVAATAATTTAAAAATTTATTSAAAAATTAAAPPALDIFGDLF

DSAPEVAAAPKPDAAPSIDLFGTDAFSSPPRGASPVPESSLTADLLSVDAFAAPSPAS

TASPAKAESSGVIDLFGDAFGSGASETQPAPQAVSSSSASADLLAGFGGSFMAPSTTP

VTPAQNNLLQPSFEAAFGTTPSTSSSSSFDPVFDGLGDLLMPTMAPSGQPAPVSMVP

PSPAMAASKGLGSDLDSSLASLVGNLGISGTTSKKGDLQWNAGEKKLTGGANWQPKVT

(4) (5)

PATWSAGVPPQGTVPPTSSVPPGAGAPSVGQPGAGFGMPPSGTGMTMMSQQPVMFAPQ

MMRPPFGAAVPGTQLSPSPTPATQSPKKPPAKDPLADLNIKDFL "

Figure 13. The purified NP185 proteins from bovine brains can be recognized by both anti-NP185 mAbs and anti-F1-20 mAb. NP185 was shown by the bar and NP185. Panel A: immunoblot was probed with anti-F1-20 mAb; lane 1, the proteins of CCVs purified from bovine brains; lane 2: the purified NP185 protein from bovine brains; lane 3, molecular weight markers (Sigma: 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48.5 kDa, 36.5 kDa, 26.6 kDa). Panel B: immunoblot was probed with anti-NP185 mAb, 8G8; lane 1, the proteins of CCVs purified from bovine brains; lane 2, the purified NP185 from bovine brains; lane 3, molecular weight markers (Sigma: 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48.5 kDa, 36.5 kDa, 26.6 kDa).

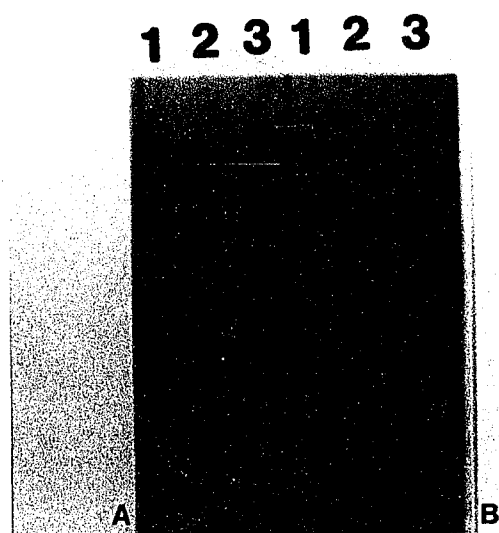


Figure 14. 2.8 kb cDNA generated by reverse transcriptase polymerase chain reaction with primers flanking the ORF of Fl-20 cDNA, as shown by ethidium bromide stained 1.0% agarose gel. Panel A: Lane 1, PCR reaction with the forward primer but without reverse primer. Lane 2, PCR with the reverse primer, but, without the forward primer. Lane 3-6, positive control for the PCR. Lane 7, molecular weight markers, HindIII-lambda-DNA: 23,130 bp, 9,416 bp, 6,557 bp, 4,361 bp, 2,322 bp, 2,027 bp, 564 bp, 125 bp. Lane 8-11, PCR products with both the forward and the reverse primers. Panel B: Lane 1, 2, PCR products with both the forward and the reverse primers. Lane 3, a positive control for the PCR. Lane 4, a PCR with the forward primer, but, without the reverse primer. Lane 5, molecular weight markers, HindIII-lambda DNA (BRL).



Figure 15. Northern blot analyses using ^{32}P -labelled 2.8 kb cDNA as a probe indicate that NP185 mRNA is tissue-specific, and about 6 kb in size. Panel A: 17 ug of total RNA was electrophoresed in 1.7% agarose formaldehyde gel, blotted and probed with the ^{32}P -cDNA 2.8 kb. Lane 1, from brain; Lane 2, from liver. Panel B: Similarly, 15 ug of mouse total RNA were prepared from variety of tissues, as shown: Lane 1, brain; lane 2, kidney; lane 3, liver; lane 4, brain (from Dr. Wei Chen as a control for preparation of total RNA); lane 5, lung; lane 6, spleen. A Beta-actin probe (pAC 1.8, from Dr. Brosius; Kirchaussen et al., 1987) were used as a control for the integrity of the RNA (not shown) to detect both blots. The size of mRNA were determined using a coelectrophoresed RNA ladder (BRL); or determined by the migration of 28S and 18S RNA as shown by arrowheads.

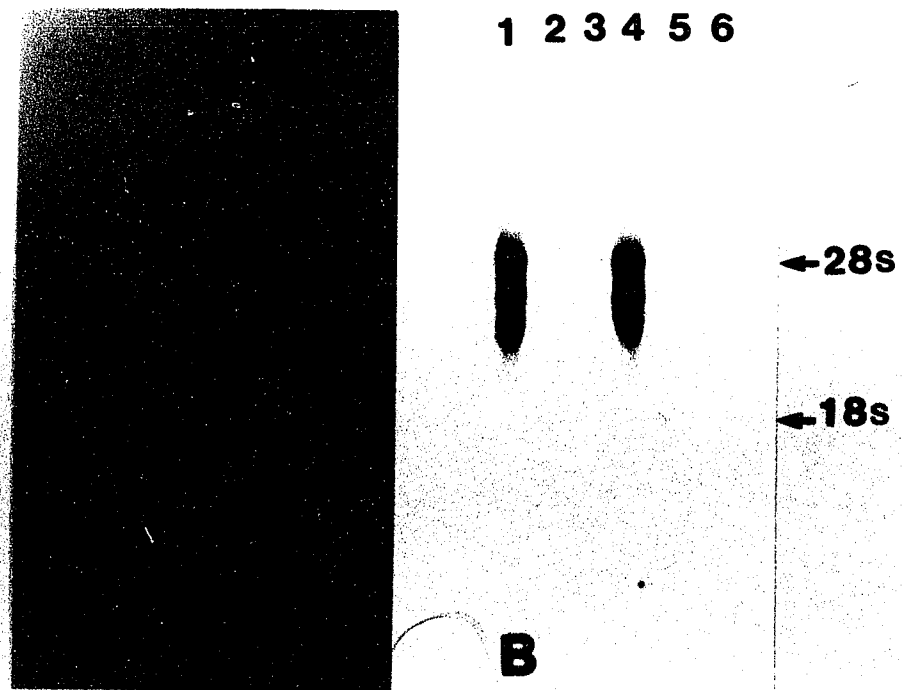


Figure 16. The 2.8 kb F1-20 ORF cDNA was subcloned into pGEX3X vector to construct recombinant plasmid pSL185. The construct was transformed into *E.coli.*, the orientation was checked by restriction mapping, and the reading frame was confirmed by DNA sequencing and protein expression as well as Western blotting analyses.

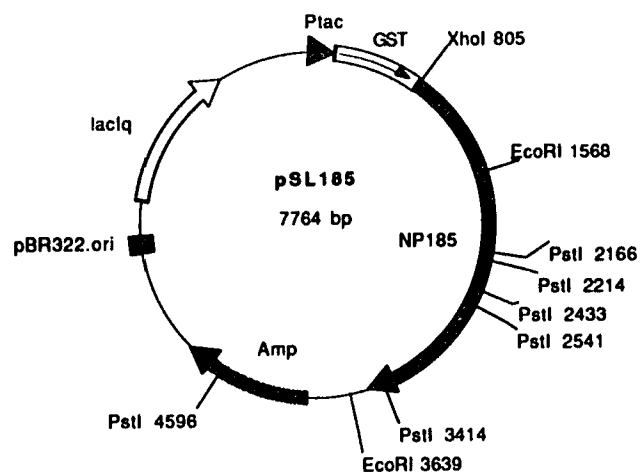


Figure 17. Time courses of GST-NP185/F1-20 protein overexpression induced by 1 mM IPTG in *E.coli.*, stained with Coomassie blue. M: molecular weight markers (Sigma; 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48.5 kDa, 36.5 kDa, 26.6 kDa); lane 1-7: cells containing the expression vector, GST with molecular weight of (26 kDa) was indicated by a bar; lane 1'-7': cells containing the recombinant plasmid, fusion protein (F.P.) of GST-NP185/F1-20 with molecular weight of 200 kDa was indicated by a bar.

Lane 1,1': cultures incubated for 1 hour at 37 °C after diluted 1:100 of overnight culture.

Lane 2,2': cultures incubated for 2 hour at 37 °C (O.D.₆₀₀ is about 0.3-0.5).

Lane 3,3': cultures from 0.5 hour after induction with 1 mM IPTG .

Lane 4,4': cultures from 1.0 hour after induction.

Lane 5,5': cultures from 1.5 hour after induction.

Lane 6,6': cultures from 2.0 hour after induction.

Lane 7,7': cultures from 2.5 hour after induction.

(see Methods and Experimental Design for details).

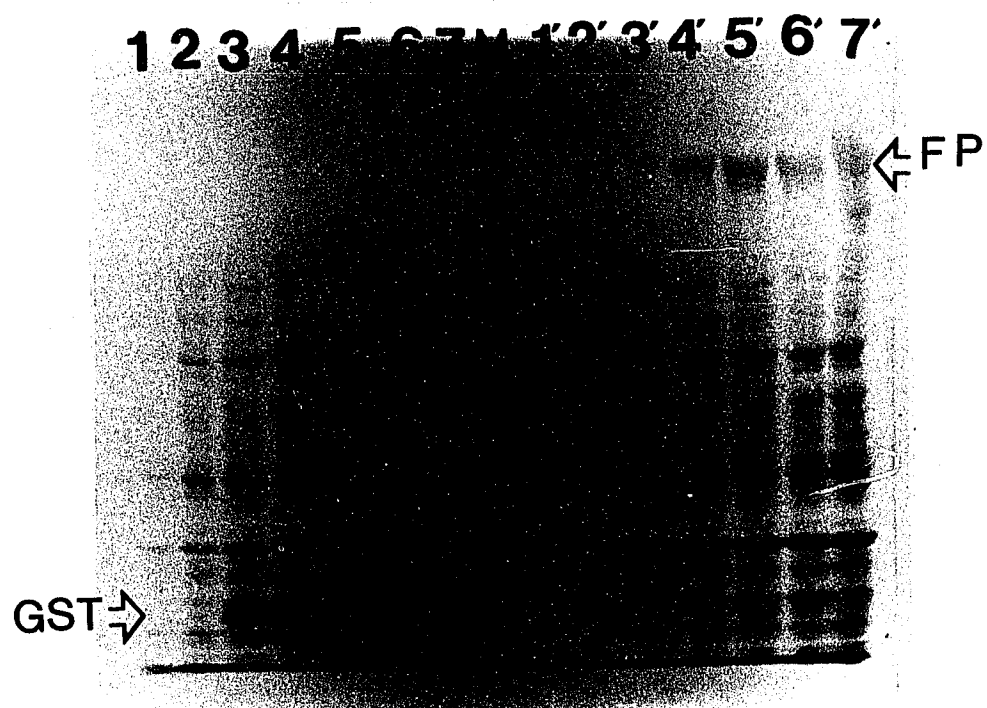


Figure 18. Expressed protein produced in *E.coli*. can be specifically recognized by both anti-F1-20 mAb and anti-NP185 mAb. For all panels, lane 1: total proteins from the cells containing expression vector without IPTG induction; lane 2: total proteins from the cells containing expression vector with IPTG induction, showing 26 kDa GST as indicated; lane 3: total proteins from the cells containing pGEX-NP185/F1-20 recombinant plasmid without IPTG induction; lane 4: total proteins from the cells containing recombinant plasmid with IPTG induction, showing GST-NP185/F1-20 protein with molecular weight 200 kDa, as indicated.

Panel A: gel stained with Coomassie blue.

Panel B: immunoblot was probed with anti-F1-20 mAb.

Panel C: immunoblot was probed with anti-NP185 mAb, 8G8.

Panel D: immunoblot was probed with anti-clathrin, 2D9.

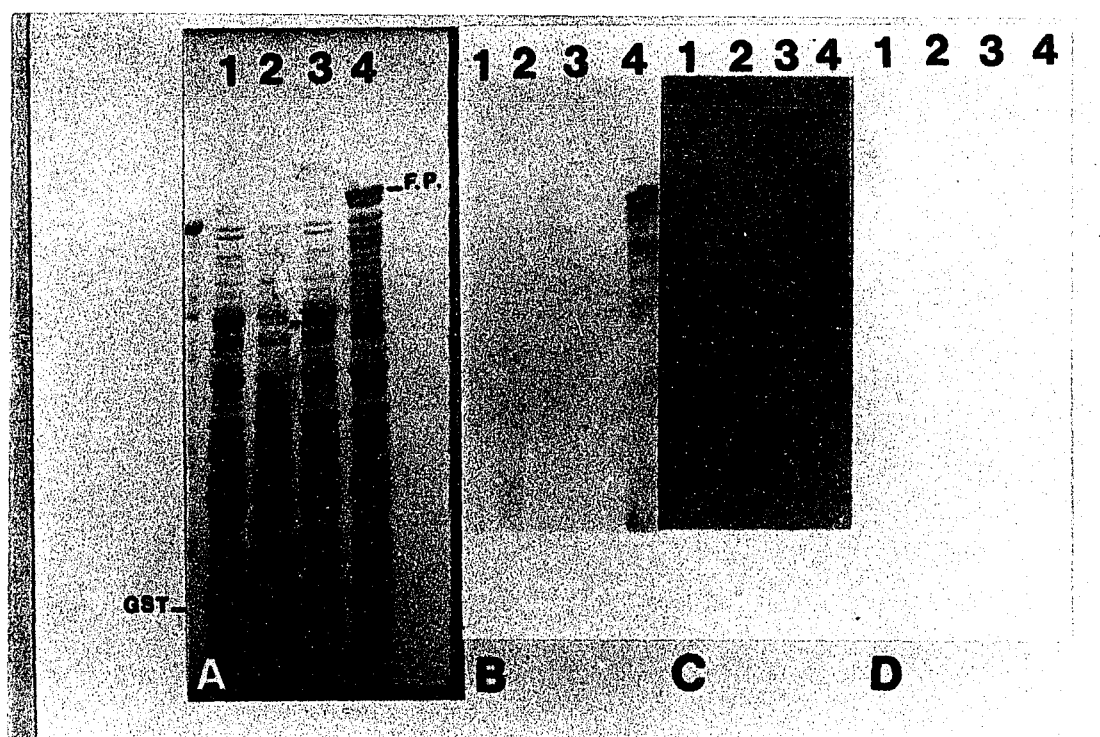


Figure 19. Purification of GST-F1-20 protein with Glutathione Sepharose 4B column (Pharmacia). For both panels, lane 1: total proteins from cells containing the recombinant plasmid, pSL185, induced by IPTG; lane 2: GST-NP185/F1-20 fusion protein purified from the column; lane 3: molecular weight markers (Sigma: 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48.5 kDa, 36.5 kDa, 26.6 kDa).

Panel A: gel stained with Coomassie blue;

Panel B: immunoblot was probed with anti-NP185 mAb, 8G8.



Figure 20. Negative staining under scanning electron microscopy showing clathrin cages induced by the bacterially expressed NP185 protein. Clathrin was incubated with NP185 in Tris buffer pH 7.5, and the protein mixture was layered on top of a 10% sucrose solution and centrifuged at high speed, air-fuge, for 2 hours. The pellet formed under the sucrose layer was resuspended and an aliquot deposited on a Formvar-coated grid. X120,000.

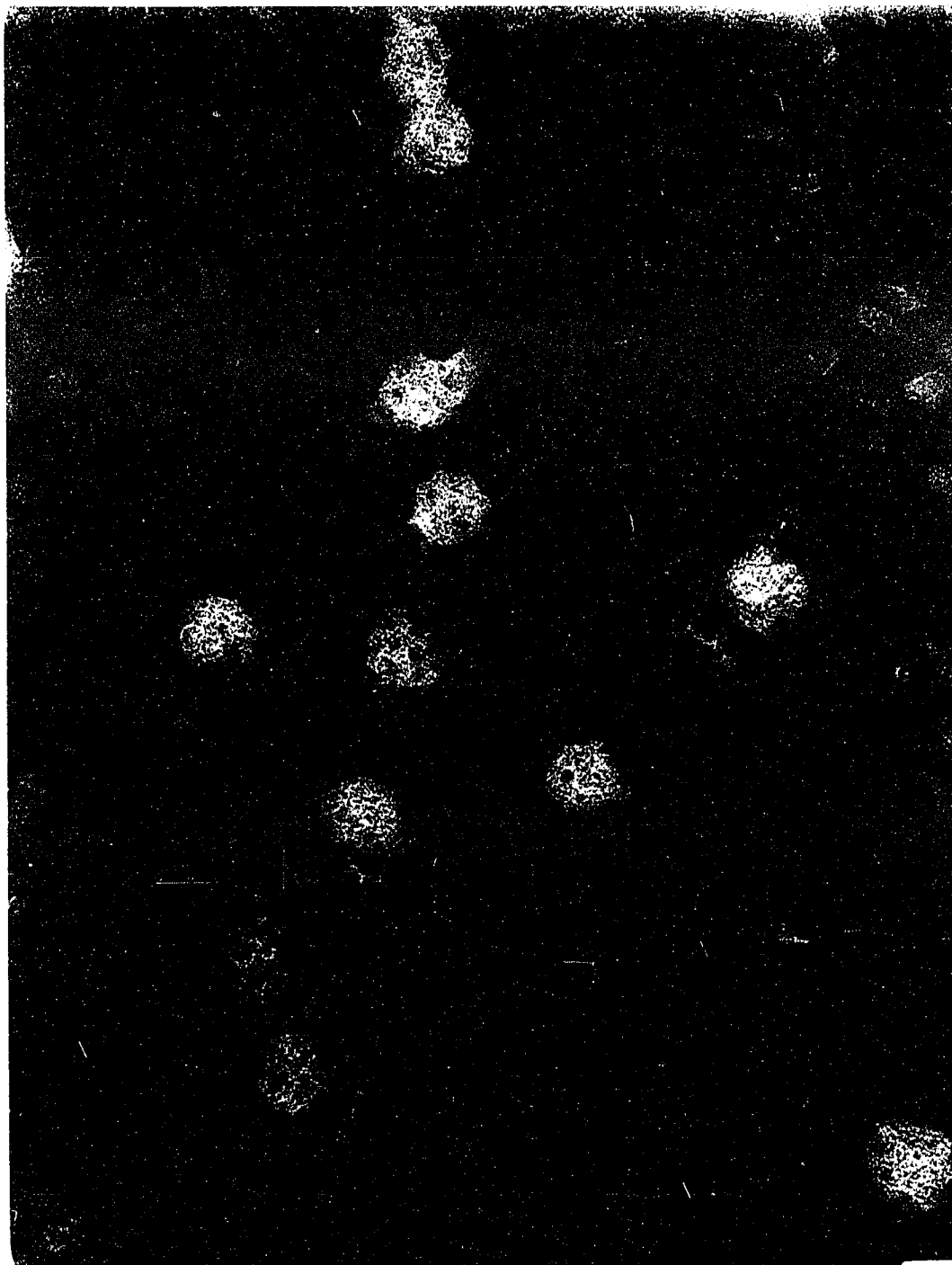


Figure 21. Schematic diagram for truncation of the NP185/F1-20 cDNA ORF. pSLEN was constructed by self-ligation after excising a 2 kb fragment with EcoRI. pSLEC was made of that the 2 kb EcoRI was subcloned into pGEX1-lambda-T vector using EcoRI site. All other constructs were from the partial digestion of pSLEC DNA with PstI, and religated as indicated. The constructs were confirmed by restriction mapping and DNA sequencing.

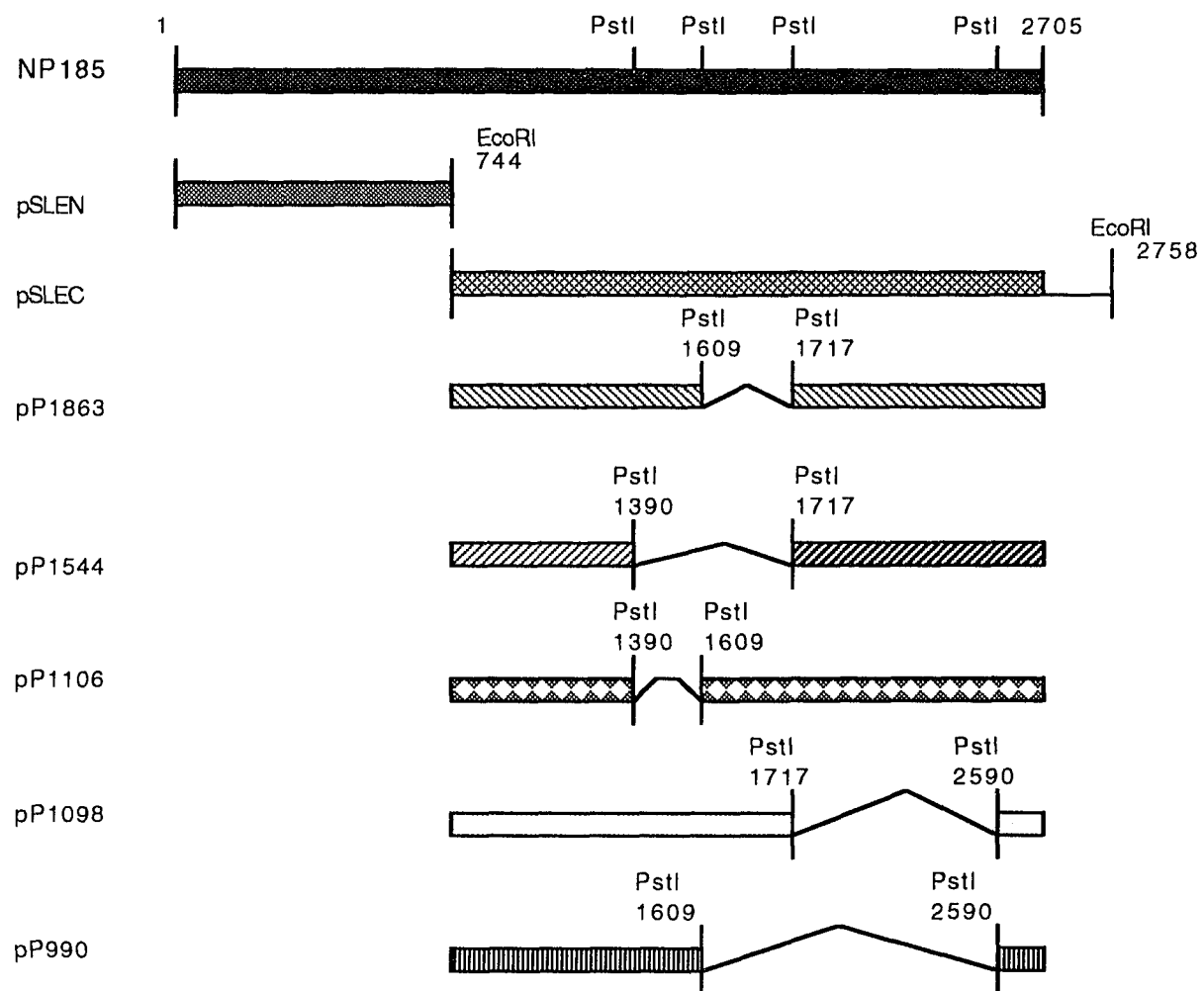


Figure 22. Schematic diagram for the construction of pSLEC. 2 kb fragment was excised out from pSL185 plasmid with EcoRI, isolated by GeneClean II kit (Bio 101), and subcloned into pGEX1λT using EcoRI site.

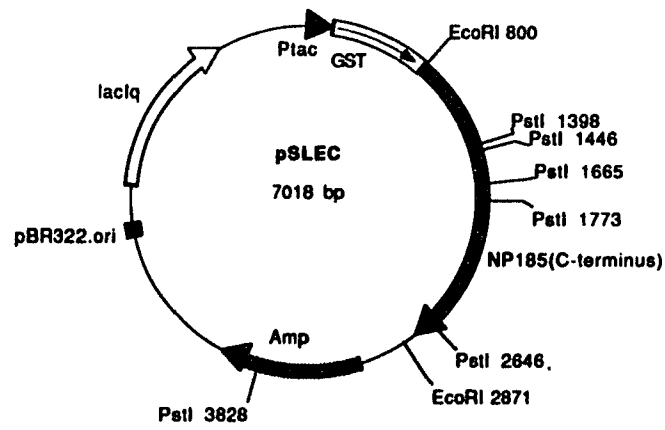


Figure 23. Western blotting analyses of the fusion protein from pSLEC and pSLEN (Fig. 21) show that both 8G8 mAb and 6G7 mAb recognize the truncated protein in the C-terminal portion of NP185/F1-20. For both panels, lane 1: molecular weight markers (Sigma high MWM: 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48.5 kDa, 36.5 kDa, 26.6 kDa); lane 2: total proteins of *E.coli* cells harboring the recombinant plasmid pSL185 (GST-NP185) (Fig. 16); lane 3: total proteins from *E.coli* cells harboring pSLEN (0.7 kb from the N-terminal portion of F1-20/NP185 (Fig. 21); lane 4: total proteins from *E.coli* cells harboring pSLEC (2 kb of the C-terminal)(Fig. 22); lane 5: total protein from *E.coli* BL21 cells harboring pGEX vector.

Panel A: blot probed with NP185 mAb 8G8.

Panel B: blot probed with NP185 mAb 6G7.



Figure 24. Expression of truncated NP185/F1-20 cDNA ORF GST fusion proteins (GST-NP185), as shown on SDS-PAGE and stained with Coomassie blue. For all the lanes containing the total proteins from *E.coli* with variety of truncated fusion proteins (Fig. 33), as shown in lane 1: pP990 (990 nt); lane 2: pP1098 (1098 nt); lane 3: pP1106 (1106 nt); lane 4: pP1863 (1863 nt); lane 5: pP1544 (1544 nt); lane 6: pSLEC (2 kb); lane 7: pSLEN (0.7 kb); lane 8: pSLEC (2 kb); lane 9: pSL185 (the full-length of cDNA); lane 10: molecular weight markers (Sigma: 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48.5 kDa, 36.5 kDa, 26.6 kDa).

1 2 3 4 5 6 7 8 9 10

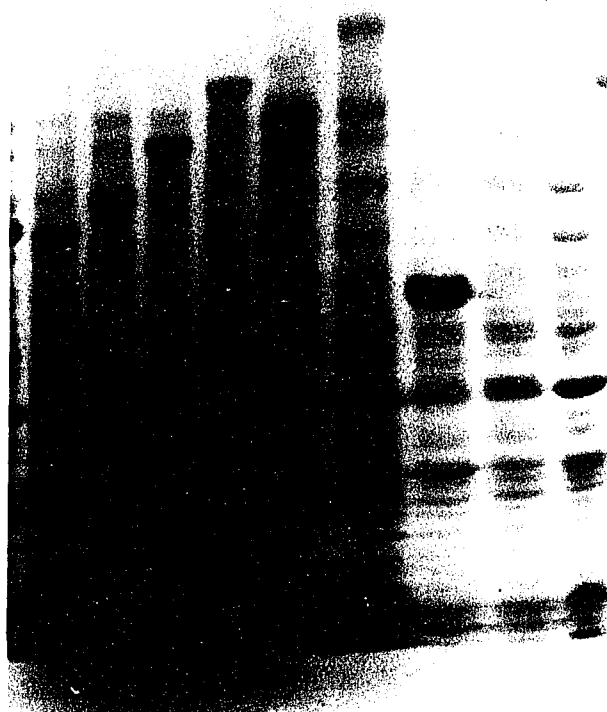


Figure 25. Immunoblot of truncated GST-NP185/F1-20-cDNA fusion proteins (GST-NP185/F1-20) probed with NP185 mAb, 6G7. For all the lanes containing the total proteins from E.coli with variety of truncated fusion proteins (Fig. 33), as shown in lane 1, 7, 11: pP990; lane 2: pP1098; lane 3: pP1106; lane 4: pP1863; lane 5, 13: pP1544; lane 6: pSLEN; lane 8, 10, 12: pGEX vector; lane 14: molecular weight markers.

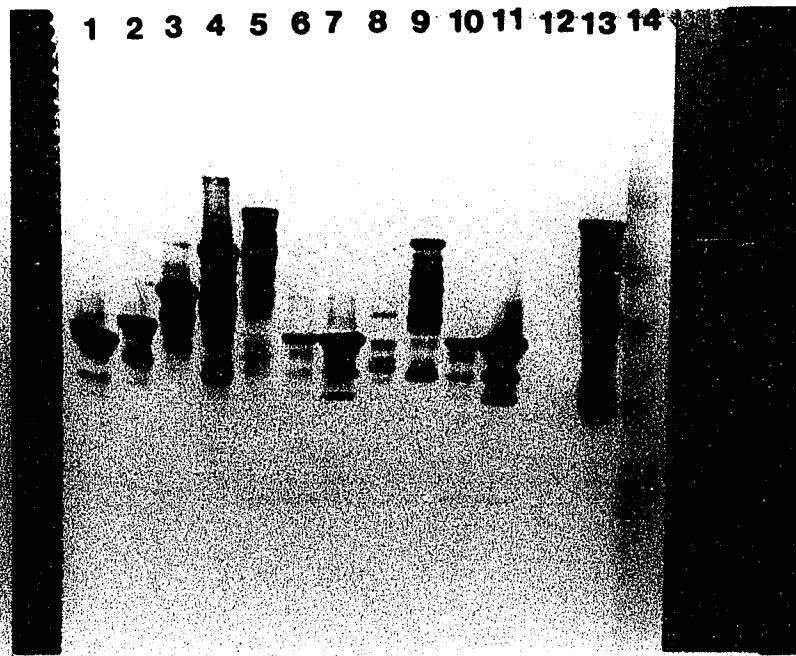


Figure 26. Schematic diagram of deletion mutants by polymerase chain reaction (PCR). Fragments amplified by PCR using F1-20 cDNA sequence were subcloned into pGEX1-lambda-T using EcoRI site. The constructs are shown as the following: pPCR244 encodes 244 residues (aa) in between nt 744 and nt 1416. pPCR124 encodes 124 residues (aa) in between nt 744 and nt 1114. pPCR28 encodes 28 aa in between nt 1029 and nt 1114. pPCR129 encodes 129 aa in between nt 1029 and nt 1416. pPCR66 encodes 66 aa in between nt 1029 and nt 1229. pPCR60 encodes 60 aa in between nt 1233 and nt 1416. pPCR56 encodes 56 aa in between nt 2535 and nt 2705.

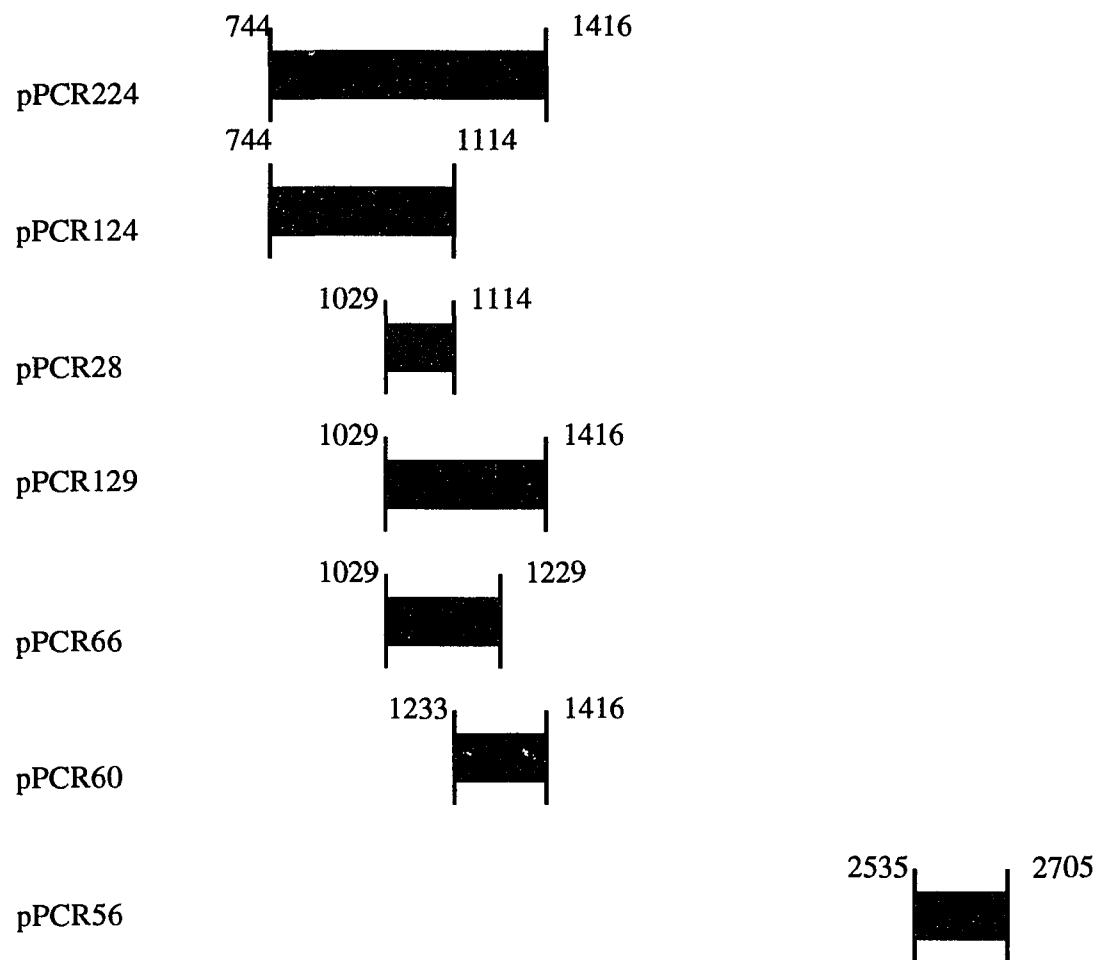


Figure 27. Expression of deletion mutants from the GST-NP185 fusion proteins deleted by PCR in *E.coli*, the total proteins from *E.coli* cells harboring different deletion mutant constructs were run on 5-15% gradient SDS-PAGE gel, stained with Coomassie blue, harboring variety of PCR-fragment constructs as shown as the following: Panel A: lane 1, molecular weight markers (Sigma high MWM: 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48.5 kDa, 36.5 kDa, 26.6 kDa); lane 2, pGEX vector; lane 3, pPCR56; lane 4, pPCR224. Panel B: lane 1, pGEX vector; lane 2, pPCR124; lane 3, pPCR129; lane 4, pPCR28. Panel C: lane 1, pPCR66; lane 2, pPCR60; lane 3, pGEX vecotor.

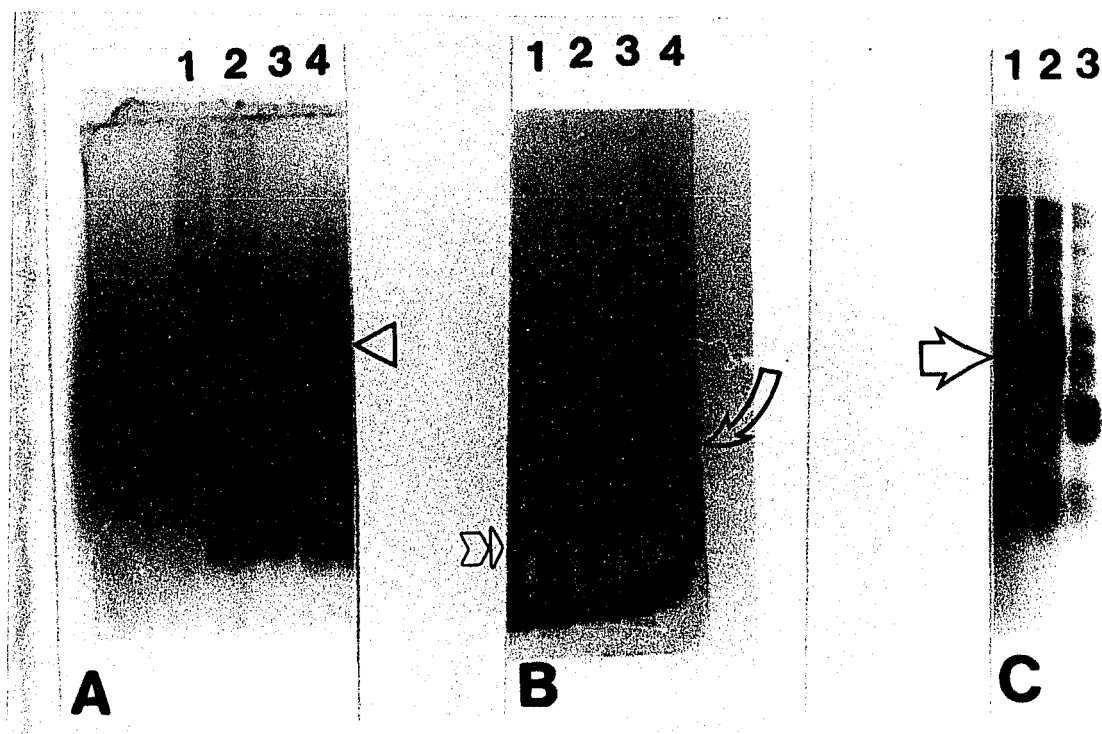


Figure 28. GST-NP185/F1-20 fusion protein binds to brain tubulin as shown by forward and reverse Far-Western blotting. Panel A: a forward Far-Western blotting. Lane 1: total proteins from *E.coli* cells containing pGEX vector; lane 2: brain tubulin; lane 3: total proteins from *E.coli* cells containing pGEX-NP185/F1-20 recombinant plasmid; lane 4: molecular weight markers (Sigma high MWM: 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48.5 kDa, 36.5 kDa, 26.6 kDa). Far-Western blotting: Protein samples as described above were resolved on 5-15% gradient SDS-PAGE gel, electrotransferred to nitrocellulose membrane. The membrane was treated with denatural and renatural buffers, incubated with GST-NP185/F1-20 fusion protein. After washing the membrane, the membrane was incubated with anti-NP185 mAb, 8G8. Washed again, the signal was detected by goat anti-mouse IgG conjugated with alkaline phosphatase, followed by adding substrates, NBT and BCIP. Panel B: Conversely, a reverse Far-Western blotting was performed as following. Lane 1: total proteins from *E.coli* cells containing pGEX vector; lane 2: total proteins from *E.coli* cells contain pGEX-NP185/F1-20 construct; lane 3: clathrin purified from bovine brain; lane 4: rat brain tubulin (50 kDa); land the proteins as described above were resolved on 5%-15% SDS-PAGE gradient gel, electrotransferred to nitrocellulose membrane. The membrane was incubated with tubulin, and then incubated with mouse anti-tubulin mAb. The signal was developed by adding goat anti-mouse IgG antibody conjugated with alkaline phosphatase (Sigma), following substrates, NBT and BCIP.

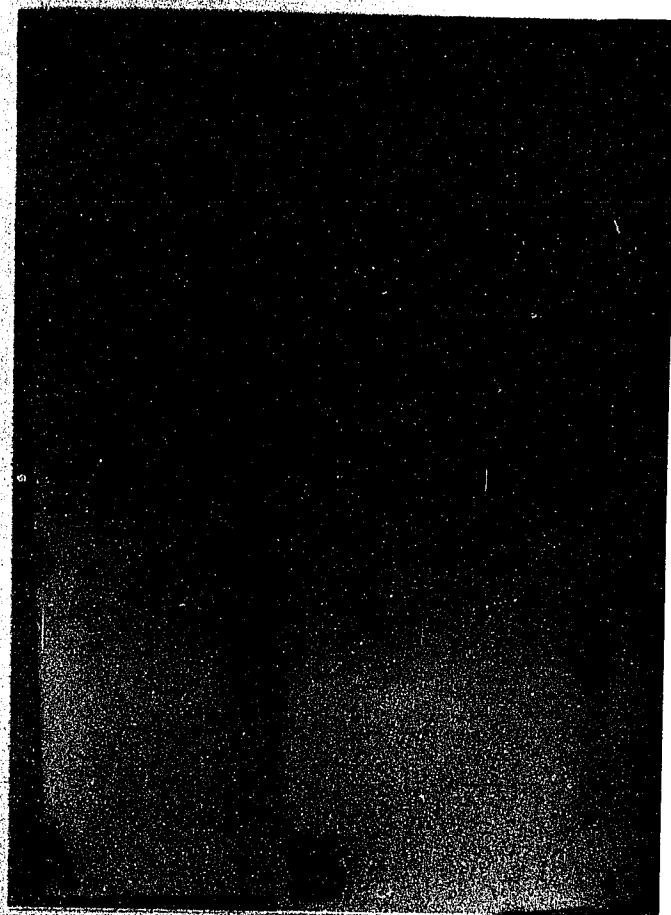


Figure 29. pPCR124 mutant was found to be a tubulin binding domain as shown by Far-Western blotting on different mutants. For all the lanes, total *E.coli* proteins harboring NP185 cDNA mutant constructs were resolved on SDS-PAGE gel, blotted onto nitrocellulose membrane, overlaid with tubulin, and detected with anti-tubulin mAb as described in standard method. The constructs were from: Lane 1, molecular weight markers (Sigma high MWM: 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48.5 kDa, 36.5 kDa, 26.6 kDa); lane 2, pSLEN; lane 3, pPCR 60; lane 4, pPCR124; lane 5, pPCR28; lane 6, pPCR56; lane 7, clathrins; lane 8, 9, pSL185; lane 10, pGEX vector.

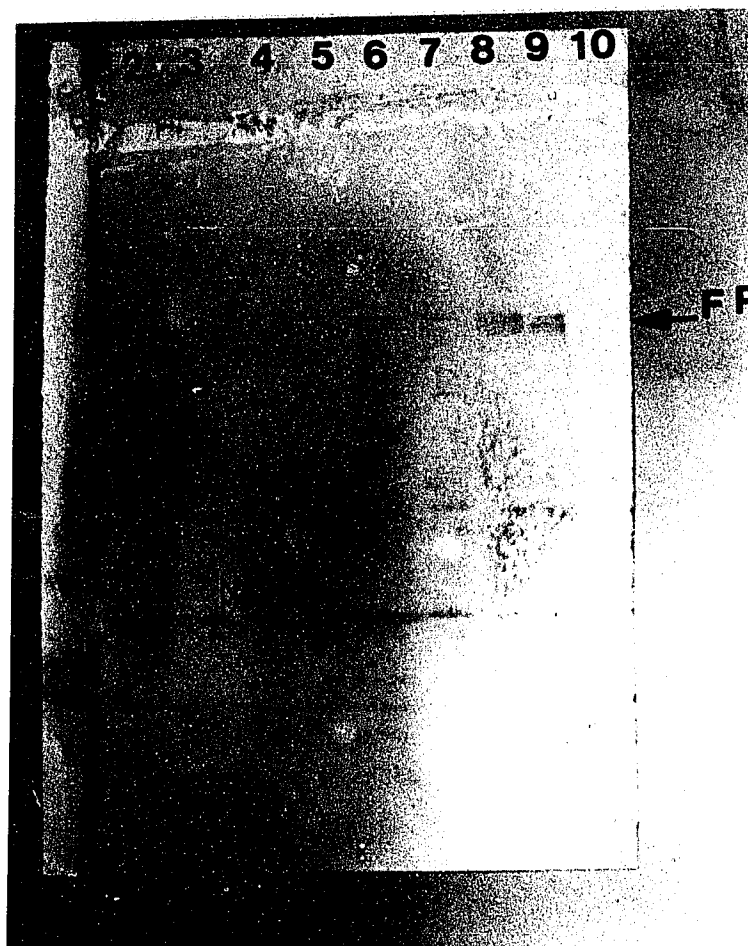


Figure 30. Far-Western blotting shows the tubulin binding domain. Panel A: lane 1, molecular weight markers (Sigma: 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48.5 kDa, 36.5 kDa, 26.6 kDa); lane 2, NP185 purified from bovine brain; lane 3, total proteins from *E.coli* cells harboring pSL185; lane 4, total proteins from *E.coli* cells harboring pPCR124; lane 5, purified clathrins with tubulins. Panel B: Far-Western blotting of the proteins in Panel A, overlaid with tubulin, then, added anti-tubulin mAb, and detected as described elsewhere.

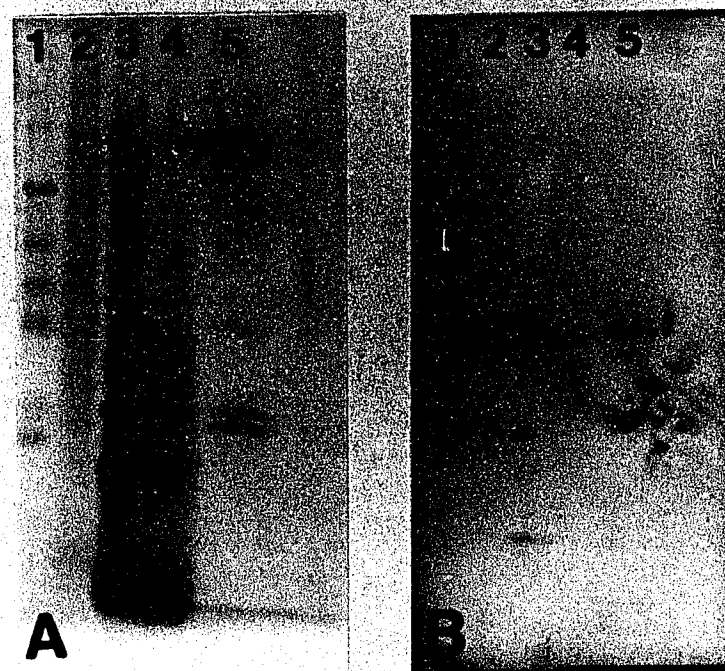


Figure 31. Far-Western blotting shows that NP185 protein purified from bovine binds to non-phosphorylated clathrin light chains (CLCs), but not bacterially expressed fusion proteins of GST-NP185/F1-20. Panel A: lane 1, NP185 proteins purified from bovine brain; lane 2, total proteins of *E.coli* harboring pGEX vector; lane 3, total proteins of *E.coli* harboring pSL185 plasmid; lane 4, purified clathrins, CHCs and CLCs; lane 5, molecular weight markers (Sigma high MWM: 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48.5 kDa, 36.5 kDa, 26.6 kDa). Panel B: Far-Western blotting of the proteins in panel A, overlaid with phosphorylated CLCs, detected by anti-CLC mAbs, 7H12, 4E5, 10B2, as described elsewhere (Kohtz et al., 1987). Panel C: Far-Western blotting of the proteins in panel A, overlaid with non-phosphorylated CLCs, detected as described above.

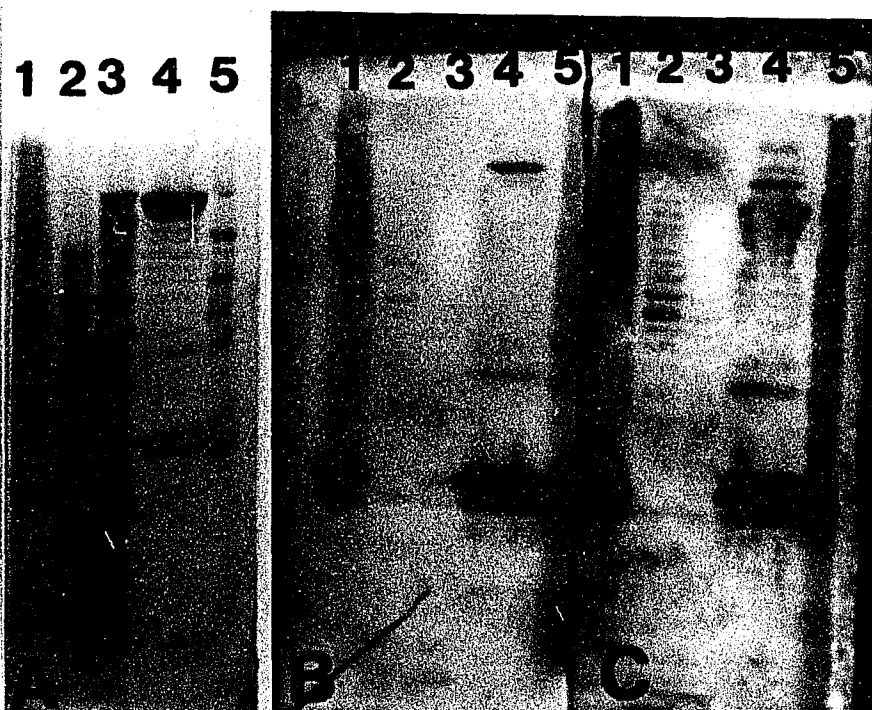


Figure 32. The amino acid composition of tubulin binding domain of NP185/F1-20 protein was mapped to 124 residues (amino acid) in the encoding region of F1-20 cDNA*^{1,2,3}.

EFLKVAEQVG IDKGDIPDLT

QAPSSLMETL EQHLNTLEGK

KPGNNEGSGA PSPLSKSSPA

TTVTSPNSTP AKTIDTSPPV

DIFATASAAA PVSSAKPSSD

LLDLQPDFSG AAAGAAPVV

PPSG

* ¹Double-underlined is the consensus (motif) for casein kinase II (CKII) S-X-X-D/E (X = any aa) (Kuenzel et al., 1987).

²Underlined is the consensus (motif) for protein kinase C (PKC) S/T-X-R/K (X is an uncharged aa) (Woodgett et al., 1986). ³tubulin binding region was found to be hydrophobic, a protein-protein interaction sites (Rienitz et al., 1988; Lewis et al., 1989; Kirsch et al., 1990); As indicated by bold letters are uncharged amino acids or hydrophobic amino acids.

Figure 33. The antigenic determinants-epitopes for the mAbs 8G8 and 6G7 of anti-NP185 were mapped to a 60 residues (amino acids) region (pPCR60, Fig. 26) as shown in Coomassie blue stain (Panel A); or blotted with 8G8 mAb (Panel B), and with 6G7 mAb (Panel C). For all the panels: lane 1, the total proteins from *E.coli* BL21 cells harboring pPCR60 constructs; lane 2, the total proteins of *E.coli* BL21 cells harboring pGEX vector alone as indicated by GST fragment; lane 3, the total proteins from *E.coli* cells harboring pSL185; lane 4: molecular weight markers (Sigma high MWM: 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48.5 kDa, 36.5 kDa, 26.6 kDa).

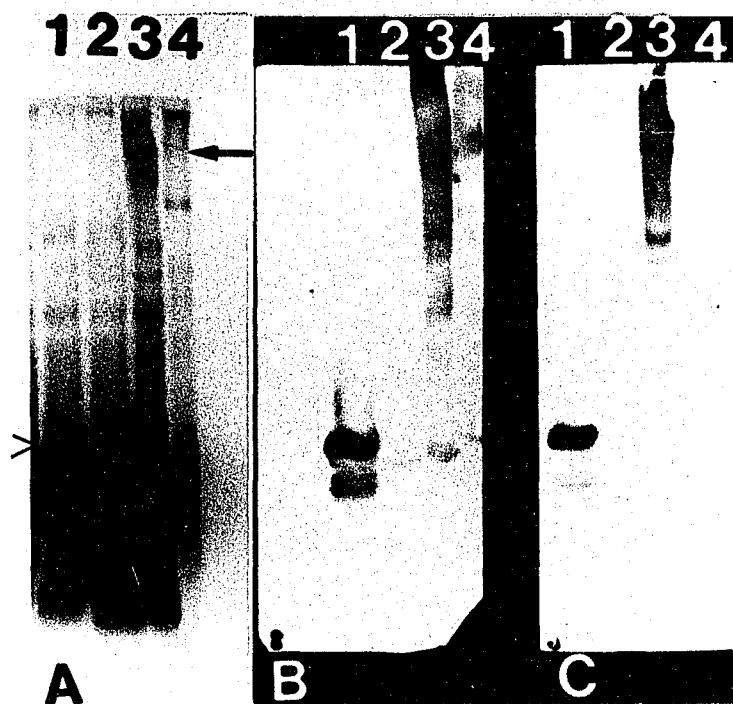


Figure 34. The amino acid composition of antigenic determinants (epitopes) for the mAbs 8G8 and 6G7 of anti-NP185 protein were mapped to 60 residues (amino acids) between nucleotide 1378 and nucleotide 1558 of F1-20 cDNA*

TTEPASASASTTTAVTAVTT

EVDLFGDAFAASSPGEAPAAS

EGATAPATPAPVAAALDACS(GND)

* Double-underlined were the consensus (motif) for casein kinase II S-X-X-D/E (X = any aa)

Figure 35. The pPCR60 (Fig. 26; containing 60 amino acid residues) fusion protein (epitopes for both 8G8 and 6G7) was purified by glutathione sepharose 4B column for further separation of epitopes, as indicated on SDS-PAGE by Coomassie blue stain. Lane 1: the total proteins from *E.coli* cells harboring pGEX vector. Lane 2: the total proteins from *E.coli* cells harboring pPCR60. Lane 3-10 were fraction 1 to 8 (1 ml each) from elution of the column.

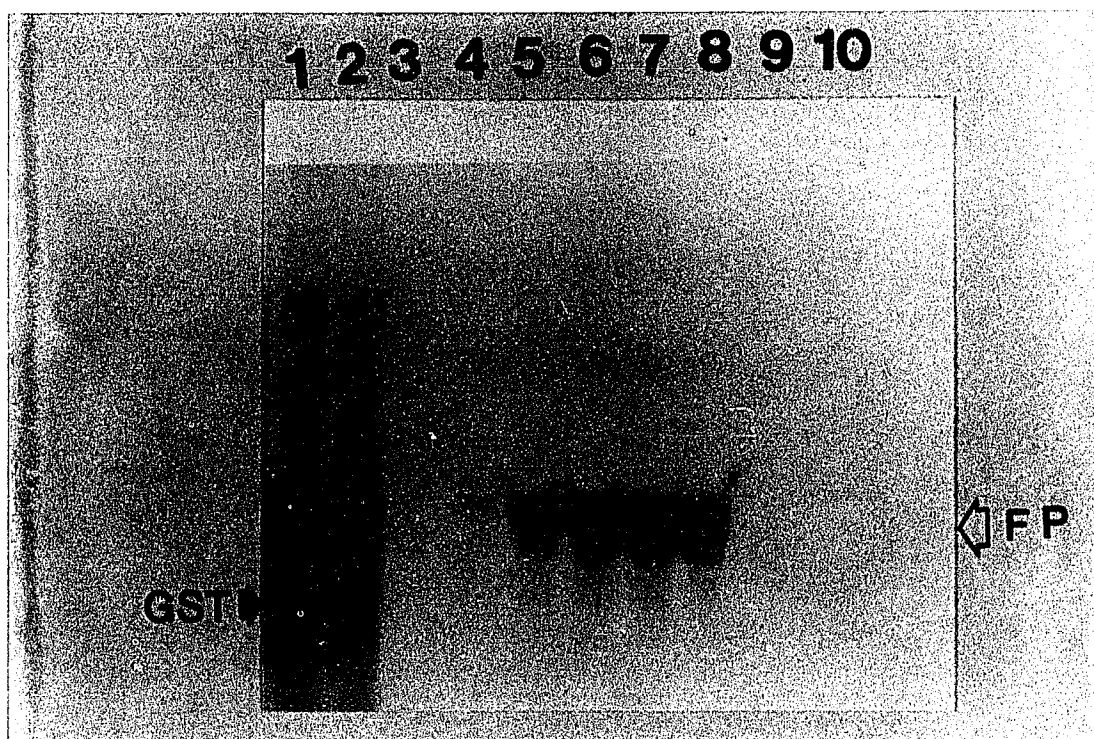
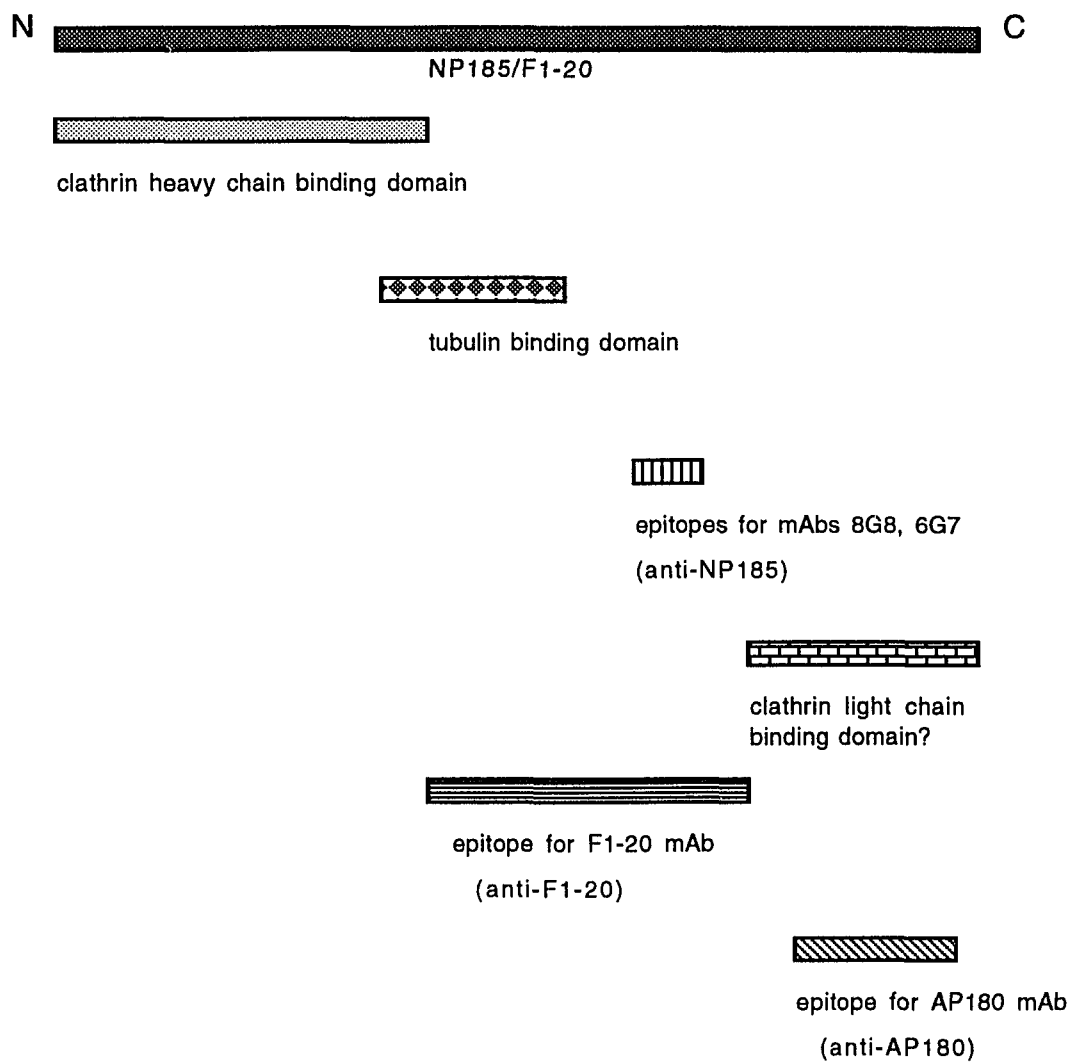


Figure 36. Schematic diagrams of domain organization of NP185/F-120/AP180 proteins. The N-terminal 300 aa residues (pI 8.7) harbor a clathrin binding site (Morris et al., 1993). The tubulin binding domain resides in the middle 124 residues between residue 249 and residue 372 of F1-20 sequence (Li et al., 1994). The epitope for NP185 was mapped to 60 residues in between residue 411 and residue 471 of the F1-20 sequence (Li et al., 1994), and the epitope for F1-20 mAb is probably present in between residue 400 and residue 766 (Zhou et al., 1992), while the epitope for AP180 mAb may reside in between residue 562 and residue 744 (Morris et al., 1993). The C-terminal region may be the CLC binding domain as speculated in the text.



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