OUKA
The University of Osaka
Institutional Knowledge Archive

Title	Purification and biochemical characterization of inositol 1,3,4,5-tetrakisphosphate-binding proteins in the mouse brain
Author(s)	Yamaguchi, Yoshihide
Citation	大阪大学, 1995, 博士論文
Version Type	VoR
URL	https://doi.org/10.11501/3100527
rights	
Note	

The University of Osaka Institutional Knowledge Archive : OUKA

https://ir.library.osaka-u.ac.jp/

The University of Osaka

Purification and biochemical characterization of inositol 1,3,4,5-tetrakisphosphate-binding proteins in the mouse brain

Yoshihide Yamaguchi

(1)

Purification and biochemical characterization of inositol 1,3,4,5-tetrakisphosphate-binding proteins in the mouse brain

Yoshihide Yamaguchi

Abbreviations 1
Abstract 2
Introduction 5
Materials and Methods 8
Materials 8
Methods 9
General methods 9
Measurement of [^{3}H]InsP_4-Binding 9
Preparation of P2/P3 membrane fraction 10
Solubilization of membrane fraction 10
Purification of IP4BP1 (synaptotagmin *II*) 11
Preparation of DE-52 flow-through fraction 12
Purification of IP4BP2a (aldolase A) 12
Purification of IP4BP2b (PLP) 13
SDS-PAGE and Immunoblot analysis 14
Determination of the partial amino acid sequences of IP4BP1 14
N-terminal sequence analysis of IP4BP2a and IP4BP2b 15
Production of antisera against synthetic C-terminal peptide and the C2A region of rat synaptotagmin 16
Immunoprecipitation of IP4BP1 (synaptotagmin *II*) 17
Monoclonal antibodies against PLP 18
Purification of AA3-IgG 18
Immunoabsorption against IP4BP2b (PLP) using Protein G-Sepharose 18
Cross-linking of IP4BP2a (aldolase A) with DMS 19
Purification of Myelin 19
Results 22
Purification of IP4BP1 22
Determination of the partial amino acid sequences of the purified IP4BP1 23
Immunological cross-reactivity of IP4BP1 and synaptotagmin 24
Characterization of the InsP_4-binding activity of the purified IP4BP1 (synaptotagmin *II*) 25
Purification of IP4BP2a 26
Cross-linking of IP4BP2a 27
N-terminal sequence analysis of IP4BP2a 27

Characterization of the $InsP_4$-binding activity of the purified IP4BP2a (aldolase A) 28
Purification of IP4BP2b 29
N-terminal sequence analysis of IP4BP2b 30
Immunological cross-reactivity of IP4BP2b and myelin proteolipid protein (PLP) 30
Characterization of the $InsP_4$-binding activity of the purified IP4BP2b (PLP) 31
Characterization of the $InsP_6$-binding activity of IP4BP2b (PLP) 32
Purification and $InsP_x$-binding activity of myelin 33
Discussion 34
IP4BP1 / synaptotagmin II 34
IP4BP2a / aldolase A 36
IP4BP2b / PLP 39
Other $InsP_4$ and $InsP_x$-binding proteins 43
The possibility that IP4BP purified in this study regulates intracellular Ca^{2+} concentration as IP4R 45
Relationships among $InsP_x$-binding proteins 46
Acknowledgments 48
References 49

Abbreviations

B_{max}	: maximum binding
BSA	: bovine serum albumin
$[Ca^{2+}]_i$	: intracellular Ca^{2+} concentration
CAPS	: 3-(cyclohexylamino)-1-propanesulfonic acid
CBB R-250	: Coomassie Brilliant Blue R-250
CHAPS	: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate
CNS	: central nervous system
DAB	: diaminobenzidine
DAG	: diacylglycerol
DMS	: dimethyl suberimidate
DTT	: dithiothreitol
EPPS	: 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid
ER	: endoplasmic reticulum
Fura 2-AM	: 1-[6-amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid, pentaacetoxymethyl ester
GluR	: glutamate receptor
Ins-1-P	: inositol 1-phosphate
Ins-1,4-P_2	: inositol 1,4-bisphosphate
$InsP_3$	: inositol 1,4,5-trisphosphate
$InsP_4$	: inositol 1,3,4,5-tetrakisphosphate
$InsP_5$	: inositol 1,3,4,5,6-pentakisphosphate
$InsP_6$	: inositol hexakisphosphate
$InsP_x$	: inositol polyphosphate
IP3R	: specific receptor protein for $InsP_3$
IP4BP	: $InsP_4$-binding proteins
IP4R	: specific receptor protein for $InsP_4$
K_D	: dissociation constant
$[L]_{0.5}$	: values obtained by numerical fitting to the Hill equation ($[L]_{0.5}$ is substituted for K_D)
MES	: 2-morpholinoethanesulfonic acid, monohydrate
nAcR	: nicotinic acetylcholine receptor
PAGE	: polyacrylamide gel electrophoresis
PBS	: phosphate-buffered saline
PEG	: polyethylene glycol
PLC	: phosphatidylinositol specific phospholipase C
PMSF	: phenylmethylsulfonyl fluoride
PVDF	: poly(vinylidene difluoride)
sulfo-SMCC	: sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate
TFA	: trifluoroacetic acid
T-PBS	: PBS containing 0.1% Tween-20

Abstract

Inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) has been considered to be a second messenger that regulates intracellular Ca^{2+} concentration like inositol 1,4,5-trisphosphate ($InsP_3$). While many observations suggesting the physiological role of $InsP_4$ have been reported, actual function of $InsP_4$ is still a matter of debate mainly because the specific receptor proteins for $InsP_4$ (IP4R) have not been well characterized yet. Therefore, to clarify physiological functions of $InsP_4$, I tried to purify and characterize the IP4R from mouse cerebellum .

Some of $InsP_4$-binding proteins were solubilized by treatment with 1% Triton X-100 from mouse cerebellar P2/P3 membrane fraction, and purified by sequential column chromatographies (ion-exchange, affinity, and gel filtration chromatography). [^{3}H] $InsP_4$-binding activity was detected using PEG precipitation method. Now, I have succeeded in purifying three $InsP_4$-binding proteins (IP4BP) from mouse cerebella, and characterized these proteins biochemically. One of IP4BP from DE-52 absorbed fraction was called IP4BP1 and the others from DE-52 flow-through fraction were called IP4BP2a and IP4BP2b.

IP4BP1 was detected by SDS-PAGE analysis as two protein bands with molecular weights of 140K and 65K. Reverse phase HPLC after limited proteolysis by lysyl endopeptidase yielded almost the same elution profile on the 140K and 65K bands, strongly suggesting that the 140K molecule is a dimer of the 65K monomer. Amino acid sequences of seven peptides derived from the 140K molecule were identical to partial sequence of synaptotagmin *II*, which is considered to play a significant role in the docking and fusion of synaptic

considered to play a significant role in the docking and fusion of synaptic vesicles at the presynaptic release sites. Immunoblot analysis of IP4BP1 using two polyclonal antibodies against synaptotagmin *II* revealed that both 140K and 65K molecules were recognized by the antibodies. Furthermore, the $InsP_4$-binding activity of the purified sample was immunoprecipitated by anti-synaptotagmin antibody.

IP4BP2a gave 42K band on SDS-PAGE analysis, and was shown to form tetramer structure by the cross-linking experiment. N-terminal sequence of IP4BP2a was nearly equal to that of aldolase A, which is one of glycolytic fructose-1,6-bisphosphate aldolases.

IP4BP2b gave 26K band on SDS-PAGE analysis. N-terminal sequence analysis of IP4BP2b revealed that it is identical to myelin proteolipid protein (PLP), which is the most abundant component and the major integral membrane protein of CNS myelin. Immunoblot analysis of IP4BP2b revealed that two monoclonal antibodies against PLP cross-react with the 26K molecule. Furthermore, the $InsP_4$-binding activity and 26K protein band were immunoabsorbed by anti-PLP monoclonal antibody.

When [^{3}H] $InsP_4$-binding activity was measured under hypotonic (low salt) condition, IP4BP1 showed optimal activity at pH 5.0, K_D of about 160 nM, 30 nM and 30 nM, and B_{max} of 35 pmol, 2.7 pmol and 2.4 pmol/μg protein at pH 5.0, 6.2 and 7.4, respectively. IP4BP2a showed optimal activity at pH 5.8, K_D of 1.2 μM, and B_{max} of 22 pmol/μg protein at pH 5.8. IP4BP2b showed optimal activity at pH 5.5, K_D of 71 nM, and B_{max} of 30 pmol/μg protein at pH 5.5. On inhibition assays of [^{3}H] $InsP_4$-binding by various inositol phosphates, $InsP_4$-binding activities of IP4BP1/synaptotagmin *II*, IP4BP2a/aldolase A, and IP4BP2b/PLP

were strongly inhibited by inositol high-polyphosphates ($InsP_4$, $InsP_5$, and $InsP_6$).

Under isotonic condition, among $InsP_3$, $InsP_4$, and $InsP_6$-binding activities of IP4BP2b/PLP, only [^{3}H] $InsP_6$-binding activity was detected, and indicated K_D of 58 nM, and B_{max} of 13 pmol/μg protein. Further, myelin membrane, where PLP is present in abundance, also showed [^{3}H] $InsP_6$-binding activity.

Aldolase has been considered to involve in the cerebellar function, and synaptotagmin and PLP in the intracellular vesicle transport system. Therefore, above results strongly suggest that inositol high-polyphosphates ($InsP_4$, $InsP_5$, and $InsP_6$) may be important for regulating the cerebellar function and the intracellular vesicle transport system.

Introduction

The phosphoinositide cascade plays a central role in the transduction of many extracellular stimuli. Hormones, neurotransmitters, and growth factors activate this cascade by binding to specific plasma membrane receptors that are coupled to phosphatidylinositol specific phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate and generates two second messengers; inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). DAG activates protein kinase C, whereas $InsP_3$ mobilizes Ca^{2+} from the endoplasmic reticulum via binding to a specific receptor (Berridge *et al.*, 1989). By this mechanism, $InsP_3$ activates numerous Ca^{2+}-dependent processes in a wide variety of cell types.

Molecular mechanism involved in the $InsP_3$-dependent Ca^{2+} release process has recently been characterized. The receptor protein for $InsP_3$ (IP3R) has been purified from mouse and rat cerebellum, where it was found to be highly abundant in Purkinje cells (Supattapone *et al.*, 1988; Maeda *et al.*, 1990). In this laboratory, Furuichi *et al.* (1989) succeeded in cloning the cDNA of the mouse cerebellar IP3R protein, and deduced its primary structure from the nucleotide sequence. Reconstitution of the purified IP3R protein into planar lipid bilayer or lipid vesicles demonstrated that the receptor protein constitutes $InsP_3$-sensitive Ca^{2+} channels and that the $InsP_3$-induced Ca^{2+}-mobilizing is regulated by phosphorylation of IP3R (Ferris *et al.*, 1989; Ferris *et al.*, 1991; Maeda *et al.*, 1991; Nakade *et al.*, 1991; Nakade *et al.*, 1994). These findings indicate that the cerebellar IP3R protein is a Ca^{2+} permeable cation-selective channel having $InsP_3$-binding sites. Thus, significant roles for $InsP_3$ have been dramatically clarified by identification and biochemical characterization of the

specific receptor as described above.

On the other hand, $InsP_3$ is rapidly metabolized either by dephosphorylation to inositol 1,4-bisphosphate or by phosphorylation to inositol 1,3,4,5-tetrakisphosphate ($InsP_4$). Ferris *et al.* (1989) reported that in their reconstituted lipid vesicles $InsP_4$ did not bind significantly to IP3R and was inactive to the $InsP_3$-induced Ca^{2+}-mobilizing. Mainly, the specific enzyme $InsP_3$ 3-kinase plays a role to inactivate an effective second messenger. However, the coexistence of a 5-phosphatase, which also performs the function of inactivating $InsP_3$, have given speculation that $InsP_4$ has some specific function as an second messenger, since the apparent energy cost of the 3-kinase pathway is high and the half-life of cellular $InsP_4$ is short.

Recently, several reports have suggested the physiological role of $InsP_4$ from the following two points : (i) regulation of Ca^{2+}-influx phase of the response; (ii) modulation of various intracellular Ca^{2+} pools. As for the first point, Irvine and Moor (1986) suggested, on the basis of microinjection experiment in certain species of sea urchin oocytes, that $InsP_4$ could play a role in controlling Ca^{2+}-influx by acting together with $InsP_3$, as egg activation. In mouse lacrimal acinar cell, measuring transmembrane currents in single cells by voltage clamping, Morris *et al.* (1987) and Changya *et al.* (1989) showed that $InsP_4$ and $InsP_3$ in combination evoke a sustained increase in Ca^{2+}-activated-K^+ current which is dependent on external Ca^{2+}. In addition , Lückhoff and Clapham (1992) reported that $InsP_4$ enhances the activity of the Ca^{2+}-permeable channel when exposed to intracellular surface of endotherial cell inside-out patches.

At the secondary point, Hill *et al.* (1988) suggested that $InsP_4$ may induce Ca^{2+} sequestration in rat liver epithelial cell and also Yoo *et al.* (1991) reported $InsP_4$ induce rapid uptake of $^{45}Ca^{2+}$ into bovine adrenal-medullary secretory vesicles. On the

other hand, Ely *et al.* (1990) showed that $InsP_4$ stimulates Ca^{2+}-release from bovine adrenal microsomes by a mechanism independent of the IP3R. Although many observations have been made which is indicative of $InsP_4$-fuction, the actual function of $InsP_4$ is still a matter of debate, partly because the specific receptor proteins for $InsP_4$ (IP4R) have not been well characterized yet.

We have detected [^{3}H]$InsP_4$-binding activity during sequential purification process of IP3R from mouse cerebella, which indicates the existence of IP4R. Cerebellum was used as the source of IP4R, because of the accumulating of evidences showing that the phosphoinositide cascade is actively involved in cerebellar functions: (i) the IP3R is abundant in Purkinje cells alone (Worley *et al.*, 1987; Maeda *et al.*, 1990); (ii) high levels of $InsP_3$ 3-kinase are also localized in Purkinje cells (Mailleux *et al.*, 1991; Yamada *et al.*, 1993), suggesting that $InsP_4$ may be easily generated in Purkinje cells; (iii) $InsP_4$ binding activity has been found to be present in relatively high levels in the cerebellum (Theibert *et al.*, 1987; Donié *et al.*, 1989). These findings strongly suggest the possible involvement of inositol polyphosphates (such as $InsP_3$ and $InsP_4$) in the neuronal functions of Purkinje cells.

In this study, to clarify physiological functions of $InsP_4$, I tried to purify and characterize the IP4R. Now, I have succeeded in isolating three $InsP_4$-binding proteins from mouse cerebella; IP4BP1 (synaptotagmin *II*), IP4BP2a (aldolase A), IP4BP2b (myelin proteolipid protein : PLP) and characterized these proteins biochemically.

Materials and Methods

Materials

[^{3}H]InsP_4 (17 Ci/mmol), [^{3}H]InsP_3 (17 Ci/mmol), [^{3}H]InsP_6 (12 Ci/mmol), SOLVABLE and AQUASOL-2 were obtained from Du Pont-New England Nuclear. Ins-1,4,5-P_3, Ins-1,3,4,5-P_4, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate (CHAPS) were from Dojindo Laboratories. Ins-2,4,5-P_3, Ins-1,3,4,6-P_4, and Ins-1,2,4,5-P_4 were kindly supplied by Dr. Hirata and Dr. Ozaki. Ins-1,3,4,5,6-P_5 was from Beohringer Manheim, while Ins-1-P, Ins-1,4-P_2, InsP_6, Heparin-Agarose, and Protein G-Sepharose were from Sigma. *Achromobacter lyticus* lysyl endopeptidase and silver staining kit were obtained from Wako Pure Chemical Industries, Ltd. DE-52, CM-32, and CM-52 were purchased from Whatman. Glutathione-Sepharose 4B, Protein A-Sepharose CL-4B, CM-Sepharose CL-6B, Sepharose CL-6B, Sephacryl S-300, and gel filtration molecular weight standards were from Pharmacia. Dimethyl suberimidate (DMS), cationized BSA coupled with sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), and aluminum hydroxide adjuvant were obtained from Pierce Chemical Co. Biotinylated goat anti-rat IgG (Fc region specific) was purchased from Jackson Immunoresearch Laboratories and biotinylated goat anti-rabbit IgG and Vectastain ABC kit were from Vector Laboratories. Nitrocellulose membrane was obtained from Amersham. Rat IgG was from Inter-Cell Technologies Inc. Molecular weight standards for SDS-PAGE, poly(vinylidene difluoride) (PVDF) membrane, and Dye Reagent concentrate for protein assay were purchased from Bio Rad. All of the other chemicals were of the highest purity commercially available.

Methods

General methods

Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as standard. All the purification steps and protein handling were performed at 4 °C or on ice. The pH of all buffers was adjusted at room temperature and was not corrected for cooling to 4 °C.

Measurement of [^{3}H]$InsP_4$-Binding

$InsP_4$-binding was measured by the polyethylene glycol (PEG) precipitation method, as described previously (Maeda *et al.*, 1991), with minor modifications. To assay sequential fractions from each column chromatography, [^{3}H]$InsP_4$ was added to 50 µl aliquots (after appropriate dilution) of the samples to a concentration of 9.6 nM. The samples were incubated for 10 min at 0 °C, then mixed with 2 µl of 50 mg/ml (IP4BP1 and IP4BP2a) or 4 mg/ml (IP4BP2b) bovine γ-globulin, and equal volume of the solution containing 30% PEG 6000 in 50 mM Tris-HCl, pH 8.0 (IP4BP1 and IP4BP2a), or 20 mM HEPES-NaOH, pH 7.2 (IP4BP2b) were added to the samples, which were incubated for 5 min at 0 °C. After centrifugation at 10,000 x g for 5 min, the supernatant was removed by aspiration, and the precipitate was dissolved in 500 µl of SOLVABLE. Radioactivity was measured after adding 5 ml of AQUASOL-2 as a liquid scintillation cocktail. Nonspecific binding was determined in the presence of 10 µM unlabeled $InsP_4$ or without protein samples.

For biochemical characterization of the purified samples, $InsP_4$-binding was assayed by another method. In a total volume of 100 µl, the assay mixture contained

10 μl of [^{3}H]InsP_4 solution (final concentration: 4.8 nM), 10 μl of γ-globulin solution (final concentration: 20 mg/ml), 10 μl of 0.5 M various Good's buffers (HEPES-NaOH in the usual assay, and MES-NaOH, HEPES-NaOH and EPPS-NaOH in the study on pH-dependency), 10 μl of various inositol phosphate solutions diluted with 50 mM HEPES-NaOH, pH 7.4, at appropriate concentrations (4.8 μM of unlabeled InsP_4 was used for estimation of nonspecific binding except the inhibition study by various inositol phosphates), aliquots of the samples, and distilled water. After incubation for 10 min at 0 °C, 100 μl of 30% PEG solution was added, and then assayed as described above.

These were routinely employed assays. Minor modifications were described in figure legends.

Preparation of P2/P3 membrane fraction

Cerebella from adult male ddY mouse (18 g) were homogenized in 9 volumes of the solution containing 0.32 M sucrose, 1 mM ethylene-diamine tetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, 10 μM pepstatin A, 1 mM 2-mercaptoethanol, and 5 mM Tris-HCl, pH 7.4 (Glass-Tefron Potter homogenizer) with 10 strokes at 850 rpm. The homogenate was centrifuged at 1,000 x g for 5 min at 2 °C, and the pellet was washed again under the same conditions. The combined supernatant was centrifuged at 100,000 x g for 60 min at 2 °C to obtain P2/P3 membrane fraction.

Solubilization of membrane fraction

The P2/P3 membrane fraction was resuspended in a solution containing 1 mM EDTA, 0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A, 1 mM 2-

mercaptoethanol, and 50 mM Tris-HCl, pH 8.0, and then solubilized by addition of 20% (W/V) Triton X-100 to give final protein and detergent concentration of 3.0 mg/ml and 1.0%, respectively. The sample was stirred for 30 min on ice, and then centrifuged at 25,000 x g for 60 min at 2 °C. The supernatant (1% Triton X-100 extract) was applied to a column of DE-52 or stored at -80 °C for purification of IP4BP1.

Purification of IP4BP1 (synaptotagmin *II*)

Ten times stock of the stored supernatant (1% Triton X-100 extract) were mixed and applied to a column of Heparin-Agarose (2 x 13.7 cm) equilibrated with 0.2% Triton X-100, 10% glycerol, 10 μM pepstatin A, 10 μM leupeptin, 0.1 mM PMSF, 1 mM 2-mercaptoethanol, and 50 mM Tris-HCl, pH 8.0 (Buffer 1) containing 0.25 M NaCl. After sequentially washing the column with 430 ml of 0.25 M NaCl / Buffer 1 and 215 ml of 0.5 M NaCl / Buffer 1, the IP4BP1 was eluted with 30 ml of Buffer 1 containing 1 M NaCl. Peak fractions of the $InsP_4$ binding activity were pooled, concentrated, and applied to a gel filtration column of Sepharose CL-6B (1.0 x 60 cm) equilibrated with Buffer 1 containing 0.2 M NaCl and 1 mM EDTA. The peak fractions of the $InsP_4$ binding activity were pooled, dialyzed against Buffer 1 containing 1 mM EDTA, and applied to an anion-exchange chromatography column of DE-52 (1.0 x 7.6 cm) equilibrated with the dialyzed buffer. The column was washed with 60 ml of the equilibration buffer, and then the IP4BP1 was eluted with a 50 ml linear NaCl gradient (0-0.3 M in Buffer 1 containing 1 mM EDTA). Peak fractions of $InsP_4$ binding activity were pooled, concentrated and applied to a column of Sepharose CL-6B under the same conditions as described above. Peak fractions of $InsP_4$-binding activity were pooled, concentrated and stored at -80 °C.

Preparation of DE-52 flow-through fraction

The supernatant (1% Triton X-100 extract) (~200 ml) was applied to a column of DE-52 (2.6 x 11 cm) equilibrated with 1% Triton X-100, 10% glycerol, 1 mM EDTA, 0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A, 1 mM 2-mercaptoethanol, and 50 mM Tris-HCl, pH 8.0 (Buffer 2). The column was washed with 70 ml of Buffer 2. DE-52 flow-through fractions were stored at -80 °C.

Purification of IP4BP2a (aldolase A)

Stored DE-52 flow-through fractions from four experiments were combined (~ 800ml) and incubated with 21 ml of packed Heparin-Agarose for 1 hr at 4 °C on a rotator. These samples were poured into a column (Ø 2.0 cm), and nonadherent proteins were collected and discard. After washing the column with 400 ml of Buffer 1 containing 0.05 M NaCl, IP4BP2a was eluted with 60 ml of 1.0 M NaCl / Buffer 1. Peak fractions were pooled and diluted with 40 volumes of the solution containing 0.2% Triton X-100, 0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A, 1 mM 2-mercaptoethanol, and 50 mM MES-NaOH, pH 6.5 (Buffer 3). They were applied to a cation-exchange chromatography column of CM-32 (2.0 x 9.5 cm) equilibrated with 0.05 M NaCl / Buffer 3. The column was washed with 150 ml of equilibration buffer and IP4BP2a was eluted with a of linear gradient of 0.05-0.5 M NaCl / Buffer 3 (300 ml total) at 0.6 ml/min. Peak fractions of the $InsP_4$-binding activity were pooled, concentrated, and applied to a gel filtration column of Sephacryl S-300 (1.0×57 cm) equilibrated with 0.2M NaCl / Buffer 3. Peak fractions were pooled and diluted with 4 volumes of Buffer 3, and applied to a cationic-exchange chromatography column of CM-Sepharose CL-6B (1.0 x 3.8 cm) equilibrated with 0.05 M NaCl / Buffer 3.

IP4BP2a was eluted with a linear gradient of 0.05-0.5 M NaCl / Buffer 3 (30 ml total) at 0.4 ml/min. Peak fractions of the $InsP_4$ binding activity were pooled, concentrated, and applied to a column of Sephacryl S-300 (1.0×57 cm) equilibrated with Buffer 3 containing 0.2 M NaCl. Peak fractions of $InsP_4$ binding activity were pooled and stored at -80 °C.

Purification of IP4BP2b (PLP)

Stored DE-52 flow-through fractions from two experiments were combined (~ 400 ml) and incubated with 5 ml of packed Heparin-Agarose for 1 hr at 4 °C on a rotator. The Heparin-Agarose was poured into a column (Ø 1.0 cm), and nonadherent proteins were collected and discarded. The column was washed with 40 ml of Buffer 4 (10 mM CHAPS, 0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A, 1 mM 2-mercaptoethanol, and 50 mM EPPS-NaOH, pH 8.0) containing 0.05 M NaCl and 40 ml of Buffer 4 containing 0.25 M NaCl, and the $InsP_4$-binding activities were eluted with a linear gradient of 0.25-1.0 M NaCl / Buffer 4 (50 ml total) at 0.5 ml/min. Peak fractions of the $InsP_4$-binding activity were pooled and diluted with 15 volumes of the solution containing 0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A, 1 mM 2-mercaptoethanol, 50 mM HEPES-NaOH, pH 7.5 (Buffer 5), and incubated with 8 ml of packed cation-exchange gel CM-52 for 1 hr at 4 °C on a rotator. They were poured into a column, and nonadherent proteins were discarded. The column was washed with 40 ml of Buffer 5 containing 10 mM CHAPS, 0.05 M NaCl and the binding activities were eluted with a linear gradient of 0.05-0.5 M NaCl / 10 mM CHAPS / Buffer 5 (50 ml total) at 0.5 ml/min. Peak fractions of the $InsP_4$ binding activity were pooled, concentrated, and applied to a column of Sephacryl S-300 (1.0× 57 cm) equilibrated with 10 mM CHAPS / 0.5 M NaCl / Buffer 5. Peak fractions of

$InsP_4$ binding activity were pooled, concentrated and stored at -80 °C.

SDS-PAGE and Immunoblot analysis

SDS-PAGE for protein profile analysis or immunoblotting were carried out by the method of Laemmli (1970). The proteins were visualized with Coomassie Brilliant Blue R-250 (CBB R-250) or by silver staining with Silver Stain *II* kit (Wako).

After electrophoresis, the proteins were transferred to a nitrocellulose or PVDF membranes with semidry system (ATTO) or tank transfer system (Bio-Rad). The membrane was soaked in 5% skim milk in PBS containing 0.1% Tween-20 (T-PBS) for 1 hr at room temperature to block non-specific binding and then incubated in the antisera or the culture supernatant of hybridoma producing monoclonal antibody for 60 min at room temperature. After washing with T-PBS, the membrane was incubated with biotinylated goat anti-rabbit IgG (Vectastain) or biotinylated goat anti-rat IgG (Jackson) (1:200) in 5% skim milk / T-PBS. Immunoreactive bands were visualized using Vectastain ABC kit according to the manufacturer's protocol. Finally, the membrane was treated with 0.1% diaminobenzidine (DAB) / 0.02% hydrogen peroxide / PBS.

Determination of the partial amino acid sequences of IP4BP1

The purified IP4BP1 (approximately 0.3 mg) was separated by SDS-PAGE on 3.75-12.5% linear gradient gel according to the method of Laemmli (1970). Generation and purification of the peptides from IP4BP1 was carried out by the method of Iwamatsu (1991). After separation, the proteins were transferred to a PVDF membrane (0.2 μm) and visualized with Ponceau S. Two protein bands with molecular weight of 140K and 65K were cut from the blots and destained with 0.5

mM NaOH. They were cut into 2 mm squares, placed in separate sample-tubes, and reduced by dithiothreitol (DTT) followed by alkylation using monoiodoacetic acid. After washing with 2% acetonitrile, both membrane pieces were treated with lysyl endopeptidase for 24 hr at 37 °C at molar concentrations one fiftieth those of the IP4BP1 in 300 μl of 100 mM Tris-HCl, pH 8.5, containing 7.5% acetonitrile and 0.05% SDS. The membrane pieces were removed by centrifugation at 10,000 x g for 5 min. The supernatants were lyophilized, dissolved in 0.1% trifluoroacetic acid (TFA) and applied to an HPLC (Waters 600E) with a column (0.39 x 15 cm) of C8 (μBONDASPHERE, 300A, Waters) that had been equilibrated with distilled water containing 0.1% TFA. A gradient of 0-30% 2-propanol / acetonitrile (7:3) containing 0.1% TFA was run over 45 ml (0.5 ml/min). The elution profile was monitored by absorption at 214 nm and collected manually. The major peaks from 140K molecule were concentrated to a volume of less than 100 μl and applied to a gas-phase protein sequencer (Applied Biosystem). The sequences were compared to those in the SWISS PROT database.

N-terminal sequence analysis of IP4BP2a and IP4BP2b

The purified IP4BP2a (approximately 8 μg) and IP4BP2b (approximately 14.4 μg) were separated by SDS-PAGE on 12% gel. After separation, the proteins were transferred to a PVDF membrane (0.2 μm) with CAPS transfer buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) -NaOH, pH 11.0, 10% methanol]. These proteins were visualized with CBB R-250 and protein bands were cut from the blots. The membrane pieces were applied to a gas-phase protein sequencer (Applied-Biosystem). The sequences were compared to those in the SWISS PROT database.

Production of antisera against synthetic C-terminal peptide and the C2A region of rat synaptotagmin *II*

A peptide corresponding to the C-terminal region of rat synaptotagmin *II* with an additional amino acid, cysteine, on the amino-terminal side (amino acid residues, 409-422) was custom-synthesized (Applied Biosystems). The peptide was conjugated to cationized bovine serum albumin coupled to a heterobifunctional crosslinker containing a maleimide group (sulfo-SMCC-cBSA), which reacts with free sulfohydryl groups. Briefly, 5 mg of C-terminal peptide and 5 mg of sulfo-SMCC-cBSA were dissolved in 1 ml of 0.1 M phosphate buffer, pH 7.2, containing 0.1 M EDTA and 0.15 M NaCl, and the mixture was incubated for 3 hr at room temperature with constant stirring. The remaining unreacted peptide was separated by gel filtration on a Sephadex G-25 column equilibrated with 0.083 M phosphate buffer, pH 7.2, containing 0.15 M NaCl. The void fractions containing peptide-cBSA conjugates were collected, homogenized with an equal volume of aluminum hydroxide at a concentration of 1 mg/ml protein, and then subcutaneously injected into the back of New Zealand White rabbits. Two weeks later, the rabbits were boosted with homogenate containing the same amount of adjuvant and the peptide-cBSA conjugates. Booster injections were performed every two weeks until the titer of the antiserum was saturated.

Antisera against C2A region were kindly prepared by Niinobe and Fukuda. The procedure is described below. A fusion protein of the C2A region (amino acid residues, 139-267) of rat synaptotagmin *II* with glutathione S-transferase was expressed in *Escherichia coli* and purified as reported previously (Smith *et al.*, 1988). Briefly, cDNA fragments encoding the C2A domain of rat synaptotagmin *II* were

amplified by the polymerase chain reaction as described by McPherson *et al.* (1991). The amplified fragment was sub-cloned into pGEX-2T vector, and the structure of the resultant plasmid was verified by sequencing. The fusion protein was expressed in *Escherichia coli*, JM109. The membranes were disrupted by sonication for 5 min at 0 °C and the protein was extracted by treatment with Triton X-100 at a final concentration of 1%. After centrifugation at 10,000 x g for 15 min it was purified by affinity chromatography using glutathione-Sepharose 4B. The purity of the preparation was judged by SDS-PAGE on 10% gel. The purified fusion protein was concentrated, dialyzed against phosphate-buffered saline, and then used to immunize New Zealand White rabbits by the same procedures as described above, except for the use of Freund's complete adjuvant.

Immunoprecipitation of IP4BP1 (synaptotagmin *II*)

Ten microliters of the anti-C2A polyclonal antibody solutions (concentrations of antibody used were 2, 5, and 10 μg as IgG protein per 10 μl of 50 mM HEPES-NaOH, pH 7.4, containing 0.1 M NaCl) purified using Protein A column kit from Amersham were added to 10 μl of the purified IP4BP1 (0.35 μg) dialyzed with 50 mM HEPES-NaOH, pH 7.4, containing 0.1 M NaCl. The mixtures were then incubated for 30 min at room temperature. Thirty microliters of protein A-Sepharose CL-4B suspension (0.3 mg of Protein A Sepharose powder) in the same buffer was added, and the sample tubes were gently rotated for 30 min at 4 °C. The protein A-Sepharose CL-4B particles were precipitated by centrifugation at 10,000 x g for 10 min, and the residual [^{3}H]InsP_4 binding activity of the supernatant was estimated. A control experiment was also performed by the same method using the IgG fraction from the serum before immunization.

Monoclonal antibodies against PLP

The hybridoma of anti-PLP monoclonal antibodies (AA3 and AH7-2a) were kindly supplied from Dr. Marjorie B. Lees (Yamamura *et al.*, 1991). The hybridomas were cultured in HYBRIDOMA-SFM (GIBCO) medium. The culture supernatant was used for immunoblot analysis or IgG-purification.

Purification of AA3-IgG

Ammonium sulfate (313 g/l of the culture supernatant) was gradually added to the culture supernatant of AA3-hybridoma with stirring on ice. Fifteen minutes after the addition, mixing was stopped and the mixture was kept still on ice for 1 hr. Then the mixture was centrifuged at 13,800 x g for 10 min. The pellet was resuspended in 20 ml of phosphate-buffered saline (PBS). Half volume of saturated ammonium sulfate solution (pH 7.2 adjusted by KOH) was added dropwise to the suspension with constant mixing on ice. After standing on ice, the mixture was centrifuged as above. The pellet was resuspend in a least volume of PBS and the suspension was dialyzed in 20 mM HEPES-NaOH, pH 7.2 containing 0.15 M NaCl. The IgG solution was stored at -80 °C.

Immunoabsorption against IP4BP2b (PLP) using Protein G-Sepharose

The AA3-IgG solution or normal Rat IgG solution were diluted with PBS to give a final protein concentration of 2 mg/ml. To each 500 µl of the above IgG solution (1 mg protein), equal volume of the binding-solution: 1 M acetate buffer, pH 4.6 containing 3 M NaCl was added, and incubated with 200 µl of packed Protein G-Sepharose equilibrated with the binding-solution, overnight at 4 °C on a rotator.

Each of these solutions was poured into a column (Ø 5 mm), and washed with 2 ml of the washing-solution containing 0.15 M NaCl / 20 mM HEPES-NaOH, pH 7.5. The washed gel particles were transferred into sample-tubes and incubated with 20 µl of the purified IP4BP2b (PLP) (13.6 µg) for 1 hr at 4 °C on rotator. After incubation , these were re-poured into columns (Ø 5 mm) and washed four times with 100 µl of the washing solution . Nonadherent proteins were collected at approximately 100 µl/fraction. A control experiment was also performed by the same method without IgG. Each of these five fractions were used for [^{3}H]InsP_4-binding assay or SDS-PAGE analysis.

Cross-linking of IP4BP2a (aldolase A) with DMS

The purified IP4BP2a was dialyzed against 0.2% Triton X-100, 1 mM 2-mercaptoethanol, 50 mM MES-KOH, pH 6.5. Thirty microliters of the dialyzed sample were mixed with 1 % Triton X-100 and 1 M carbonate buffer, pH 9.9 to make a final concentration of 0.2% Triton X-100 and 50 mM carbonate. Aliquots (200 µl) of the solution were treated with various concentrations (0, 40, 80, 200 µg) of dimethyl suberimidate (DMS) at the protein concentration of 0.3 mg/ml. The reactions were allowed to proceed for 4 hr on ice and then were quenched by the addition of 25 µl of 1 M glycine. The samples were concentrated by the ethanol precipitation method. The concentrated samples were analyzed by SDS-PAGE (Weber and Osborn, 1969).

Purification of Myelin

Myelin was purified from the medulla oblongata and the spinal cord of adult male ddY mouse by a modification of the procedure of Lucas *et al.* (1994). The

medulla oblongatas and the spinal cords (1 g) were mixed with 20 volumes of 10 μM pepstatin A, 10 μM leupeptin, 0.1 mM PMSF, 1 mM 2-mercaptoethanol, and 5 mM Tris-HCl, pH 7.5 (Buffer 7) containing 0.85M sucrose and homogenized (Glass-Tefron Potter homogenizer) with 10 strokes at 850 rpm. The homogenate was overlaid with 16.5 ml of 0.32 M sucrose / Buffer 7 and centrifuged at 28,000 rpm for 60 min in an SW-28 rotor (Beckman). Three fractions were separated as the crude interface, the crude precipitate, and the crude middle-layer between them. Each of them was homogenized in 0.85 M / Buffer 7 (final total volume: 19 ml) overlaid with 16.5 ml of 0.32 M sucrose / Buffer 7 and centrifuged as above.

The crude interface was resuspended in 20 ml ddH_2O and centrifuged at 30,000 rpm in a Type 50.2 Ti rotor (Beckman). The pellet was resuspended in 20 ml of 10 mM O,O'-bis(2-aminoethyl)ethyleneglycol-N,N,N'-N'-tetraacetic acid (EGTA) (pH 7.5) and homogenized. The suspension was kept at 2 °C and centrifuged at 10,000 rpm for 15 min in a JA-17 fixed angle rotor (Beckman). The pellet was washed again under the same conditions. The pellet was homogenized in 17 ml of 0.85 M sucrose / 2 mM EGTA (pH 7.5), overlaid with 18 ml of 0.32 M sucrose / Buffer 7 and centrifuged at 28,000 rpm for 60 min in an SW-28 rotor. The interface was collected by centrifugation, and washed repeatedly. The pellet was resuspended in 20 ml of 10 mM EGTA (pH 7.5) and homogenized. The suspension was kept at 2 °C and centrifuged at 10,000 rpm for 15 min in a JA-17 fixed angle rotor. The pellet (myelin fraction) was homogenized in 5 ml of 1 mM EDTA, 1 mM EGTA, 10 μM pepstatin A, 10 μM leupeptin, 0.1 mM PMSF, 1 mM 2-mercaptoethanol, and 20 mM HEPES-KOH, pH 7.2 (Buffer 8), snap frozen in liquid N_2 and stored at -80 °C.

The crude middle-layer was diluted with 3 volumes of ddH_2O and centrifuged at 30,000 rpm in a Type 50.2 Ti rotor. The pellet was kept in 5 ml of 10 mM EGTA

(pH 7.5) and homogenized. The suspension was kept at 2 °C and centrifuged at 10,000 rpm for 15 min in a JA-17 fixed angle rotor. The pellet was washed again under the same conditions. The pellet (middle-layer fraction) was homogenized in 2 ml of Buffer 8, snap frozen in liquid N_2 and stored at -80 °C.

The crude precipitate was resuspended in 9 ml of 0.32 M sucrose/Buffer 7, and centrifuged at 2,500 rpm for 10 min in a JA-17 fixed angle rotor. The pellet was washed again under the same condition. The combined supernatant was diluted with 3 volumes of ddH_2O and centrifuged at 30,000 rpm in a Type 50.2 Ti rotor. The pellet was suspended in 5 ml of 10 mM EGTA (pH 7.5) and homogenized. The suspension was kept at 2 °C and centrifuged at 10,000 rpm for 15 min in a JA-17 fixed angle rotor. The pellet was washed again under the same condition. The pellet (P2/P3 membrane-myelin fraction) was homogenized in 5 ml of Buffer 8, snap frozen in liquid N_2 and stored at -80 °C.

Results

Three [^{3}H] $InsP_4$- binding proteins (IP4BP) were purified from mouse cerebella. One of IP4BP from DE-52 absorbed fraction was called IP4BP1 and the others from DE-52 flow-through fraction were called IP4BP2a and IP4BP2b. The sequential purification procedure is shown as a flow chart (Fig. 1).

$InsP_4$-binding activity was efficiently solubilized by treatment with 1% Triton X-100 (Table 1). It seemed that many substances which affect $InsP_4$ binding activity, such as the $InsP_3$ receptor, inositol polyphosphate phosphatases and kinases, were extracted by the treatment with detergent. However, most of kinases which are in cytosol, may not be present in P2/P3 membrane fraction and the other proteins may have been eliminated by chromatographic procedure : during IP4BP1-purification procedure, phosphatases were in the flow through and 0.5 M NaCl fractions, and IP3R was in the 0.5 M NaCl fraction on Heparin-Agarose chromatography; during IP4BP2-purification procedure, phosphatases and IP3R were in DE-52 absorbed fraction.

Purification of IP4BP1

During the purification procedure of IP4BP1, Heparin-Agarose was most efficient to concentrate the $InsP_4$-binding activity (Table 1). Low levels of $InsP_4$-binding activity were also detected in the void fraction during gel filtration chromatography on Sepharose CL-6B (Fig. 2, a). Although most of the contaminants were eliminated by ion-exchange chromatography on DE-52, small amounts of low molecular weight substances still remained (Fig. 2, b), which were removed by the second gel filtration chromatography (Fig. 2, c).

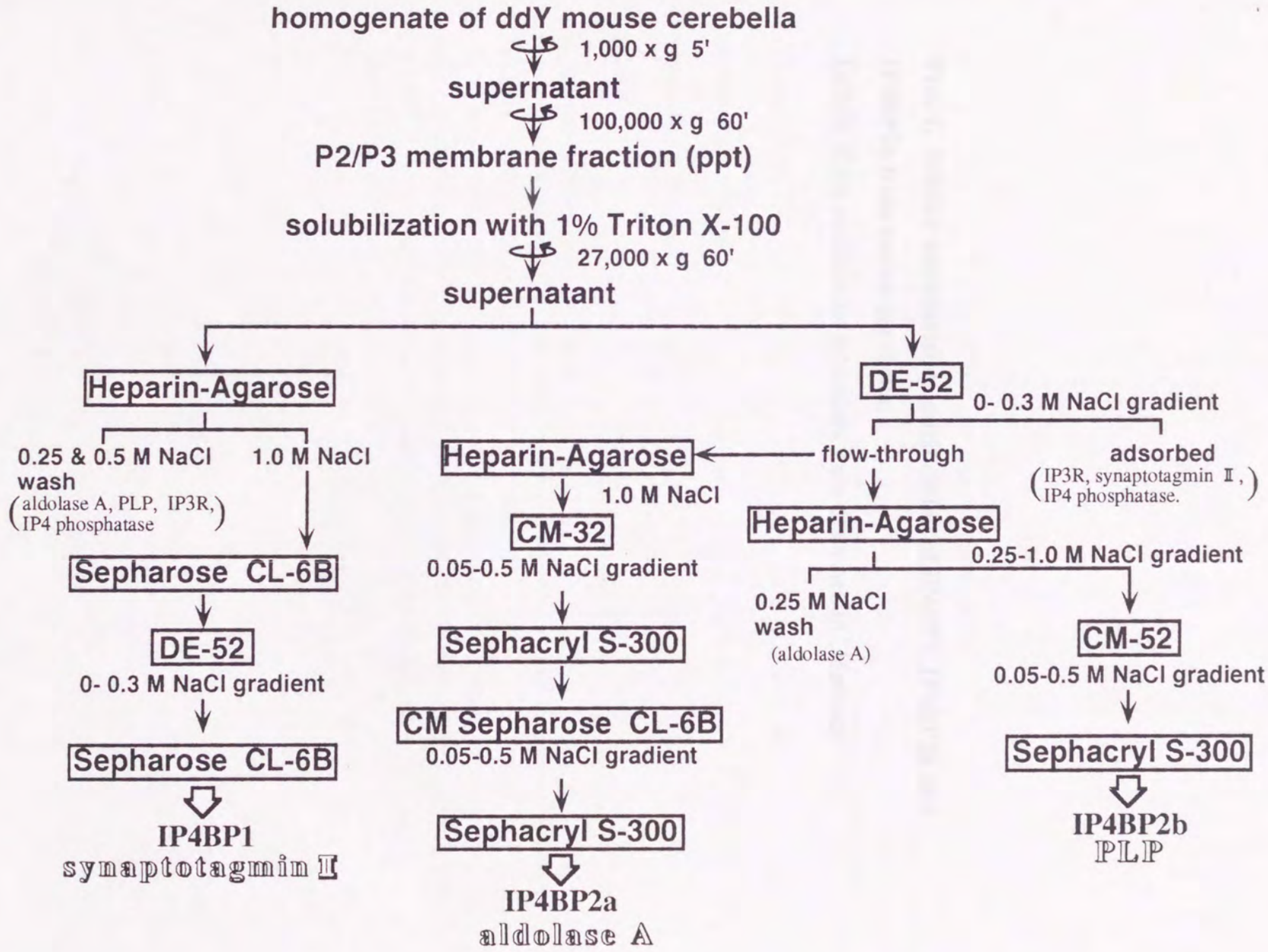

homogenate of ddY mouse cerebella
1,000 x g 5'
supernatant
100,000 x g 60'
P2/P3 membrane fraction (ppt)
solubilization with 1% Triton X-100
27,000 x g 60'
supernatant
Heparin-Agarose
0.25 & 0.5 M NaCl wash
(aldolase A, PLP, IP3R, IP4 phosphatase)
1.0 M NaCl
Sepharose CL-6B
DE-52
0- 0.3 M NaCl gradient
Sepharose CL-6B
IP4BP1
synaptotagmin II
DE-52
0- 0.3 M NaCl gradient
flow-through
adsorbed
(IP3R, synaptotagmin II, IP4 phosphatase.)
Heparin-Agarose
1.0 M NaCl
CM-32
0.05-0.5 M NaCl gradient
Sephacryl S-300
CM Sepharose CL-6B
0.05-0.5 M NaCl gradient
Sephacryl S-300
IP4BP2a
aldolase A
Heparin-Agarose
0.25 M NaCl wash
(aldolase A)
0.25-1.0 M NaCl gradient
CM-52
0.05-0.5 M NaCl gradient
Sephacryl S-300
IP4BP2b
PLP

FIG. 1

FIG. 1. Scheme summarizing purification of IP4BP1, IP4BP2a and IP4BP2b from mouse cerebella.

Details of the purification procedure were described in "Methods."

TABLE 1
Purification summary of IP4BP1

Fraction	Protein[a] (mg)	$InsP_4$-Binding[b] (μmol)	Specific Act. (μmol/mg)	Yield (%)	Purification (fold)
P2/P3 membrane	3740	12	0.00[c]	100	1
Triton extract	2797	28	0.01	85	3.3
Heparin-Agarose pool	14	4.8	0.34	15	113
Sepharose CL-6B pool	4.2	2.7	0.64	8.2	213
DE-52 pool	1.3	1.4	1.08	4.2	360
Sepharose CL-6B pool	0.45	1.1	2.44	3.3	813

The result presented is for a typical preparation.

[a]Protein was determined by using the Bio-Rad protein assay reagent.

[b]$InsP_4$-binding activities were measured by routine assay with 4.8 nM of [^{3}H]$InsP_4$ at pH 7.4. Nonspecific binding was determined without samples.

[c]Specific activity was estimated to be 0.003.

FIG. 2

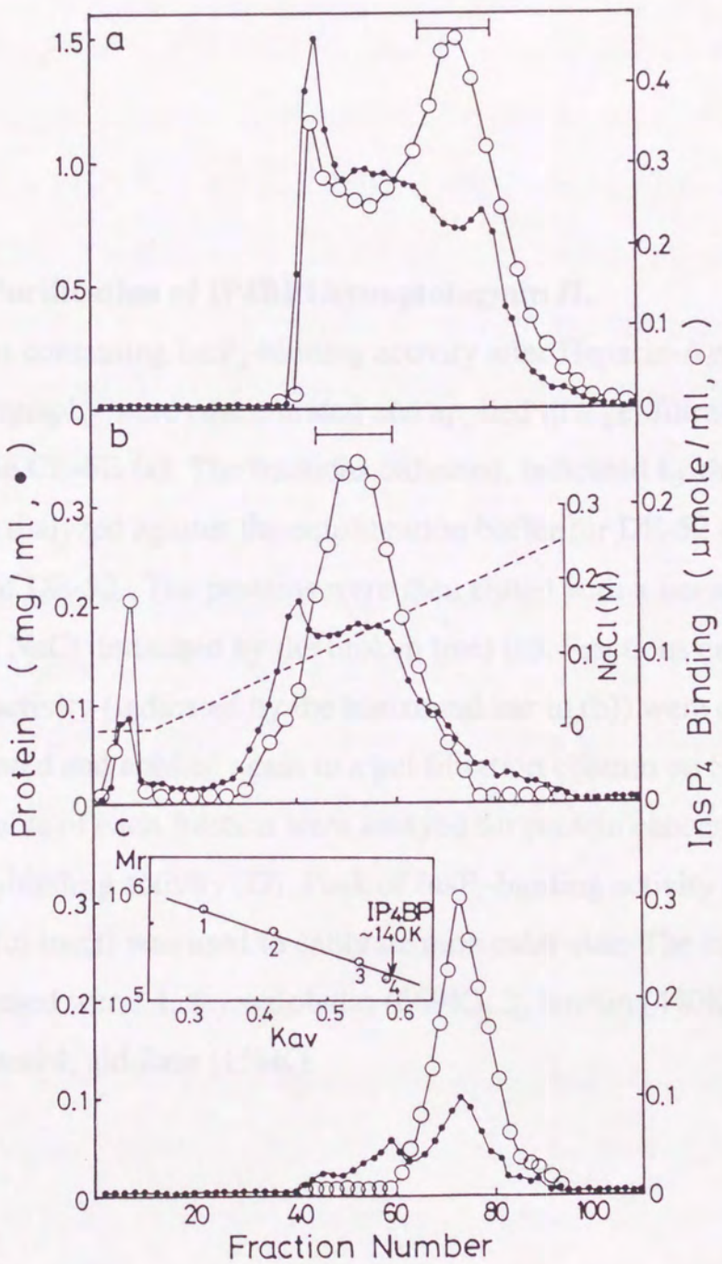

a
b
c
Protein (mg / ml , •)
InsP4 Binding (μmole / ml , ○)
NaCl (M)
Mr
IP4BP
~140K
Kav
Fraction Number

FIG. 2. Purification of IP4BP1/synaptotagmin *II*.

Fractions containing $InsP_4$-binding activity after Heparin-Agarose chromatography were concentrated and applied to a gel filtration column on Sepharose CL-6B (a). The fractions collected, indicated by the horizontal bar in (a), were dialyzed against the equilibration buffer for DE-52 and applied to a column of DE-52. The proteins were then eluted with a linear NaCl gradient (0-0.3 M NaCl indicated by the broken line) (b). Fractions containing $InsP_4$-binding activity (indicated by the horizontal bar in (b)) were collected, concentrated and applied again to a gel filtration column on Sepharose CL-6B (c). Aliquots of each fraction were assayed for protein concentration (•) and for $InsP_4$-binding activity (O). Peak of $InsP_4$-binding activity on Sepharose CL-6B [(c) inset] was used to calibrate molecular size. The molecular weight markers used were: 1, thyroglobulin (669K); 2, ferritin (440K); 3, catalase (232K); and 4, aldolase (158K).

The protein profile of each purification step was characterized by SDS-PAGE (Fig. 3, A). Most of the contaminating proteins after Heparin-Agarose fraction were removed efficiently by the first Sepharose CL-6B gel filtration chromatography (Fig. 3, A, lane c and d). After the final step on Sepharose CL-6B, two protein bands with molecular weights of 140K and 65K were detected (Fig. 3, A, lane f, and Fig. 3, C). $InsP_4$-binding activity perfectly coincided with the intensities of CBB R-250 staining of each fraction (Fig. 2, c, and Fig. 3, B). In the second gel filtration, the molecular size of the IP4BP1 was also estimated to be about 140K in the presence of detergent (Fig. 2, c), indicating that the 140K molecule may be a dimer of the 65K molecule. Table 1 summarizes the purification of IP4BP1. Approximately 0.45 mg of the binding protein can be obtained from 150 g of mouse cerebellum.

Determination of the partial amino acid sequences of the purified IP4BP1

The purified IP4BP1 from Sepharose CL-6B gel filtration chromatography was applied to SDS-PAGE on 3.75-12.5% linear gradient gel. The proteins were transferred to a PVDF membrane and visualized by Ponceau S staining. The purified IP4BP1-140K and 65K bands cut from the membrane were hydrolyzed with lysyl endopeptidase in the presence of 0.05% SDS. The resultant peptides were separated by reverse phase column chromatography (Fig. 4, A). Elution profile of the 140K bands (Fig. 4, A, a) was quite similar to that of 65K band (Fig. 4, A, b). Therefore, I determined the amino acid sequence of seven peptides derived from the 140K molecule (Fig. 4, B). The amino acid sequences of each peptide were checked against the SWISS PROT database, revealing 100% identity with that of rat synaptotagmin *II*. Synaptotagmin is an integral membrane protein of synaptic vesicles considered to play a significant role in the docking and fusion of synaptic vesicles at the presynaptic

FIG. 3

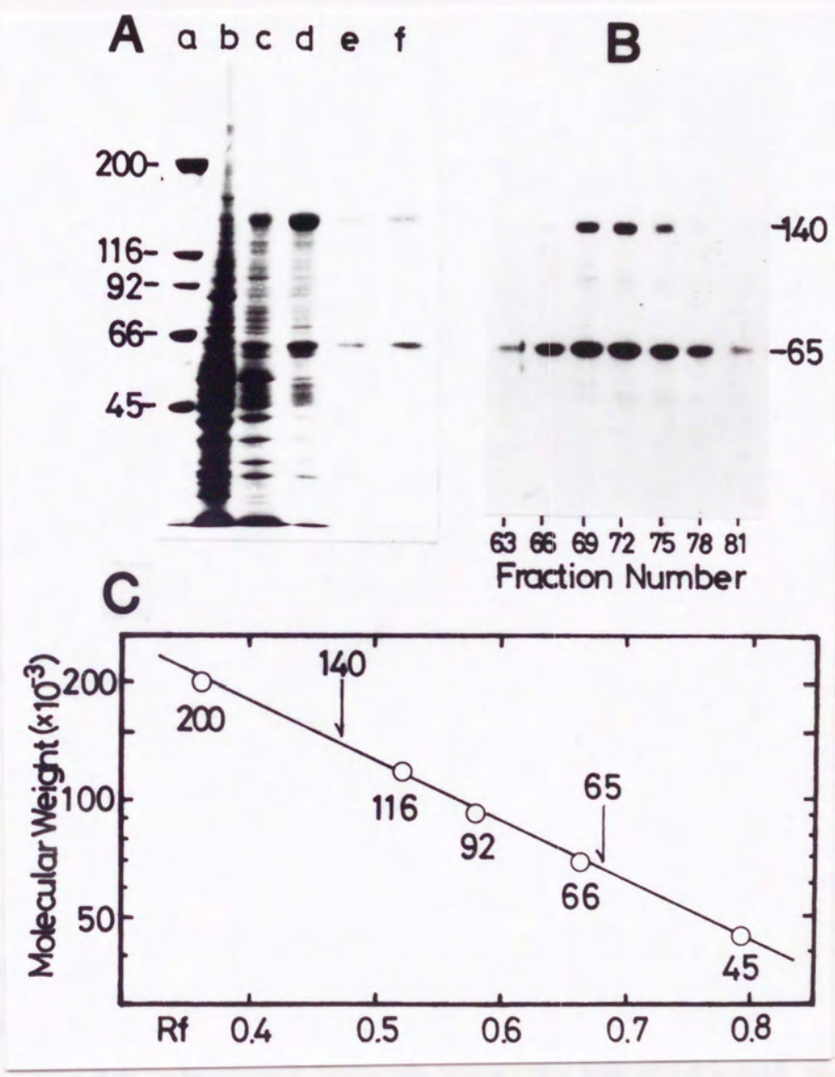

A
a b c d e f
200-
116-
92-
66-
45-
B
140
65
63 66 69 72 75 78 81
Fraction Number
C
Molecular Weight (x10⁻³)
200
100
50
140
65
200
116
92
66
45
Rf 0.4 0.5 0.6 0.7 0.8

FIG. 3. SDS-PAGE analysis of each purification step of IP4BP1 (A) and the fractions after the second gel filtration column chromatography of IP4BP1 on Sepharose CL-6B (B).

Electrophoresis was carried out according to Laemmli (1970) on a 3.75-12.5% linear gradient gel. The gel was visualized by CBB R-250 staining. (A) : molecular weight marker (*lane* a), proteins of 80 μg from solubilized supernatant (Triton X-100 extract) (*lane* b), 30 μg from Heparin-Agarose (*lane* c), 10 μg from 1st Sepharose CL-6B (*lane* d), 2 μg from DE-52 (*lane* e) and the second Sepharose CL-6B (*lane* f). (B) : protein profile of fractions eluted from the second Sepharose CL-6B gel filtration column (10 μl each). (C) : molecular weight calibration of the purified IP4BP1/synaptotagmin. Molecular weight standards (A, *lane* a) (200K, myosin; 116K, β-galactosidase; 97.4K, phosphorylase b; 66K, bovine serum albumin; 45K, ovalbumin) with corresponding Mr values ($x10^{-3}$) are shown to the left of (A), and in (C).

FIG. 4

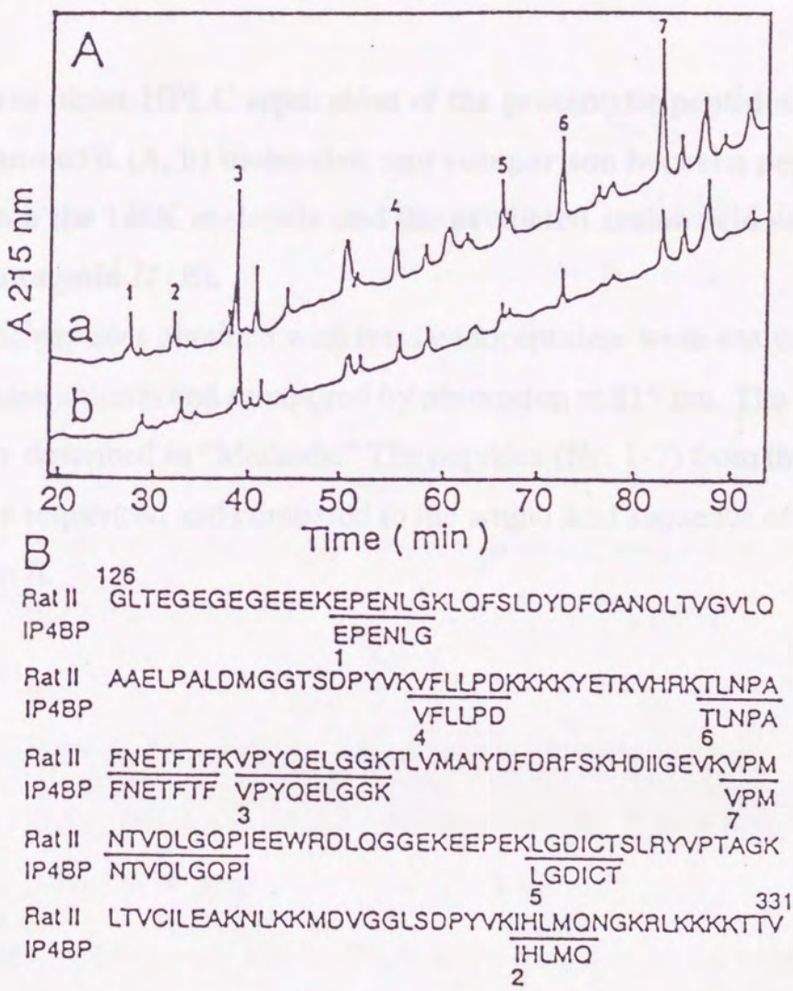

A
A 215 nm
a
b
1
2
3
4
5
6
7
20
30
40
50
60
70
80
90
Time (min)
B
126
Rat II GLTEGEGEGEEEKEPENLGKLQFSLDYDFQANQLTVGVLQ
IP48P EPENLG
1
Rat II AAELPALDMGGTSDPYVKVFLLPDKKKKYETKVHRKTLNPA
IP48P VFLLPD TLNPA
4 6
Rat II FNETFTFKVPYQELGGKTLVMAIYDFDRFSKHDIIGEVKVPM
IP48P FNETFTF VPYQELGGK VPM
3 7
Rat II NTVDLGQPIEEWRDLQGGEKEEPEKLGDICTSLRYVPTAGK
IP48P NTVDLGQPI LGDICT
5
Rat II LTVCILEAKNLKKMDVGGLSDPYVKIHLMQNGKRLKKKKTTV 331
IP48P IHLMQ
2

FIG. 4. Reverse phase HPLC separation of the proteolytic peptides of the 140K (A, a) and 65K (A, b) molecules, and comparison between peptide sequences from the 140K molecule and the predicted amino acid sequence of rat synaptotagmin *II* (B).

The proteolytic peptides obtained with lysyl endopeptidase were analyzed on a C8 reverse phase column and monitored by absorption at 215 nm. The detailed procedures are described in "Methods." The peptides (No. 1-7) from the 140K molecule were sequenced and compared to the amino acid sequence of rat synaptotagmin *II*.

release sites (Südhof *et al.*, 1991; Greengard *et al.*, 1993). Native rat brain synaptotagmin has been reported to form dimers that are organized into high molecular weight complexes with other presynaptic membrane proteins (Perin *et al.*, 1991, a). As shown in Figure 4, A, reverse phase column chromatography after limited proteolysis by lysyl endopeptidase yielded almost the same elution profile on the 140k and 65K bands, strongly suggesting that the 140K molecule is a dimer of the 65K monomer.

Immunological cross-reactivity of IP4BP1 and synaptotagmin *II*

We prepared two antisera against synaptotagmin *II*, one is against C-terminal and another is against C2A domain. The former was produced by immunization of a rabbit against conjugates of synthetic C-terminal peptide (amino acid residues, 409-422) of rat synaptotagmin *II* with cationized BSA. The latter was produced by immunization of a rabbit against a fusion protein of the C2A region (amino acid residue, 136-267) of rat synaptotagmin *II* with glutathione S-transferase, which was expressed in *Escherichia coli.* The C2A domain is one of two regions of synaptotagmin *II*, homologous to the C2 domain in the regulatory region of protein kinase C (PKC) (Geppert *et al.*, 1991).

Sample of the supernatant (Triton X-100 extract) (Fig. 5, a) and purified IP4BP1 from the second Sepharose CL-6B gel filtration chromatography (Fig. 5, b) were applied to SDS-PAGE on 3.75-12.5% linear gradient gel, and the proteins were transferred to PVDF membranes. The membranes were either stained with amido black (Fig. 5, A) or analyzed immunochemically using antisera against synthetic C-terminal peptide (Fig. 5, B) or the C2A region (Fig. 5, C). This immunoblot analysis revealed that polyclonal antibodies against synaptotagmin *II* cross-react with

FIG. 5

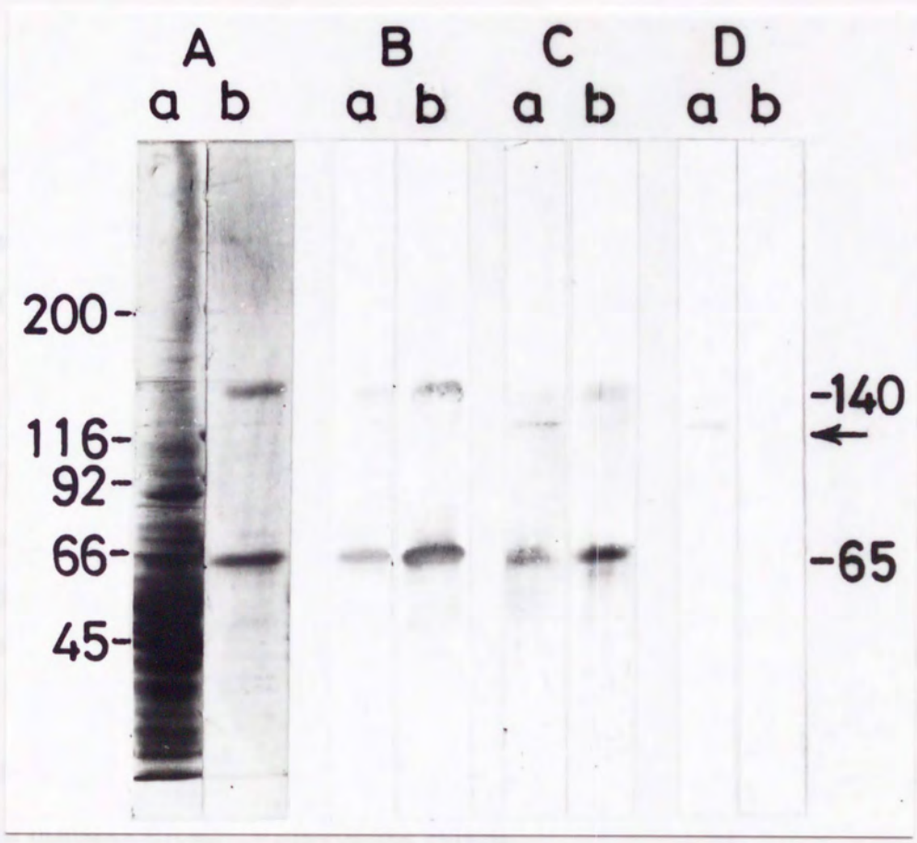


FIG. 5. Immunoblot analysis of IP4BP1/synaptotagmin *II* in crude extract and purified preparation.

The solubilized supernatant (Triton X-100 extract; 50 μg) (*lane* a) and 3 μg of sample after the second Sepharose CL-6B (*lane* b) were applied to SDS-PAGE on a Laemmli gel with a 3.75-12.5% linear gradient. Proteins were transferred to PVDF membranes, and the blots were then stained with amido black (A) and probed with polyclonal anti-sera against the C2A domain (B), C-terminal peptide (C) of rat synaptotagmin *II*, and preimmune serum serving as a control (D). Peroxidase-coupled detection was performed by the DAB staining method using a Vectastain ABC kit. Molecular weight markers (Mr x 10^{-3}) are shown to the left as described in the Fig. 3 legend. Arrow indicates position of the band stained nonspecifically with control serum.

each of the 140K and 65K molecules. Furthermore, the $InsP_4$ binding activity of the purified sample was immunoprecipitated in a dose-dependent manner by anti-C2A antibody (Fig. 6). These results together with the partial amino acid sequences strongly indicate that the purified IP4BP1 is the mouse synaptotagmin *II*.

Characterization of the $InsP_4$-binding activity of the purified IP4BP1 (synaptotagmin *II*)

The dependence of $InsP_4$-binding on pH is demonstrated in Fig. 7 . In the presence of unlabeled $InsP_4$ at a concentration of 4.8 μM the maximum binding of [^{3}H]$InsP_4$ was observed around pH 5.0. However, the nonspecific binding component defined in the presence of unlabeled $InsP_4$ was significantly higher at this pH or lower, i.e., to 90% and 60% of total binding at pH 4.5 and pH 5.0, respectively, whereas it was less than 10% at pH 7.0 or higher. Specific [^{3}H]$InsP_4$ binding between pH 6.25 (50 mM HEPES-NaOH) and pH 8.25 (50 mM EPPS-NaOH) did not change much, which was about 30% of specific [^{3}H]$InsP_4$-binding at pH 5.0. Displacement of specifically bound [^{3}H]$InsP_4$ from the purified samples by unlabeled $InsP_4$ at pH 5.0, 6.2 and 7.4 is shown in Fig. 8. Half-maximal reduction of binding occurred at about 30 nM, 40 nM and 170 nM at pH 6.2, 7.4 and 5.0, respectively. Each Scatchard analysis indicates a single binding site with a K_D of about 30 nM, 30 nM and 160 nM, and a B_{max} of 2.7 pmol, 2.4 pmol and 35 pmol/μg protein at pH 6.2, 7.4 and 5.0, respectively. The Hill coefficients at each pH were not significantly different from 1.0. These findings indicate that acidic conditions at pH 5.0 are unsuitable for Scatchard analysis of [^{3}H]$InsP_4$-binding activity because of the high level of nonspecific binding and relatively lower affinity. The specificity of the Ins 1,3,4,5-P_4 binding site was characterized by adding several inositol polyphosphates (Fig.9). Ins

FIG. 6

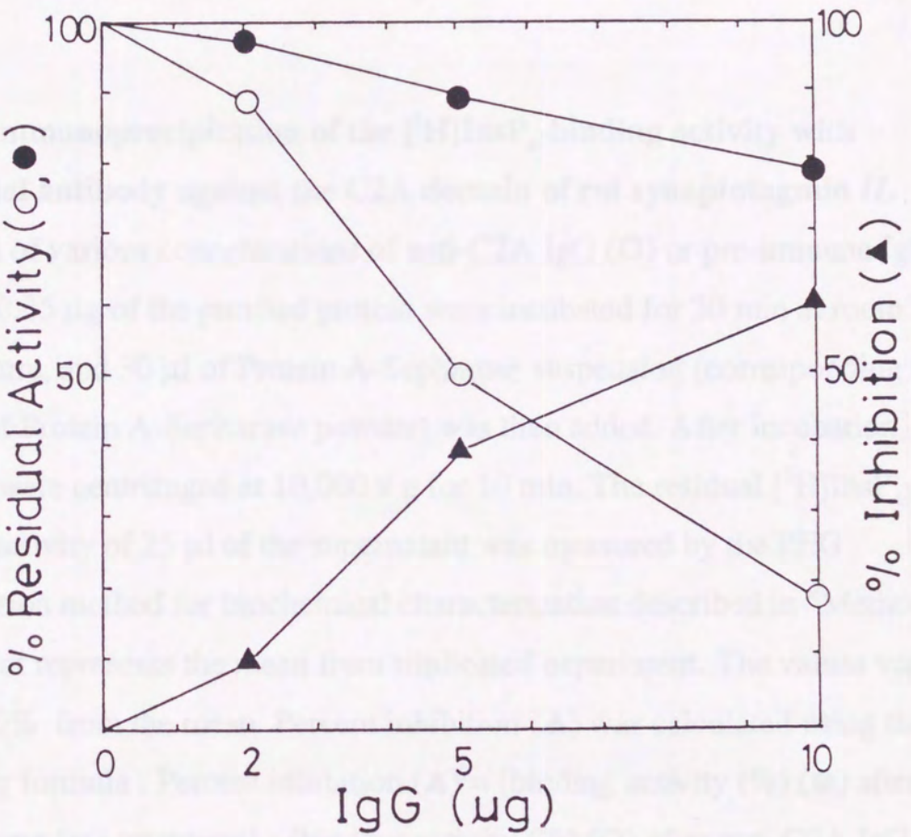


FIG. 6. Immunoprecipitation of the [^{3}H]InsP$_4$-binding activity with polyclonal antibody against the C2A domain of rat synaptotagmin *II*. Mixtures of various concentrations of anti-C2A IgG (O) or pre-immune IgG (●) and 0.35 μg of the purified protein were incubated for 30 min at room temperature, and 30 μl of Protein A-Sepharose suspension (corresponding to 0.3 mg of Protein A-Sepharose powder) was then added. After incubation, the samples were centrifuged at 10,000 x g for 10 min. The residual [^{3}H]InsP$_4$-binding activity of 25 μl of the supernatant was measured by the PEG precipitation method for biochemical characterization described in "Methods." Each point represents the mean from triplicated experiment. The values varied within 10% from the mean. Percent inhibition (▲) was calculated using the following formula : Percent inhibition (▲) = [binding activity (%) (●) after pre-immune IgG treatment] - [binding activity (%) (O) after anti-C2A IgG treatment].

FIG. 7

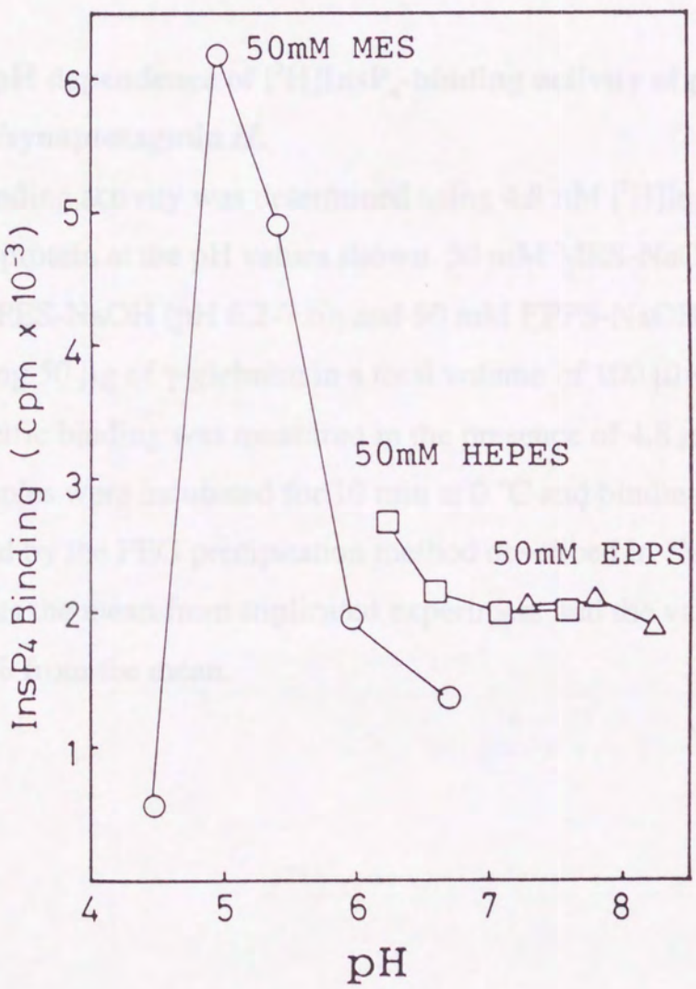

50mM MES
50mM HEPES
50mM EPPS
InsP4 Binding (cpm x 10-3)
1
2
3
4
5
6
4
5
6
7
8
pH

FIG. 7. pH dependence of [^{3}H]$InsP_4$-binding activity of purified IP4BP1/synaptotagmin *II*.

$InsP_4$-binding activity was determined using 4.8 nM [^{3}H]$InsP_4$ and 0.35 μg of purified protein at the pH values shown. 50 mM MES-NaOH (pH 4.5-6.7), 50 mM HEPES-NaOH (pH 6.2-7.6), and 50 mM EPPS-NaOH (pH 7.3-8.3) buffers containing 50 μg of γ-globulin in a total volume of 100 μl were used. Nonspecific binding was measured in the presence of 4.8 μM unlabeled $InsP_4$. The samples were incubated for 10 min at 0 °C and binding activity was measured by the PEG precipitation method described in "Methods." Each point represents the mean from triplicated experiment and the values differed less than 10% from the mean.

FIG. 8

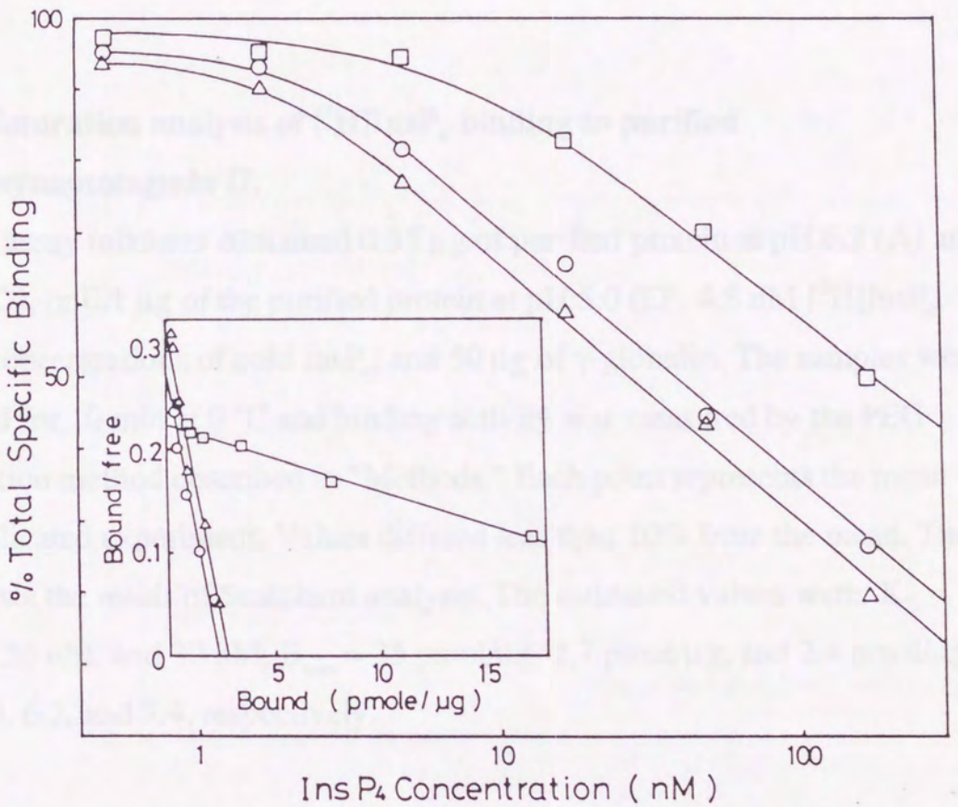

100
50
% Total Specific Binding
0.3
0.2
0.1
0
Bound/free
5
10
15
Bound (pmole/μg)
1
10
100
Ins P4 Concentration (nM)

FIG. 8. Saturation analysis of [^{3}H]InsP$_4$-binding to purified IP4BP1/synaptotagmin *II*.

Binding assay mixtures contained 0.35 μg of purified protein at pH 6.2 (△) and pH 7.4 (○), or 0.1 μg of the purified protein at pH 5.0 (□), 4.8 nM [^{3}H]InsP$_4$, various concentrations of cold InsP$_4$, and 50 μg of γ-globulin. The samples were incubated for 10 min at 0 °C and binding activity was measured by the PEG precipitation method described in "Methods." Each point represents the mean from triplicated experiment. Values differed less than 10% from the mean. The inset shows the result of Scatchard analysis. The estimated values were: K_D = 160 nM, 30 nM, and 30 nM; B_{max} = 35 pmol/μg, 2.7 pmol/μg, and 2.4 pmol/μg, at pH 5.0, 6.2, and 7.4, respectively.

FIG. 9

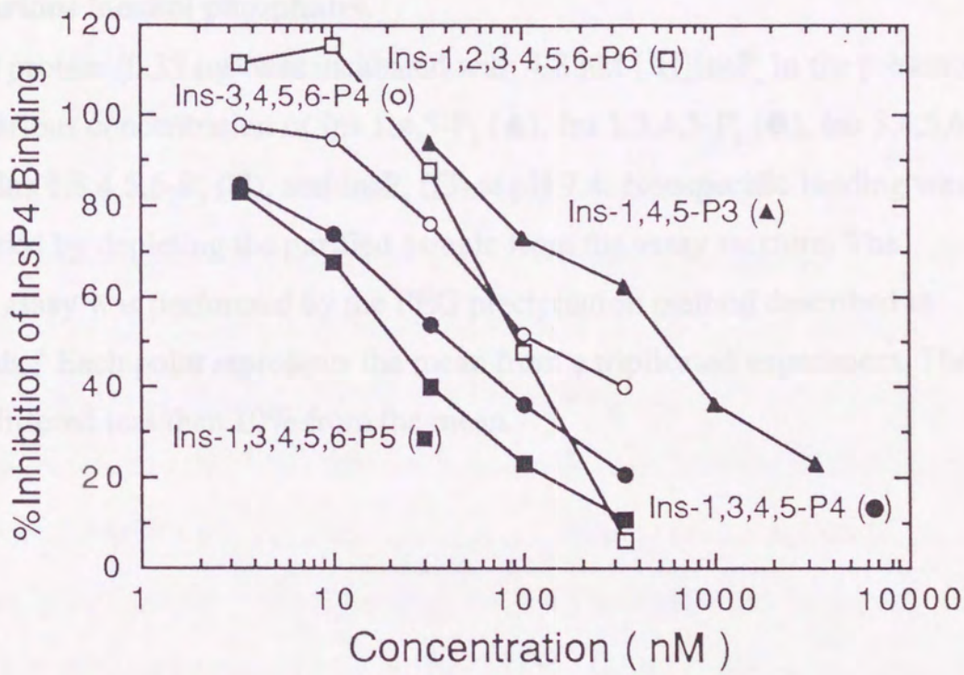

%Inhibition of InsP4 Binding
120
100
80
60
40
20
0
1
10
100
1000
10000
Concentration (nM)
Ins-1,2,3,4,5,6-P6 (□)
Ins-3,4,5,6-P4 (○)
Ins-1,4,5-P3 (▲)
Ins-1,3,4,5,6-P5 (■)
Ins-1,3,4,5-P4 (●)

FIG. 9. Inhibition of [^{3}H]InsP_4-binding to purified IP4BP1/synaptotagmin *II* by various inositol phosphates.

Purified protein (0.35 μg) was incubated with 4.8 nM [^{3}H]InsP_4 in the presence of the various concentration of Ins 1,4,5-P_3 (▲), Ins 1,3,4,5-P_4 (●), Ins 3,4,5,6-P_4 (○), Ins 1,3,4,5,6-P_5 (■), and InsP_6 (□) at pH 7.4. Nonspecific binding was determined by depleting the purified sample from the assay mixture. The binding assay was performed by the PEG precipitation method described in "Methods." Each point represents the mean from a triplicated experiment. The values differed less than 10% from the mean.

1,3,4,5,6-pentakisphosphate displaced [^{3}H]Ins 1,3,4,5-P_4 binding with higher potency than Ins 1,3,4,5-P_4. Ins 3,4,5,6-P_4 and inositol hexakisphosphate suppressed Ins 1,3,4,5-P_4 binding half-maximally at about 100 nM. But in the case of inositol hexakisphosphate the displacement slope was relatively sharp, with an enhancing effect on [^{3}H]InsP_4 binding at about 10 nM, and strong suppression of [^{3}H]InsP_4 binding at 300 nM. Similar suppression at 300 nM of Ins 1,3,4,5-P_4 was also observed. The other inositol phosphates tested displayed much lower affinity, with Ins 1,4-P2 in particular having no influence at concentrations up to 10 μM (data not shown).

Purification of IP4BP2a

IP4BP1 and most of other concomitant proteins (IP3R, IP4BP1 and phosphatases) were separated from IP4BP2 by the first anion-exchange chromatography on DE-52 (Fig. 10). Since the volume of DE-52 flow-through fraction was large, InsP_4-binding activity was concentrated by Heparin-Agarose chromatography (Fig. 11, a). After dilution to lower the NaCl concentration and pH, the InsP_4-binding activity was concentrated and enriched by cation-exchange chromatography on CM-32 (Table 2 and Fig. 11, b). The InsP_4-binding activity was purified by sequential chromatography on the first Sephacryl S-300 (Fig. 11, c), CM Sepharose CL-6B (Fig. 11, d), and the second Sephacryl S-300 (Fig. 11, e). On the first Sephacryl S-300, the first peak of the InsP_4-binding activity is due to the presence of IP4BP2b. Table 2 summarizes the purification of IP4BP2a. Approximately 1.6 mg of the binding protein can be obtained from 80 g of mouse cerebellum.

The protein profile of each purification step was characterized by SDS-PAGE

FIG. 10

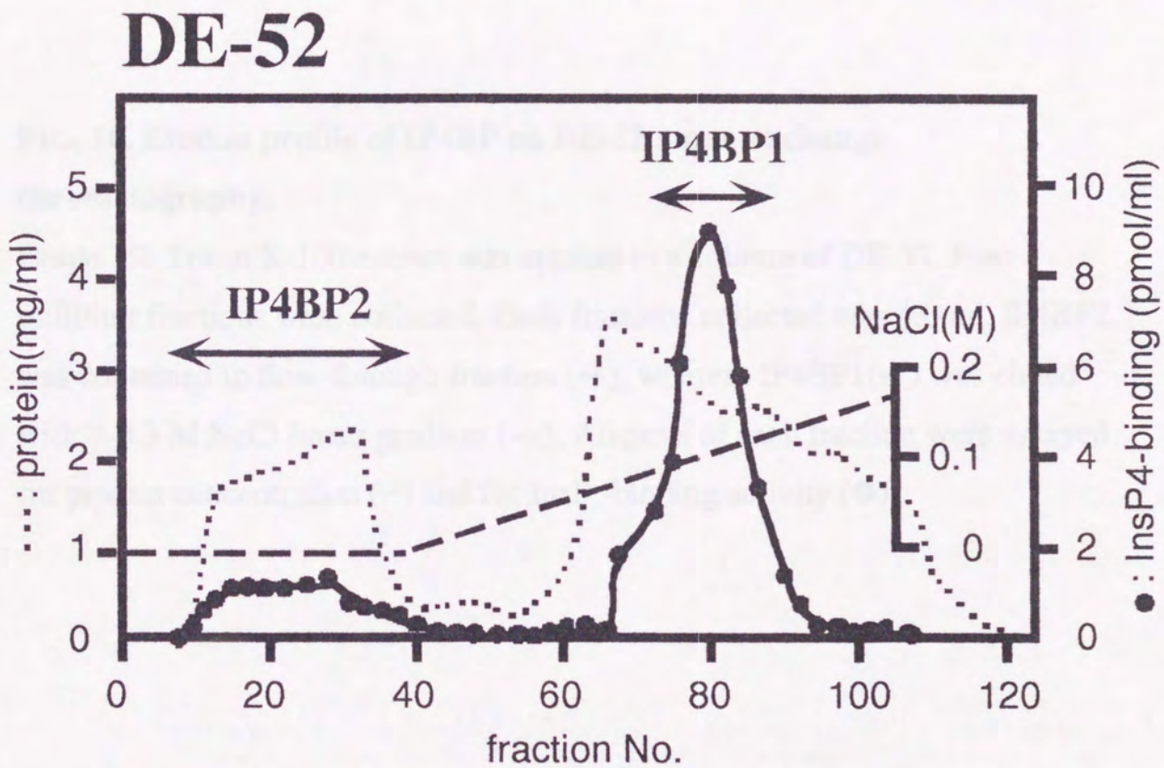

DE-52
IP4BP1
IP4BP2
NaCl(M)
0.2
0.1
0
···: protein(mg/ml)
●: InsP4-binding (pmol/ml)
0
1
2
3
4
5
0
2
4
6
8
10
0
20
40
60
80
100
120
fraction No.

FIG. 10. Elution profile of IP4BP on DE-52 anion-exchange chromatography.

Crude 1% Triton X-100 extract was applied to a column of DE-52. Four milliliter fractions were collected. Each fractions collected was 4.0 ml. IP4BP2 was contained in flow-through fraction (↔), whereas IP4BP1(↔) was eluted with 0-0.3 M NaCl linear gradient (---). Aliquots of each fraction were assayed for protein concentration (···) and for $InsP_4$-binding activity (●).

FIG. 11

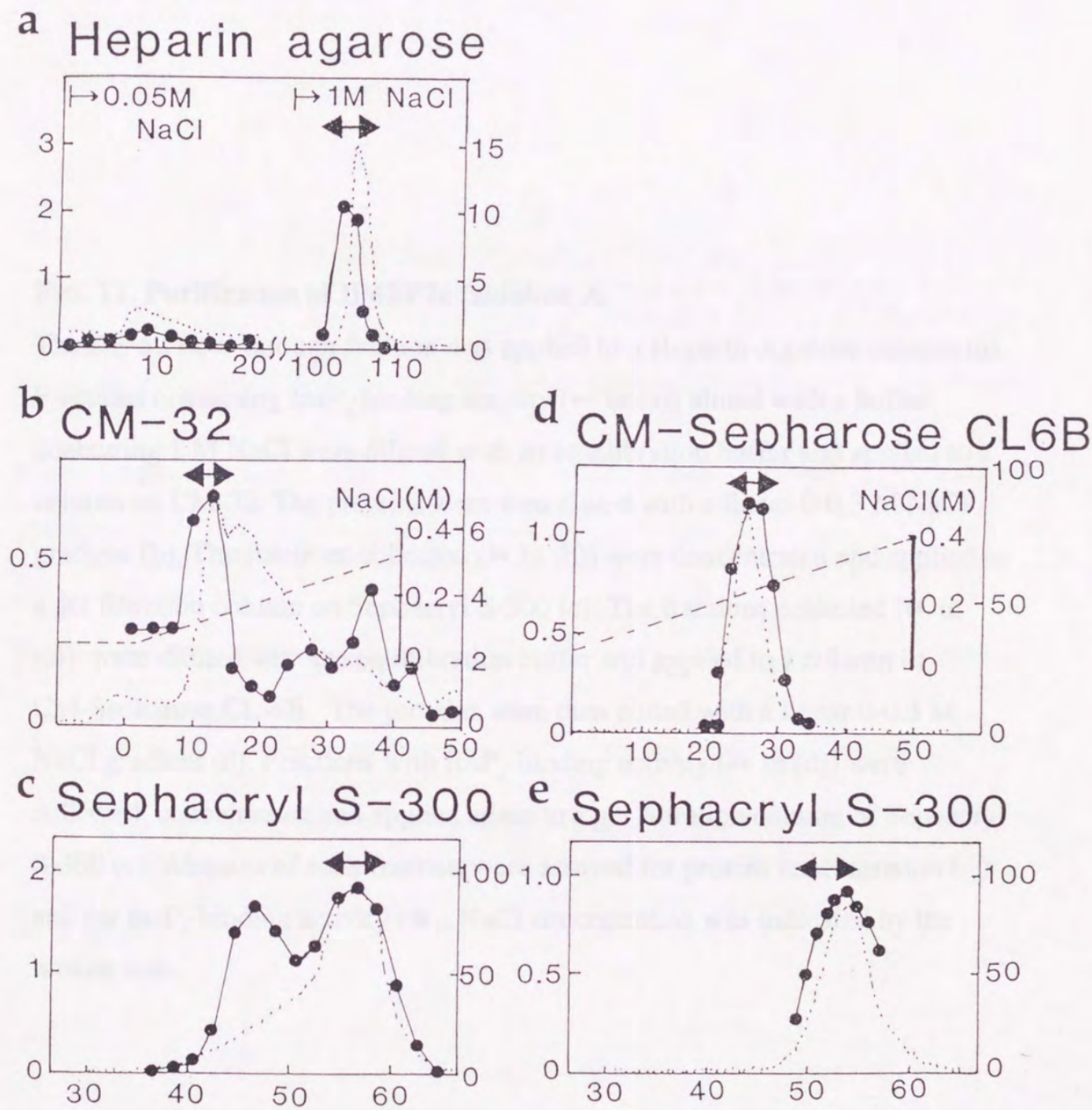

a Heparin agarose
0.05M NaCl
1M NaCl
b CM-32
NaCl(M)
c Sephacryl S-300
d CM-Sepharose CL6B
NaCl(M)
e Sephacryl S-300

FIG. 11. Purification of IP4BP2a /aldolase A.

The DE-52 flow-through fraction was applied to a Heparin-Agarose column (a). Fractions containing $InsP_4$-binding activity (↔ in (a)) eluted with a buffer containing 1 M NaCl were diluted with an equilibration buffer and applied to a column on CM-32. The proteins were then eluted with a linear 0-0.3 M NaCl gradient (b). The fractions collected (↔ in (b)) were concentrated and applied to a gel filtration column on Sephacryl S-300 (c). The fractions collected (↔ in (c)) were diluted with the equilibration buffer and applied to a column of CM-Sepharose CL-6B. The proteins were then eluted with a linear 0-0.3 M NaCl gradient (d). Fractions with $InsP_4$-binding activity (↔ in (d)) were collected, concentrated and applied again to a gel filtration column of Sephacryl S-300 (e). Aliquots of each fraction were assayed for protein concentration (···) and for $InsP_4$-binding activity (●). NaCl concentration was indicated by the broken line.

TABLE 2
Purification summary of IP4BP2a

Fraction	Protein[a] (mg)	$InsP_4$-Binding[b] (μmol)	Specific Act. (μmol/mg)	Yield (%)	Purification (fold)
DE-52 (flow-through)	436	17.2	0.04	100	1
Heparin-Agarose pool	121	17.7	0.15	103	3.7
CM-32 pool	10.6	4.0	0.38	23	9.6
Sephacryl S-300 pool	5.3	2.7	0.51	16	13
CM-Sepharose CL-6B pool	3.1	2.3	0.74	13	19
Sephacryl S-300 pool	1.6	1.6	1.0	9	25

The result presented is for a typical preparation.

[a]Protein was determined by using the Bio-Rad protein assay reagent.

[b]$InsP_4$-binding activities were measured by routine assay with 9.6 nM of [^{3}H]$InsP_4$ at pH 5.8. Nonspecific binding was determined without samples.

(Fig. 12). Most of the contaminating proteins after Heparin-Agarose fraction were removed efficiently by CM-32 chromatography (Fig. 12, lane 2 and 3). After the final step on Sephacryl S-300, a protein band with molecular weight of 42K was detected (Fig. 12, lane 6). $InsP_4$ binding activity perfectly coincided with the intensities of CBB staining of this 42K protein (data not shown).

Cross-linking of IP4BP2a

While the molecular weight of IP4BP2a was 42K by SDS-PAGE analysis, the apparent molecular weight was 160K by molecular weight calibration on gel filtration chromatography (Sephacryl S-300) (Fig. 13). On this account, IP4BP2a was expected to be a tetramer of a single subunit . To prove this hypothesis, I carried out cross-linking of IP4BP2a. The purified protein was cross-linked with DMS. First, the cross-linked proteins were analyzed by SDS-PAGE with the buffer system of Laemmli (1970) and visualized with CBB R-250. The result of the molecular weight calibration of these protein bands did not match with my estimation (data not shown). Therefore, I used the buffer system of Weber and Osborn (1969) as an alternative. Molecular weight calibration of four protein bands indicated that I, *II*, *III*, and IV corresponded approximately to 45K, 90K, 135K, and 180K (Fig. 14). This result nearly coincided with apparent molecular weight on Sephacryl S-300.

N-terminal sequence analysis of IP4BP2a

The purified IP4BP2a from the second Sephacryl S-300 gel filtration chromatography was applied to SDS-PAGE on 10% gel. The protein was transferred to a PVDF membrane with CAPS buffer to prevent the contamination of glycine. The 42K band stained with CBB R-250 was cut out from the blot and N-terminal sequence

FIG. 12

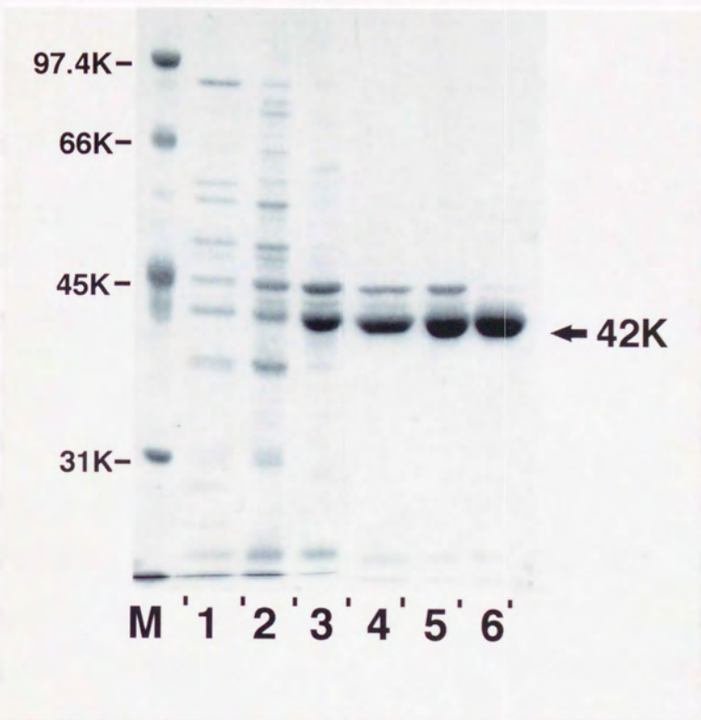


FIG. 12. SDS-PAGE analysis after each purification step of IP4BP2a.

SDS-PAGE was carried out on a 10 % gel using the buffer system of Laemmli. The gel was visualized by CBB R-250 staining. *Lane 1*, DE-52 flow-through fraction; *lane 2*, pooled fractions from the Heparin-Agarose column; *lane 3*, pooled fractions obtained from the CM-32 column; *lane 4*, pooled fractions obtained from the 1st Sephacryl S-300 column; *lane 5*, pooled fractions from the CM-Sepharose CL-6B column; *lane 6*, pooled fraction obtained from the 2nd Sephacryl S-300 column. The protein loaded onto each *lane* was 4.0 μg as determined by Bio-Rad protein assay reagent. Molecular weight standards (*lane* M) (97.4K, phosphorylase b; 66K, bovine serum albumin; 45K, ovalbumin; 31K, carbonic anhydrase) with corresponding Mr values are shown at the left.

FIG. 13

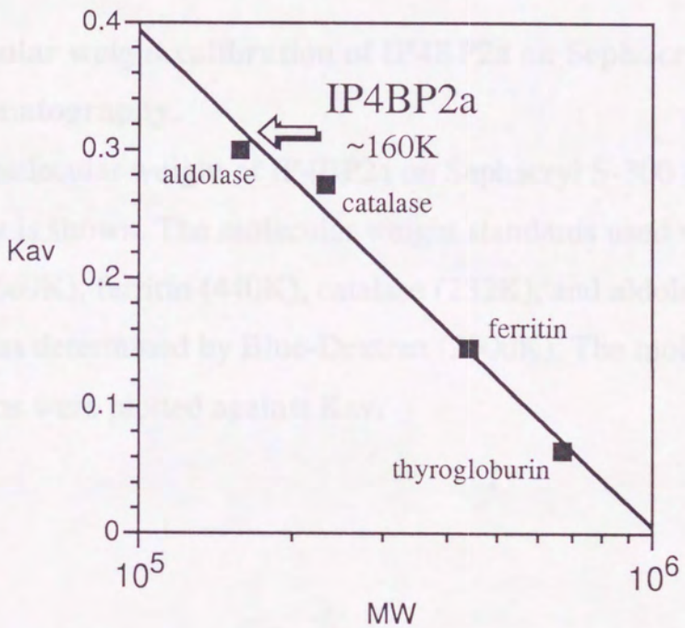

0.4
0.3
0.2
0.1
0
Kav
IP4BP2a
~160K
aldolase
catalase
ferritin
thyrogloburin
10^5
10^6
MW

FIG. 13. Molecular weight calibration of IP4BP2a on Sephacryl S-300 gel filtration chromatography.

Calibration of molecular weight of IP4BP2a on Sephacryl S-300 gel filtration chromatography is shown. The molecular weight standards used were thyroglobulin (669K), ferritin (440K), catalase (232K), and aldolase (158K). Void volume was determined by Blue-Dextran (2000K). The molecular weights of theses proteins were plotted against Kav.

FIG. 14



FIG. 14. SDS-PAGE analysis of cross-linked IP4BP2a using Weber and Osborn buffer system.

Purified IP4BP2a (0.3 mg/ml) was cross-linked with DMS at reagent concentrations of 0 (*lane 1*), 40 (*lane 2*), 80 (*lane 3*), 200 (*lane 4*) μg/ 200 μl. Cross-linked proteins were analyzed with SDS-PAGE on a 10% gel using the Weber and Osborn buffer system (1969). The proteins were stained with CBB R-250. Molecular weight standards (200K, myosin; 116K, β-galactosidase; 97.4K, phosphorylase b; 66K, bovine serum albumin; 45K, ovalbumin) with corresponding Mr values are shown at the left. *I* , *II*, *III*, and *IV* correspond to the position of the monomeric, dimeric, trimeric and tetrameric forms of IP4BP2a, respectively.

of this protein was determined by a gas-phase protein sequencer. The N-terminal sequence of IP4BP2a was checked against SWISS PROT database, revealing close identity with mouse aldolase A (Table 3). At the amount of IP4BP2a used for sequencing, histidine (H) and threonine (T) residues were difficult to identify. Aldolase A is one of the three isoforms of fructose-1,6-bisphosphate aldolase, which is a ubiquitous glycolytic enzyme and catalyzes the reversible aldol cleavage of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxy acetone phosphate.

Characterization of the $InsP_4$-binding activity of the purified IP4BP2a (aldolase A)

The pH dependence of $InsP_4$-binding is shown in Fig. 15. The maximum binding of [^{3}H]$InsP_4$ was observed around pH 5.8. Nonspecific binding component in the absence of purified IP4BP2a was significantly higher at pH value lower than 5.0 as in the case of IP4BP1. It seemed that this nonspecific binding is caused by abundant γ-globulin in PEG-precipitation methods. Thus following $InsP_4$-binding analyses were carried out at pH 5.8.

Scatchard analysis of the $InsP_4$-binding to the purified IP4BP2a indicated that the K_D was 1.2 μM and the B_{max} was 22 pmol/μg of protein (Fig. 16). The Hill coefficient was about 1.0. The specificity of the Ins 1,3,4,5-P_4-binding site was characterized by adding several inositol polyphosphates (Fig. 17). Ins 1,3,4,6-P_4, Ins 1,2,4,5-P_4, Ins 1,3,4,5-P_4, and Ins 1,3,4,5,6-P_5 suppressed [^{3}H]Ins 1,3,4,5-P_4 binding with higher potency than Ins 2,4,5-P_3, Ins 1,4,5-P_3, and $InsP_6$. The IC_{50} of Ins 1,3,4,6-P_4, Ins 1,2,4,5-P_4, Ins 1,3,4,5-P_4, and Ins 1,3,4,5,6-P_5 were about 1 μM. The IP4BP2a appeared to have higher affinity against $InsP_4$ and $InsP_5$. Ins 1-P and Ins 1,4-P_2

TABLE 3

Comparison of N-terminal amino acid sequences from IP4BP2a and mouse muscle aldolase A, IP4BP2b and mouse PLP/DM20

mouse	1	10	20
aldolase A :	P H P Y P A P L T P E Q K K E L S D I A H R		
IP4BP2a :	P*? P Y P A P L ? P E Q K K ? L ? ? ? A		

mouse	1	10
PLP/DM20 :	G L L E C C A R C L V G A P F A	
IP4BP2b :	G L L E ? ? A ? ? L V G A P	

Amino acid sequencing was done as described in methods.
*? refers to an amino acid that was not positively identified.

FIG. 15

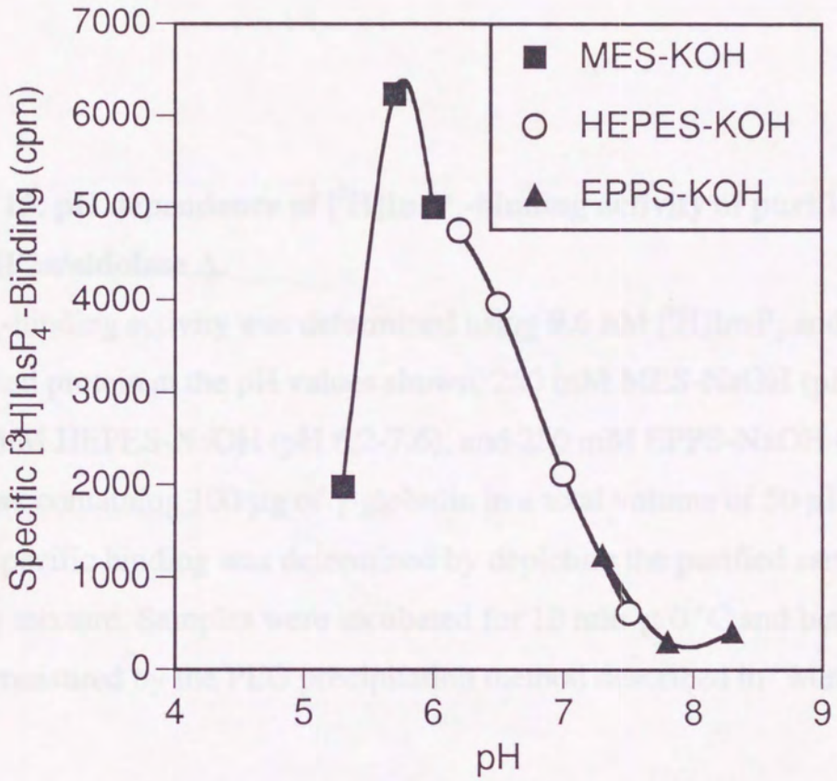

7000
6000
5000
4000
3000
2000
1000
0
4
5
6
7
8
9
pH
Specific [3H]InsP4-Binding (cpm)
MES-KOH
HEPES-KOH
EPPS-KOH

FIG. 15. pH dependence of [^{3}H]$InsP_4$-binding activity of purified IP4BP2a/aldolase A.

$InsP_4$-binding activity was determined using 9.6 nM [^{3}H]$InsP_4$ and 1.6 μg of purified protein at the pH values shown. 250 mM MES-NaOH (pH 4.7-6.1), 250 mM HEPES-NaOH (pH 6.2-7.6), and 250 mM EPPS-NaOH (pH 6.9-8.5) buffers containing 100 μg of γ-globulin in a total volume of 50 μl were used. Nonspecific binding was determined by depleting the purified sample from the assay mixture. Samples were incubated for 10 min at 0 °C and binding activity was measured by the PEG precipitation method described in "Methods."

FIG. 16

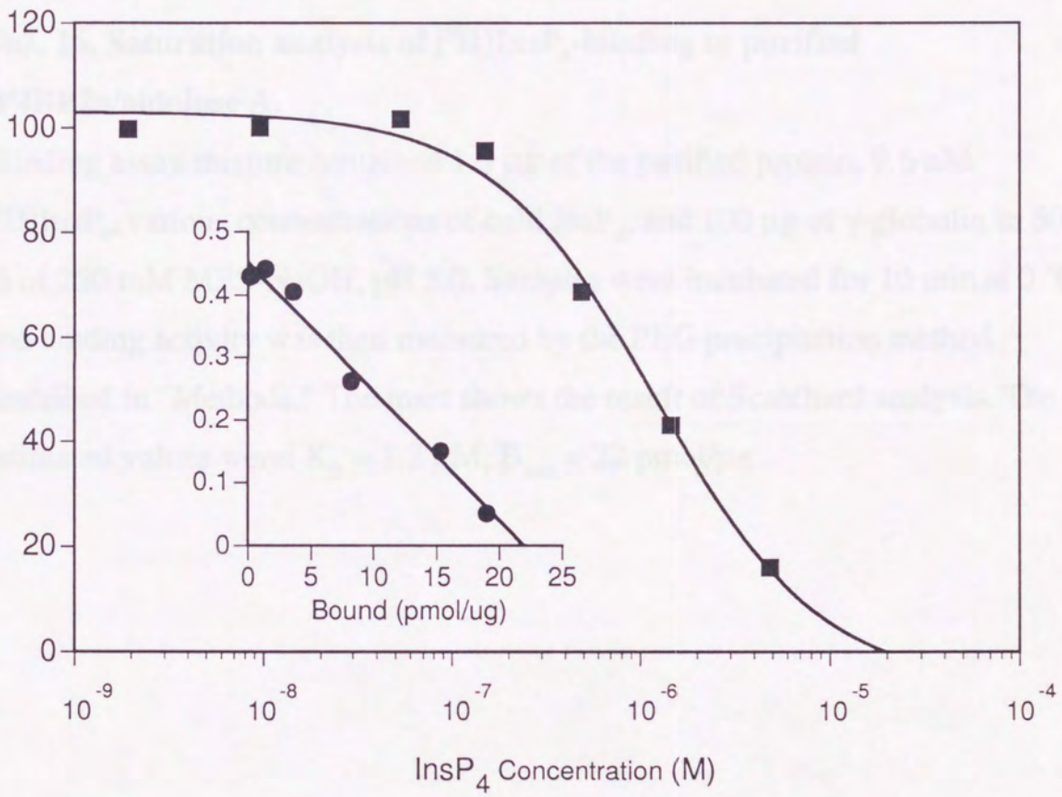

120
100
80
60
40
20
0
0.5
0.4
0.3
0.2
0.1
0
0 5 10 15 20 25
Bound (pmol/ug)
10^-9
10^-8
10^-7
10^-6
10^-5
10^-4
InsP4 Concentration (M)

FIG. 16. Saturation analysis of [^{3}H]InsP$_4$-binding to purified IP4BP2a/aldolase A.

Binding assay mixture contained 1.3 μg of the purified protein, 9.6 nM [^{3}H]InsP$_4$, various concentrations of cold InsP$_4$, and 100 μg of γ-globulin in 50 μl of 250 mM MES-NaOH, pH 5.0. Samples were incubated for 10 min at 0 °C and binding activity was then measured by the PEG precipitation method described in "Methods." The inset shows the result of Scatchard analysis. The estimated values were: K_D = 1.2 μM, B_{max} = 22 pmol/μg.

FIG. 17

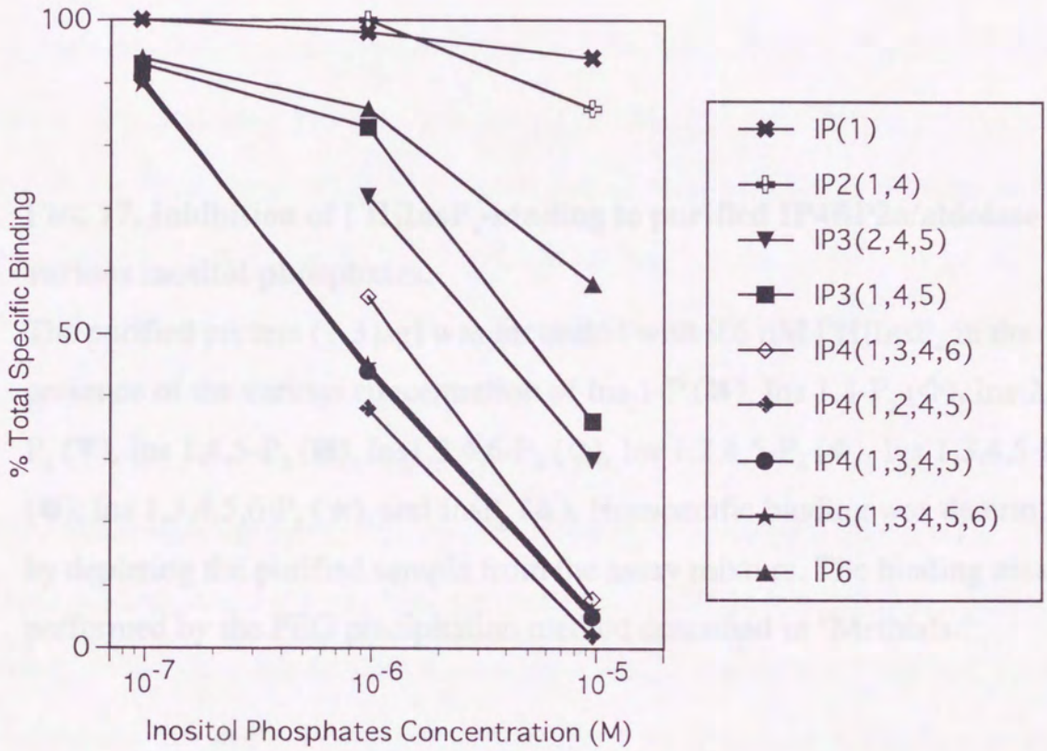

100
50
0
% Total Specific Binding
10^{-7}
10^{-6}
10^{-5}
Inositol Phosphates Concentration (M)
IP(1)
IP2(1,4)
IP3(2,4,5)
IP3(1,4,5)
IP4(1,3,4,6)
IP4(1,2,4,5)
IP4(1,3,4,5)
IP5(1,3,4,5,6)
IP6

FIG. 17. Inhibition of [^{3}H]InsP_4-binding to purified IP4BP2a/aldolase A by various inositol phosphates.

The purified protein (1.3 μg) was incubated with 9.6 nM [^{3}H]InsP_4 in the presence of the various concentration of Ins 1-P (✖), Ins 1,4-P_2 (✣), Ins 2,4,5-P_3 (▼), Ins 1,4,5-P_3 (■), Ins1,3,4,6-P_4 (◇), Ins 1,2,4,5-P_4 (❖), Ins 1,3,4,5-P_4 (●), Ins 1,3,4,5,6-P_5 (★), and InsP_6 (▲). Nonspecific binding was determined by depleting the purified sample from the assay mixture. The binding assay was performed by the PEG precipitation method described in "Methods."

displayed much lower affinity.

Purification of IP4BP2b

From DE-52 flow-through fraction, first I purified IP4BP2a. During this procedure I detected another $InsP_4$-binding activity (IP4BP2b). To purify IP4BP2b, several modifications of the purification method were needed. The DE-52 flow-through fraction used was the same as that of IP4BP2a purification procedure. At Heparin-Agarose chromatography, detergent of sample buffer and purification buffer was changed from Triton X-100 into CHAPS because the $InsP_4$-binding activity of IP4BP2b was inhibited by Triton X-100 and because the concomitant proteins were separated from IP4BP2b.

IP4BP2a was separated from IP4BP2b by 0.25 M NaCl wash on the Heparin-Agarose chromatography (Fig. 18, A). IP4BP2b was eluted with 0.25-1.0 M NaCl linear gradient and the $InsP_4$-binding protein included in this fraction seemed to be mostly IP4BP2b. After dilution to lower the NaCl concentration and pH, the $InsP_4$-binding activity was concentrated and enriched by cation-exchange chromatography on CM-52 (Fig. 18, B). The final step was Sephacryl S-300 gel filtration (Fig. 18, C). Since the solubilized detergent was changed and $InsP_4$-binding assay was modified (described below), the purification summary can not be shown. Approximately 0.5 mg of the IP4BP2b can be obtained from 40 g of mouse cerebellum.

The protein profile of the each purification step was characterized by SDS-PAGE (Fig. 19). Particular attention has been paid not to boil the sample mixture in SDS solution and to let them stand at room temperature. Since the IP4BP2b seemed extremely hydrophobic, IP4BP2b proteins aggregated and did not enter the separation gel after boiling (data not shown).

FIG. 18

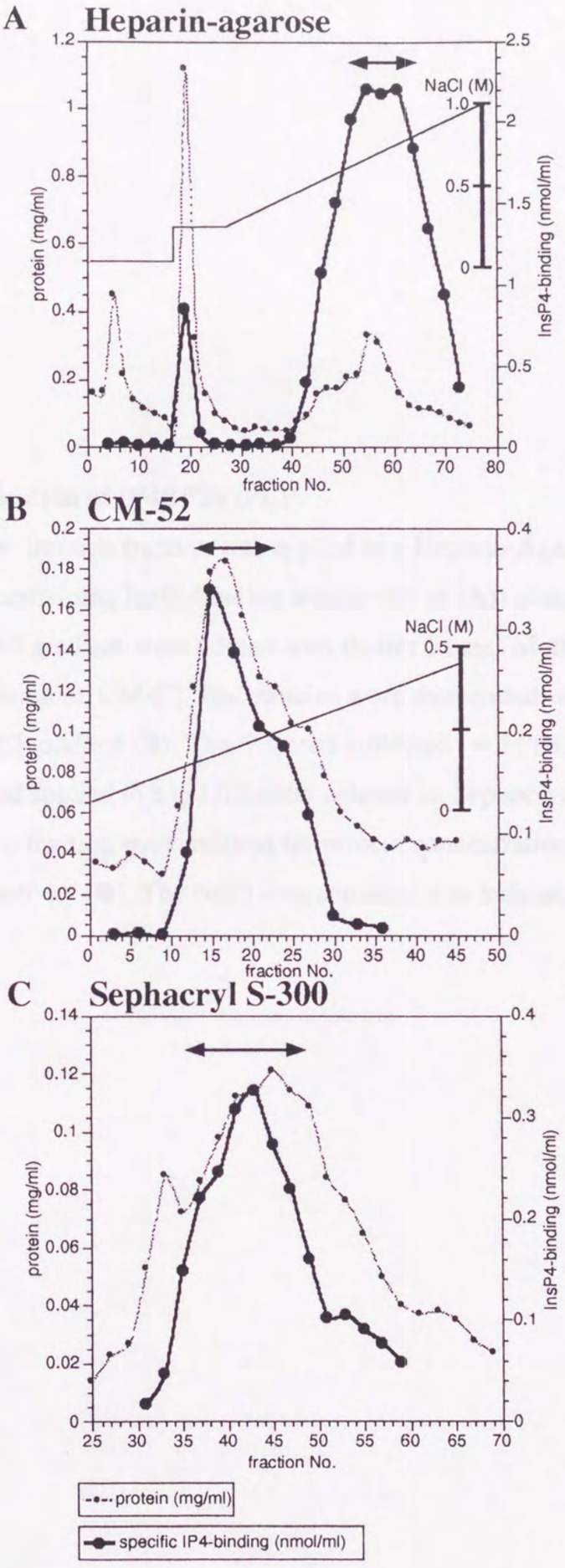

A
Heparin-agarose
NaCl (M)
1.0
0.5
0
protein (mg/ml)
InsP4-binding (nmol/ml)
fraction No.
B
CM-52
NaCl (M)
0.5
0
protein (mg/ml)
InsP4-binding (nmol/ml)
fraction No.
C
Sephacryl S-300
protein (mg/ml)
InsP4-binding (nmol/ml)
fraction No.
protein (mg/ml)
specific IP4-binding (nmol/ml)

FIG. 18. Purification of IP4BP2b /PLP.

The DE-52 flow-through fraction was applied to a Heparin-Agarose column (A). Fractions containing $InsP_4$-binding activity (↔ in (A)) eluted with linear 0.25-1.0 M NaCl gradient were diluted with Buffer 5 (see "Methods") and applied to a column on CM-52. The proteins were then eluted with a linear 0.05-0.5 M NaCl gradient (B). The fractions collected (↔ in (B)) were concentrated and applied to a gel filtration column on Sephacryl S-300 (C). Aliquots of each fraction were assayed for protein concentration (•) and for $InsP_4$-binding activity (●). The NaCl concentration was indicated by the line.

FIG. 19

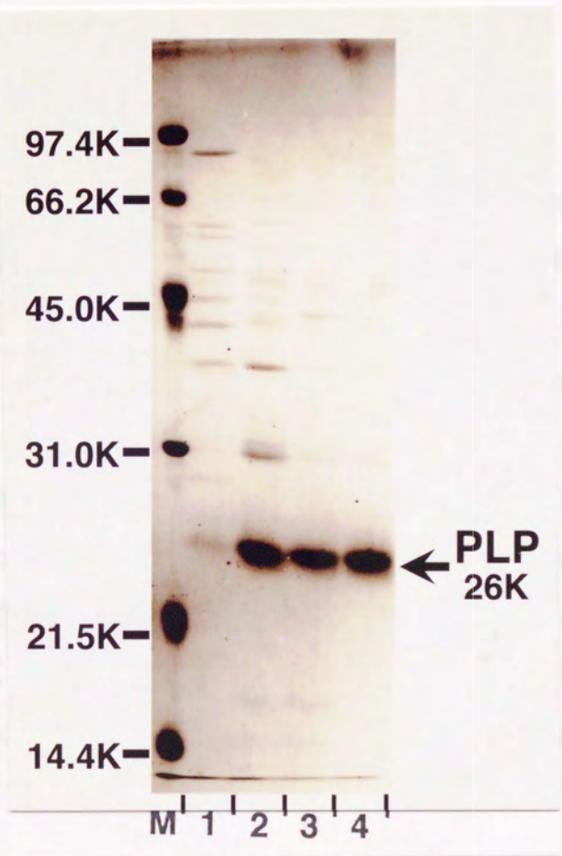


FIG. 19. SDS-PAGE analysis after each purification step of IP4BP2b. SDS-PAGE was carried out on a 15 % gel using the buffer system of Laemmli. The gel was visualized by CBB R-250 staining. *Lane 1*, DE-52 flow-through fraction; *lane 2*, pooled fractions from the Heparin-Agarose column; *lane 3*, pooled fractions obtained from the CM-52 column; *lane 4*, pooled fractions obtained from the Sephacryl S-300 column. The protein loaded onto each *lane* was 2.0 μg as determined by Bio-Rad protein assay reagent. Molecular weight standards (*lane* M) (97.4K, phosphorylase b; 66K, bovine serum albumin; 45K, ovalbumin; 31K, carbonic anhydrase; 21.5K, trypsin inhibitor; 14.4K, lysozyme) with corresponding Mr values are shown at the left.

Many of contaminating proteins after Heparin-Agarose fraction were eliminated efficiently by CM-52 chromatography (Fig. 19, lane 2 and 3). After the final step on Sephacryl S-300, a protein band with molecular weight of 26K was detected (Fig. 19, lane 4). While the molecular weight of IP4BP2b was 26K by SDS-PAGE analysis, the apparent molecular weight was about 440K-669K by gel filtration chromatography (Sephacryl S-300) (Fig. 20). Consequently, IP4BP2a was expected to be homomultimer. $InsP_4$-binding activity and the intensities of CBB staining of this 26K protein differed slightly (Fig. 18, c and Fig. 20). However, the results described below indicate that the binding activity resides in this 26K protein.

N-terminal sequence analysis of IP4BP2b

The N-terminal sequence of purified IP4BP2b from Sephacryl S-300 gel filtration chromatography was determined by a gas-phase protein sequencer as in the case of IP4BP2a. The N-terminal sequence of IP4BP2b was checked against SWISS PROT database and revealed close identity with mouse myelin proteolipid protein (PLP) (Table 3). By the sequencing method used, cysteine (C) and arginine (R) are undetectable. Generally, PLP is known to be a major intrinsic myelin protein to stabilize myelin structure in the central nervous system (CNS). Other functions will be discussed below.

Immunological cross-reactivity of IP4BP2b and myelin proteolipid protein (PLP)

Purified IP4BP2b from Sephacryl S-300 gel filtration chromatography and purified myelin sample (used below for an inositol polyphosphate-binding assay) as a positive control were applied to SDS-PAGE on 15% gel, and the proteins were transferred to PVDF membranes. The membranes were either stained with amido

FIG. 20

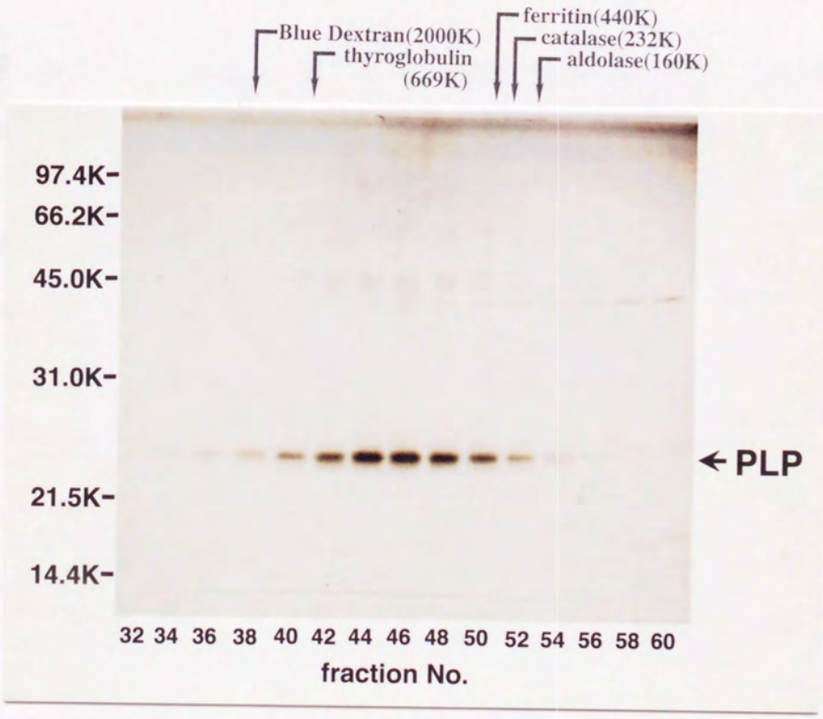

Blue Dextran(2000K)
thyroglobulin (669K)
ferritin(440K)
catalase(232K)
aldolase(160K)
97.4K-
66.2K-
45.0K-
31.0K-
21.5K-
14.4K-
← PLP
32 34 36 38 40 42 44 46 48 50 52 54 56 58 60
fraction No.

FIG. 20. SDS-PAGE analysis of the fractions obtained from gel filtration column chromatography of IP4BP2b on Sephacryl S-300.

SDS-PAGE protein profile of fractions eluted from gel filtration column (10 μl each). Molecular weight markers with corresponding Mr values are shown at the left as described in the Fig. 19 legend. Approximate elution point of molecular weight standards for gel filtration chromatography are shown at the top.

black (Fig. 21, a and d) or analyzed immunochemically using monoclonal antibodies against PLP (AH7-2a : epitope is amino acid residues number 209-217, AA3 : epitope is 264-276) (Fig. 21, b, c and e). *Lane b* and *c* revealed that monoclonal antibodies against PLP cross-react with 26K molecules. In addition, immunoblot analysis of the purified myelin showed these antibodies detect PLP (26K) and DM20 (about 20K), which is a related protein related to PLP (details of DM20 will be described in "Discussion") (Fig. 21, e).

Further, I asked whether the $InsP_4$-binding activity of IP4BP2b could be immunoabsorbed by anti-PLP antibody. The purified samples from Sephacryl S-300 gel filtration chromatography were incubated with Protein G-Sepharose resin coupled with AA3-IgG or normal rat IgG, or non-coupled. The resin were poured into columns and washed with washing solution (100 µl x 4). The nonadherent fractions were collected, and characterized by [^{3}H] $InsP_4$-binding assay and SDS-PAGE analysis with silver staining on 15% gel (Fig. 22 , A and B). The $InsP_4$-binding activity and 26K protein bands were immunoabsorbed by AA3-Protein G- Sepharose (Fig. 22 , A and B; +AA3; Fraction No. 2) but they were not absorbed by normal rat IgG-Protein G- Sepharose and Protein G- Sepharose (Fig. 22 , A and B; -IgG and +Rat IgG; Fraction No. 2). These results together with the N-terminal sequences indicate that the purified IP4BP2b is mouse PLP itself.

Characterization of the $InsP_4$-binding activity of the purified IP4BP2b (PLP)

In the $InsP_4$-binding assay of IP4BP2b, the final concentration of γ-globulin was set to 0.2-0.25 mg/ml (usual: 2 mg/ml) because when the concentration of γ-globulin was higher than 0.25 mg/ml, the $InsP_4$-binding activity of IP4BP2b was inhibited (data not shown).

FIG. 21

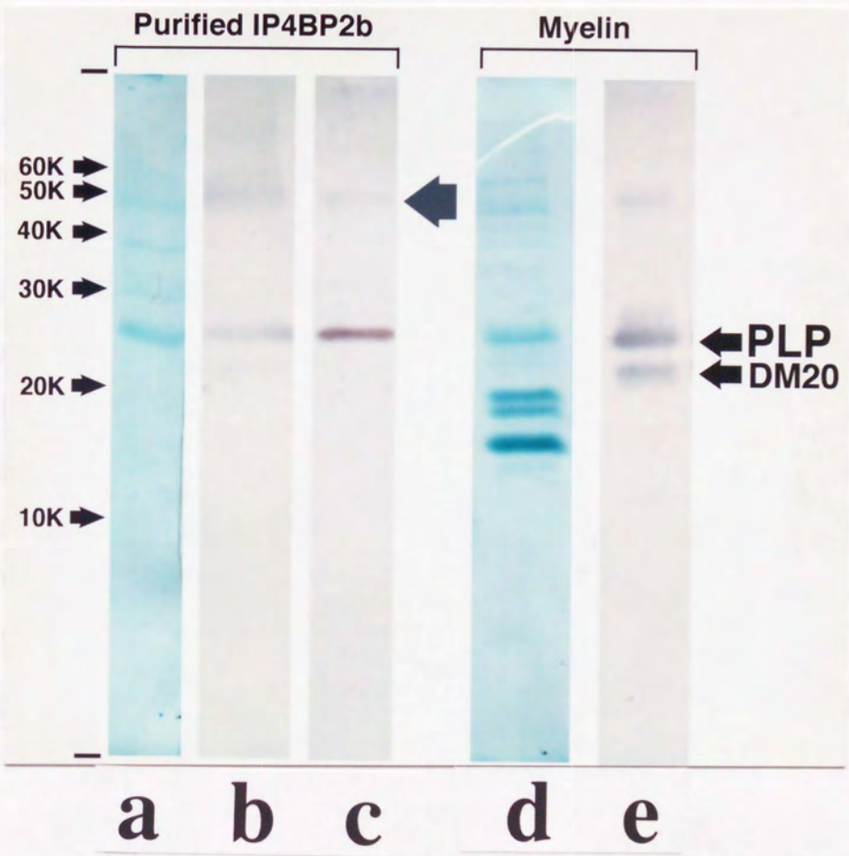
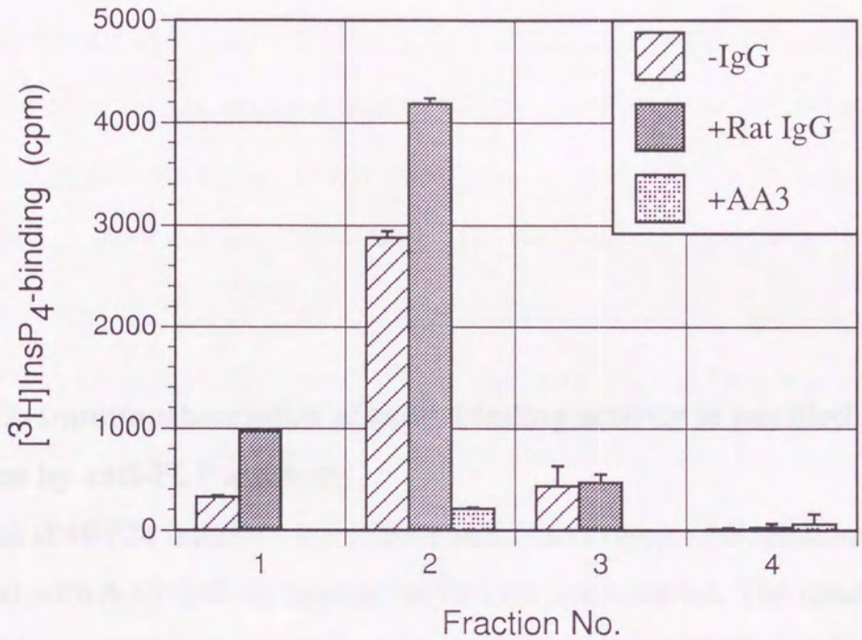

Purified IP4BP2b
Myelin
60K
50K
40K
30K
20K
10K
PLP
DM20
a
b
c
d
e

FIG. 21. Immunoblot analysis of IP4BP2b /PLP in purified preparation and of myelin fraction.

Purified IP4BP2b samples [3.4 μg (*lane a*) and 0.34 μg (*lane b* and *lane c*)] and myelin samples [2.1 μg (*lane d*) and 0.42 μg (*lane e*)] were applied to SDS-PAGE on a 15 % Laemmli gel. Proteins were transferred to PVDF membranes, and the blots were stained with amido black (*lane a* and *lane d*) and probed with monoclonal antibody against PLP, AA3 (*lane b* and *lane e*) or AH7-2a (*lane c*). Peroxidase-coupled detection was performed by the DAB staining method by using a Vectastain ABC kit. Molecular weight markers used were 10 kDa Protein Ladder (GIBCO BRL). Sizes of markers are shown at the left . The band indicated by the bold arrow seems to be aggregates of two PLP molecules.

FIG. 22

A


5000
4000
3000
2000
1000
0
[3H]InsP4-binding (cpm)
-IgG
+Rat IgG
+AA3
1
2
3
4
Fraction No.

B

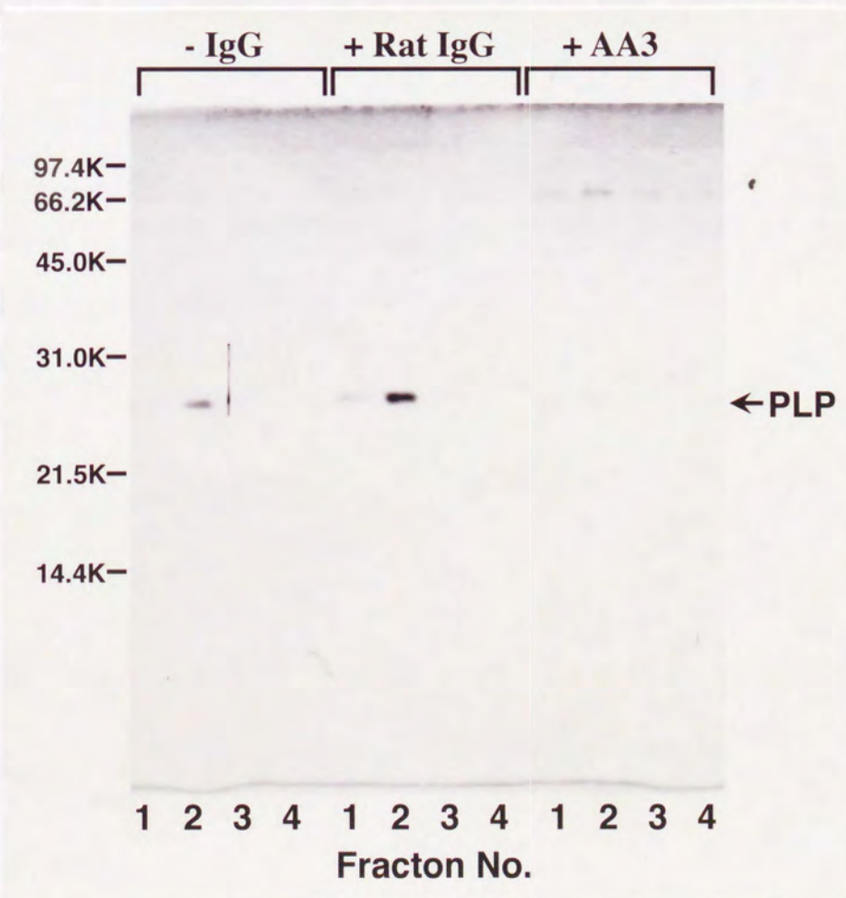

- IgG
+ Rat IgG
+ AA3
97.4K
66.2K
45.0K
31.0K
21.5K
14.4K
←PLP
1 2 3 4 1 2 3 4 1 2 3 4
Fracton No.

FIG. 22. Immunoabsorption of $InsP_4$-binding activity in purified IP4BP2b fraction by anti-PLP antibody.

Purified IP4BP2b fractions were incubated with Protein G-Sepharose residues coupled with AA3-IgG or normal rat IgG, or non-coupled. The residues were poured into columns and washed with washing solution (100 µl x 4). Nonadherent fractions were collected (fraction No. 1-4), assayed for $InsP_4$-binding activity (A) and analyzed by SDS-PAGE (B). $InsP_4$-binding activity was determined using 4.8 nM [^{3}H]$InsP_4$ in 20 mM HEPES-KOH (pH 7.2) buffer. Nonspecific binding was determined by removing the sample. Samples were incubated for 10 min at 0 °C and binding activity was measured by the PEG precipitation method described in "Methods." SDS-PAGE was carried out on a 15 % gel using the buffer system of Laemmli. The gel was visualized by silver staining. Molecular weight markers with corresponding Mr values are shown at the left as described in the Fig. 19 legend.

The pH dependence of $InsP_4$-binding is indicated in Fig. 23. The maximum binding of [^{3}H]$InsP_4$ was observed at pH range of 5.5-6.0. The nonspecific binding component defined in the absence of purified IP4BP2a was significantly higher at pH values lower than pH 5.0, which is similar to other IP4BP. Thus following $InsP_4$-binding analysises were carried out at pH 5.5.

Scatchard analysis of the $InsP_4$-binding to the purified IP4BP2b indicated that the K_D was 71 nM and the B_{max} was 30 pmol/μg of protein (Fig. 24). The Hill coefficient was about 1.0. The specificity of the Ins 1,3,4,5-P_4-binding site was characterized by adding several inositol polyphosphates (Fig. 25). Ins 1,3,4,6-P_4, Ins 1,2,4,5-P_4, Ins 1,3,4,5-P_4, and Ins 1,3,4,5,6-P_5 displaced [^{3}H]Ins 1,3,4,5-P_4 binding with higher potency than Ins 1,4,5-P_3. $InsP_6$ suppressed Ins 1,3,4,5-P_4 binding half-maximally at about 80 nM and the displacement slope was relatively sharp. Both $InsP_6$ and $InsP_4$ at 100 nM efficiently suppressed [^{3}H]$InsP_4$-binding to IP4BP2b (PLP). Ins 1-P, Ins 1,4-P_2 , and Ins 1,4,5-P_3 displayed much lower affinity. These tendency of affinities for inositol high-polyphosphate ($InsP_x$) are similar to those of IP4BP1.

Characterization of the $InsP_6$-binding activity of IP4BP2b (PLP)

From the above results, it was shown that the affinity for $InsP_6$ on $InsP_4$-binding site was unique. Analysis of $InsP_4$-binding described so far was performed under hypotonic solution (low salt), however, considering the physiological condition, I measured $InsP_3$, $InsP_4$, and $InsP_6$-binding activities of purified IP4BP2b (PLP) in the isotonic buffer containing 0.15 M KCl / 20 mM HEPES-KOH, pH 7.2. As a result, detectable binding activity was displayed only against $InsP_6$ (Fig. 26). These findings suggested that $InsP_6$ plays a physiological role for IP4BP2b (PLP). Therefore, K_D and

FIG. 23

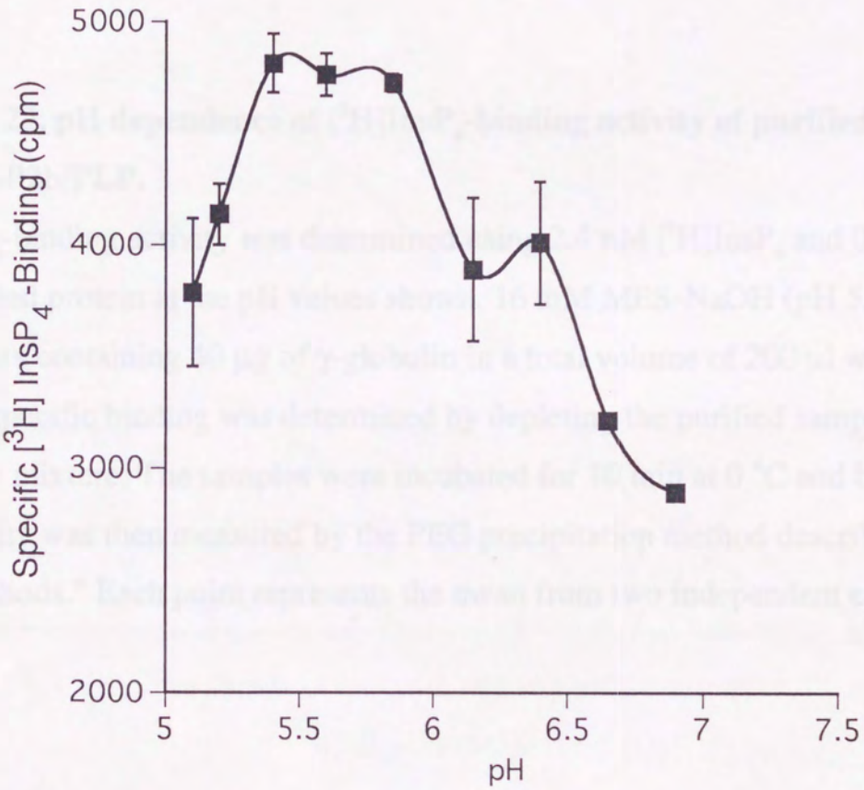

5000
4000
3000
2000
Specific [3H] InsP4-Binding (cpm)
5
5.5
6
6.5
7
7.5
pH

FIG. 23. pH dependence of [^{3}H]InsP$_4$-binding activity of purified IP4BP2b/PLP.

InsP$_4$-binding activity was determined using 2.4 nM [^{3}H]InsP$_4$ and 0.4 μg of purified protein at the pH values shown. 16 mM MES-NaOH (pH 5.3-7.4) buffers containing 40 μg of γ-globulin in a total volume of 200 μl were used. Nonspecific binding was determined by depleting the purified sample from the assay mixture. The samples were incubated for 10 min at 0 °C and binding activity was then measured by the PEG precipitation method described in "Methods." Each point represents the mean from two independent experiments.

FIG. 24

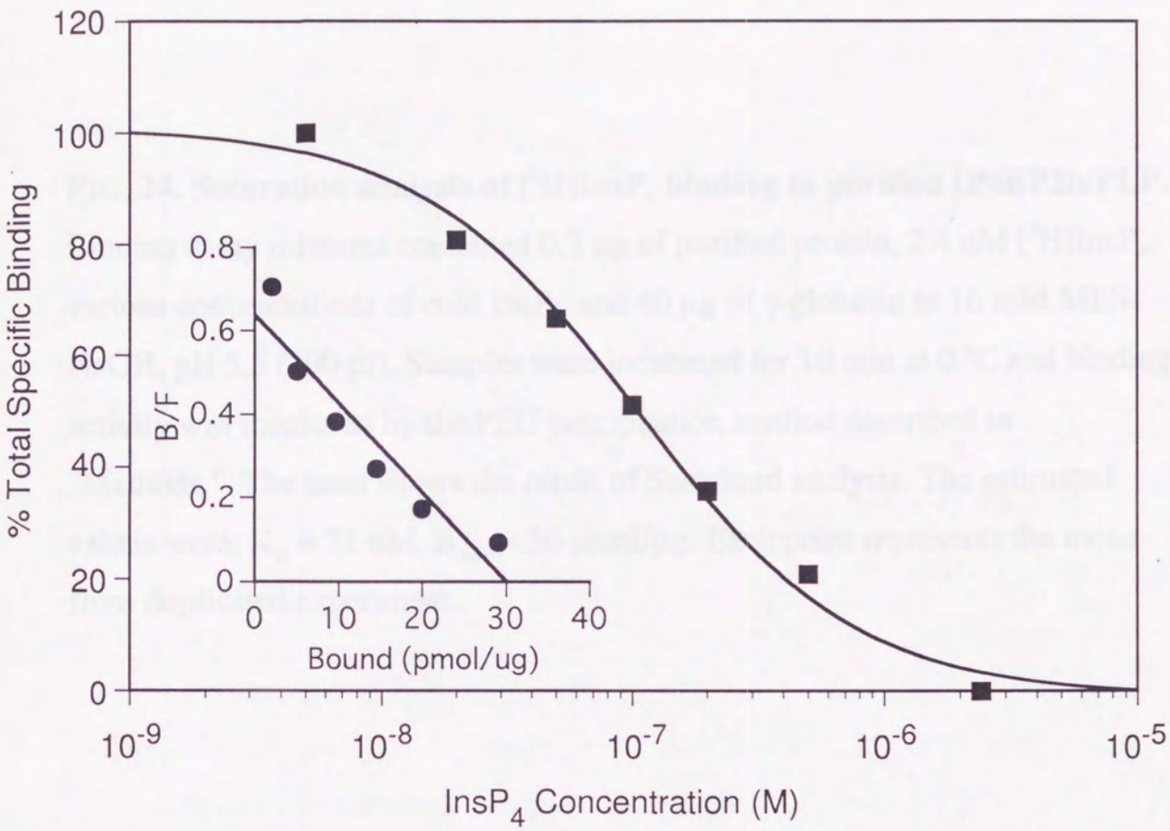

120
100
80
60
40
20
0
% Total Specific Binding
0.8
0.6
0.4
0.2
0
B/F
0
10
20
30
40
Bound (pmol/ug)
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
$InsP_4$ Concentration (M)

FIG. 24. Saturation analysis of [^{3}H]InsP$_4$-binding to purified IP4BP2b/PLP. Binding assay mixtures contained 0.3 μg of purified protein, 2.4 nM [^{3}H]InsP$_4$, various concentrations of cold InsP$_4$, and 40 μg of γ-globulin in 16 mM MES-NaOH, pH 5.5 (200 μl). Samples were incubated for 10 min at 0 °C and binding activity was measured by the PEG precipitation method described in "Methods." The inset shows the result of Scatchard analysis. The estimated values were: K_D = 71 nM, B_{max} = 30 pmol/μg. Each point represents the mean from duplicated experiment.

FIG. 25

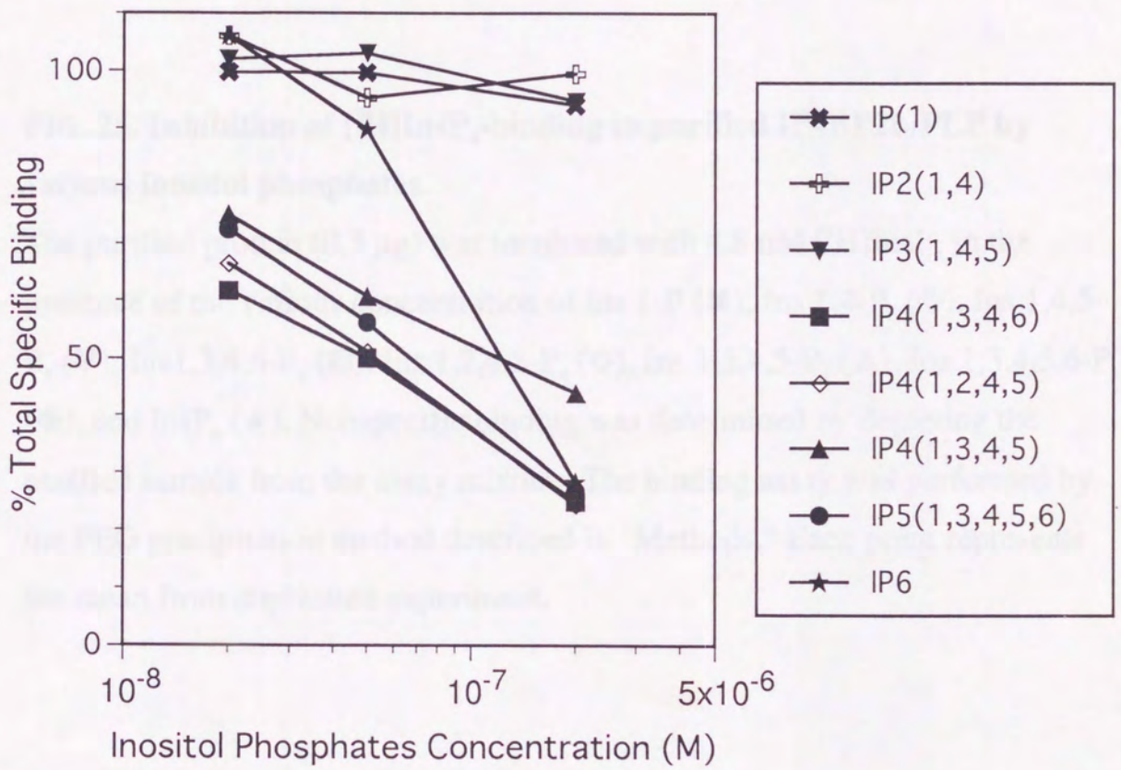

100
50
0
% Total Specific Binding
10-8
10-7
5x10-6
Inositol Phosphates Concentration (M)
IP(1)
IP2(1,4)
IP3(1,4,5)
IP4(1,3,4,6)
IP4(1,2,4,5)
IP4(1,3,4,5)
IP5(1,3,4,5,6)
IP6

FIG. 25. Inhibition of [^{3}H]InsP_4-binding to purified IP4BP2b/PLP by various inositol phosphates.

The purified protein (0.3 μg) was incubated with 4.8 nM [^{3}H]InsP_4 in the presence of the various concentration of Ins 1-P (✖), Ins 1,4-P_2 (✣), Ins 1,4,5-P_3 (▼), Ins1,3,4,6-P_4 (■), Ins 1,2,4,5-P_4 (◇), Ins 1,3,4,5-P_4 (▲), Ins 1,3,4,5,6-P_5 (●), and InsP_6 (★). Nonspecific binding was determined by depleting the purified sample from the assay mixture. The binding assay was performed by the PEG precipitation method described in "Methods." Each point represents the mean from duplicated experiment.

FIG. 26

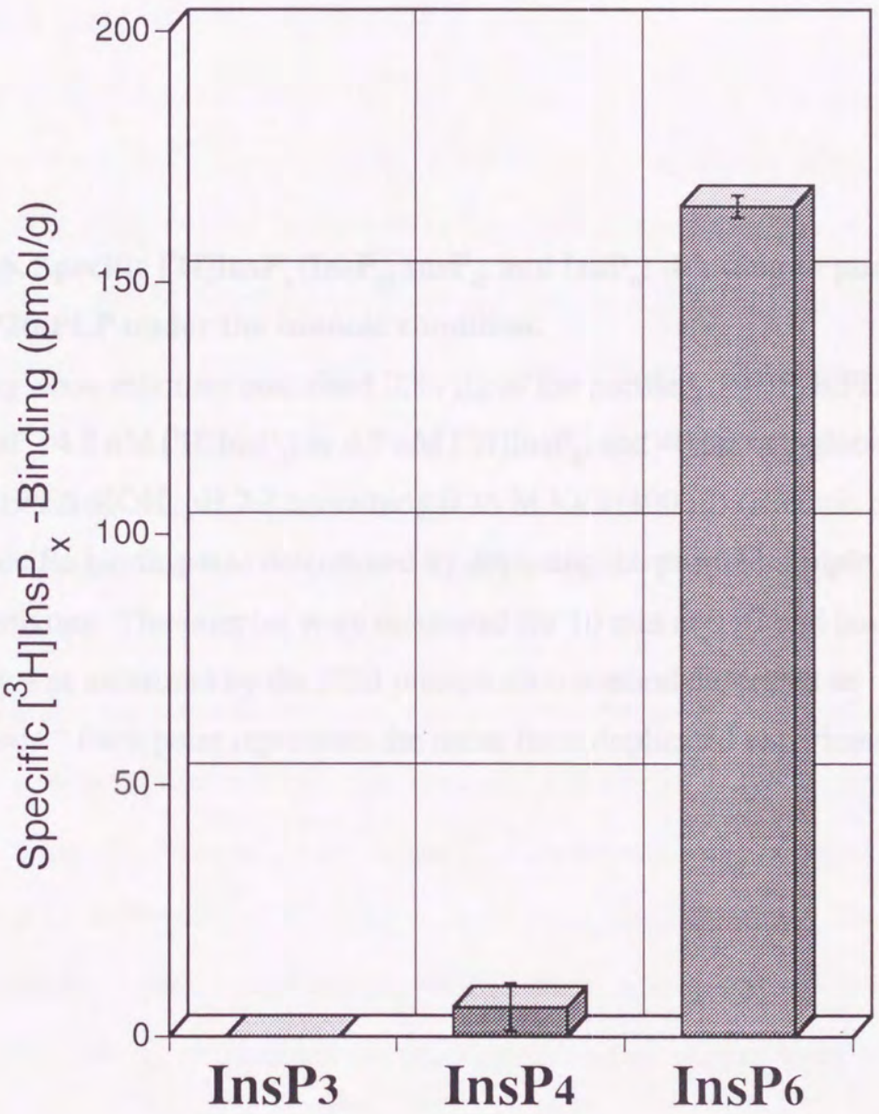

200
150
100
50
0
Specific [3H]InsPx-Binding (pmol/g)
InsP3
InsP4
InsP6

FIG. 26. Specific [^{3}H]InsP$_x$ (InsP$_3$, InsP$_4$, and InsP$_6$) -binding to purified IP4BP2b/PLP under the isotonic condition.

Binding assay mixtures contained 0.34 μg of the purified IP4BP2b/PLP, 4.8 nM [^{3}H]InsP$_3$, 4.8 nM [^{3}H]InsP$_4$, or 4.7 nM [^{3}H]InsP$_6$, and 40 μg of γ-globulin in 20 mM HEPES-KOH, pH 7.2 containing 0.15 M KCl (100 μl) (isotonic buffer). Nonspecific binding was determined by depleting the purified sample from the assay mixture. The samples were incubated for 10 min at 0 °C and binding activity was measured by the PEG precipitation method described in "Methods." Each point represents the mean from duplicated experiment.

B_{max} under isotonic condition were determined for [^{3}H]InsP_6-binding. Scatchard analysis of the InsP_6-binding to the purified IP4BP2b showed that the K_D was 58 nM and the B_{max} was 13 pmol/μg of protein (Fig. 27).

Purification and InsP_x-binding activity of myelin

The IP4BP2b was identified as PLP. Therefore, it is suggested that myelin membrane, where PLP is intrinsic, have binding activity against inositol polyphosphate (InsP_x). To prove this, I tried to purify the myelin fraction. Initially, myelin was isolated by the standard method of Norton and Poduslo (1973). The three fractions (myelin, middle layer, and P2/P3-myelin) were separated, and analyzed by SDS-PAGE and InsP_3, InsP_4, InsP_6-binding assay (data not shown). It was clear that each of these fractions contained many contaminating proteins from other fraction and had nearly the same InsP_3, InsP_4, InsP_6-binding activity. This result was possibly caused by the fact that the homogenate in 0.32 M sucrose were layered over 0.85 M sucrose and that membranes formed multilamellar. Therefore, the method (Lukas *et al.*, 1994) improving these points were applied for purifying the myelin membrane. Three fractions (myelin, middle layer, and P2/P3-myelin) were separated, and analyzed by SDS-PAGE and assayed for InsP_3, InsP_4, InsP_6-binding (Fig. 28). Many of contaminating proteins were eliminated efficiently from the myelin fraction and the InsP_6-binding activity of the myelin was significantly higher than those of other fractions.

FIG. 27

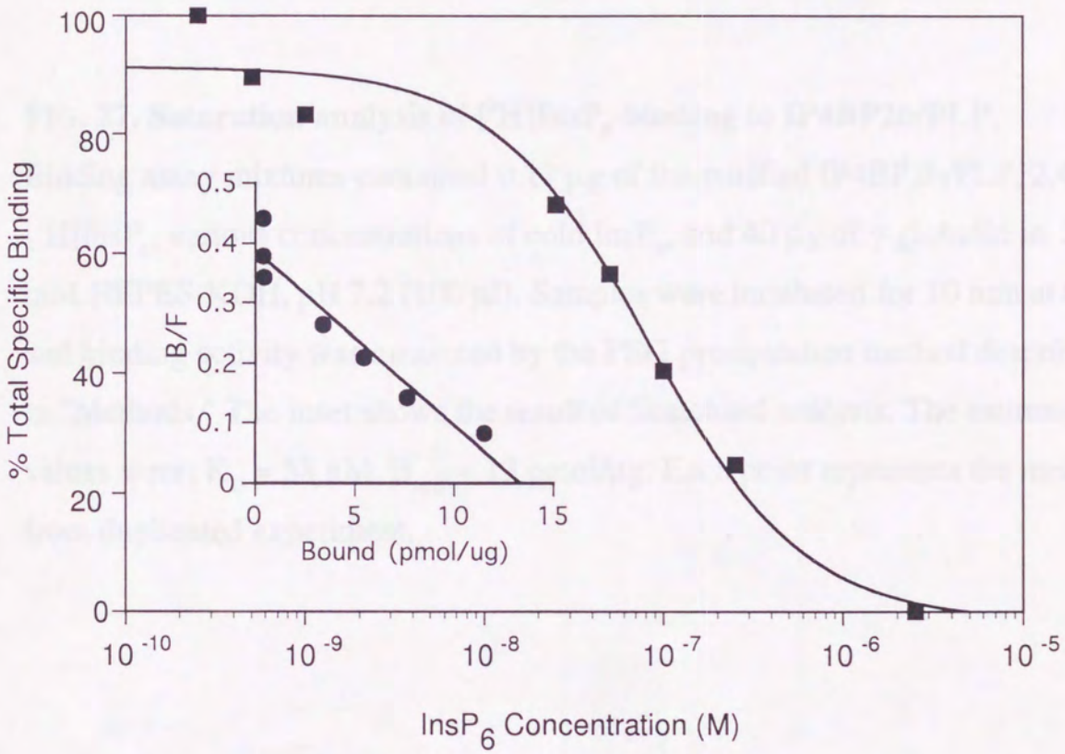

100
80
60
40
20
0
% Total Specific Binding
0.5
0.4
0.3
0.2
0.1
0
B/F
0
5
10
15
Bound (pmol/ug)
10 -10
10 -9
10 -8
10 -7
10 -6
10 -5
InsP6 Concentration (M)

FIG. 27. Saturation analysis of [^{3}H]InsP$_6$-binding to IP4BP2b/PLP.
Binding assay mixtures contained 0.17 μg of the purified IP4BP2b/PLP, 2.4 nM [^{3}H]InsP$_6$, various concentrations of cold InsP$_6$, and 40 μg of γ-globulin in 20 mM HEPES-KOH, pH 7.2 (100 μl). Samples were incubated for 10 min at 0 °C and binding activity was measured by the PEG precipitation method described in "Methods." The inset shows the result of Scatchard analysis. The estimated values were: K_D = 58 nM, B_{max} = 13 pmol/μg. Each point represents the mean from duplicated experiment.

FIG. 28

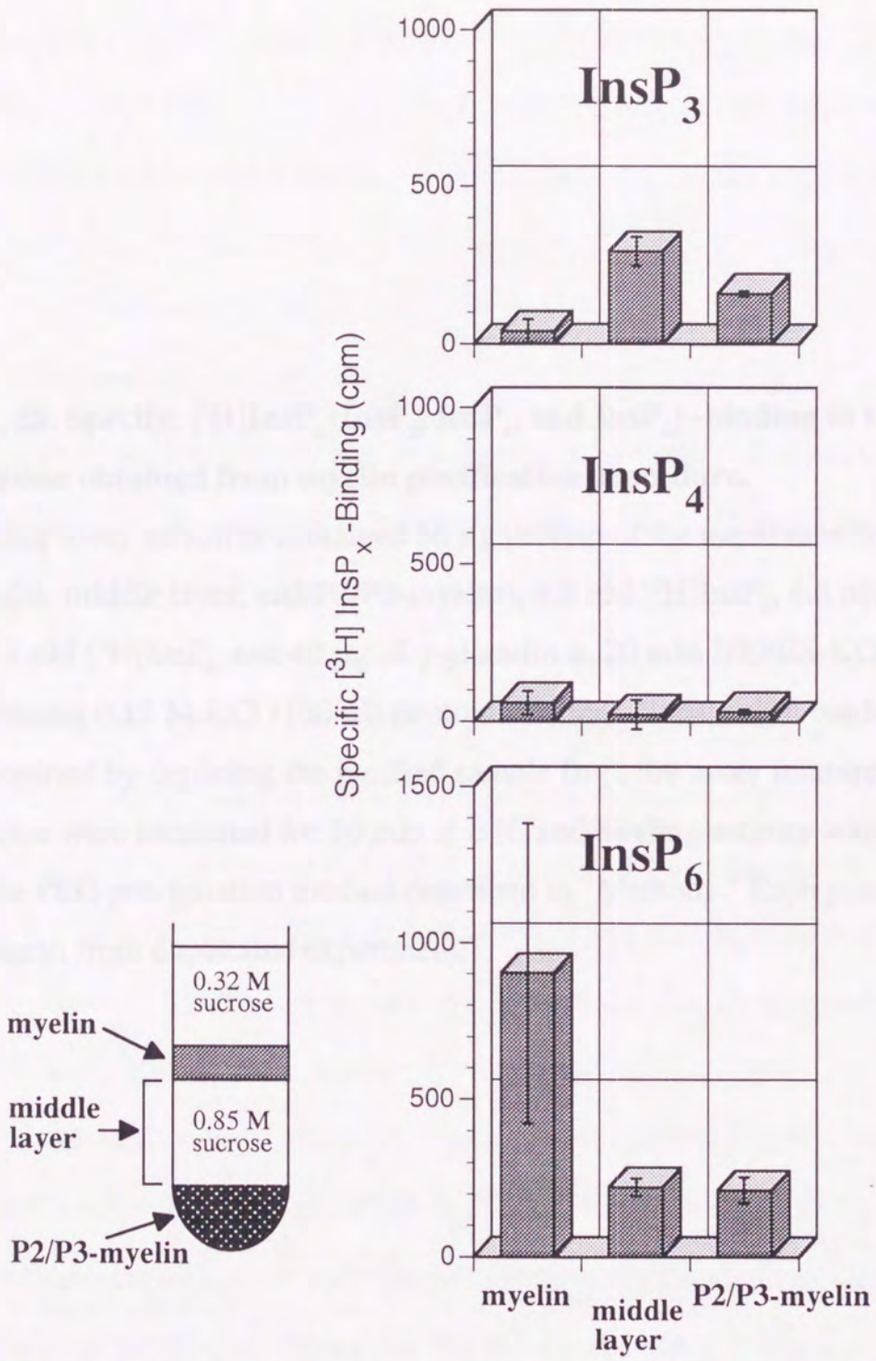

1000
500
0
InsP3
1000
500
0
InsP4
1500
1000
500
0
InsP6
Specific [3H] InsPx-Binding (cpm)
myelin
middle layer
P2/P3-myelin
0.32 M sucrose
0.85 M sucrose
myelin
middle layer
P2/P3-myelin

FIG. 28. Specific [^{3}H]InsP$_x$ (InsP$_3$, InsP$_4$, and InsP$_6$) -binding to the three fractions obtained from myelin purification procedure.

Binding assay mixtures contained 50 μg protein of the membrane fraction (myelin, middle layer, and P2/P3-myelin), 4.8 nM [^{3}H]InsP$_3$, 4.8 nM[^{3}H]InsP$_4$, or 4.1 nM [^{3}H]InsP$_6$, and 40 μg of γ-globulin in 20 mM HEPES-KOH, pH 7.2 containing 0.15 M KCl (100 μl) (isotonic buffer). Nonspecific binding was determined by depleting the purified sample from the assay mixture. The samples were incubated for 10 min at 0 °C and binding activity was measured by the PEG precipitation method described in "Methods." Each point represents the mean from duplicated experiment.

Discussion

I have purified and characterized three $InsP_4$-binding proteins (IP4BP1, IP4BP2a, and IP4BP2b) from mouse cerebella. These proteins were identified as synaptotagmin *II* (IP4BP1), aldolase A (IP4BP2a), and myelin proteolipid protein : PLP (IP4BP2b).

IP4BP1 / synaptotagmin *II*

I found that IP4BP1 showed structural characteristics identical to those of rat synaptotagmin *II*. The structural similarities between IP4BP1 and rat synaptotagmin *II* include (i) identical partial amino acid sequences of the 140K molecule (Fig. 4, B); (ii) the same 65K molecular weight as synaptotagmin, and a tendency to readily associate to form a dimer as determined by SDS-PAGE (Fig. 3 and 5); (iii) immunoreactivity of both the 140K and 65K molecules toward polyclonal antibodies produced against the C2A domain and carboxy-terminal peptide of rat synaptotagmin *II* (Fig. 5), and immunoprecipitation of $InsP_4$-binding activity by the anti-C2A antibody (Fig. 6). Based on these findings, I conclude that the purified IP4BP1 corresponds to mouse synaptotagmin *II*.

Synaptotagmin was identified as a synaptic vesicle membrane protein by an immunohistochemical approach (Matthew *et al.*, 1981). It was referred to as p65 from its molecular weight, and recently the primary sequences of synaptotagmin from several species have been determined (Geppert *et al.*, 1991; Perin *et al.*, 1991, b; Wendland *et al.*, 1991). The cDNA clones encoding synaptotagmin isolated from humans, rats, and drosophila predicted a highly conserved primary structure consisting of a small intravesicular sequence, a single transmembrane region, and a

large cytoplasmic domain containing two copies of an internal repeat homologous to the regulatory region of protein kinase C (Perin *et al.*, 1991, b). The importance of synaptotagmin has been well demonstrated by its presynaptic functions, especially in the exocytotic pathway of synaptic vesicles.

Two types of synaptotagmin (*I* and *II*) have been reported in the rat based on analysis of complementary DNA clones. RNA blotting experiments have demonstrated different distributions of the two synaptotagmins (Geppert *et al.*, 1991) with *II* being present in the phylogenetically older parts of the brain, such as the spinal cord, brain stem and cerebellum, and *I* in the newer regions, such as the cerebral cortex and hippocampus. Since cerebellum was used as a source of IP4BP, there may not be synaptotagmin *I* from the beginning of this purification process.

From the viewpoint of physiological role, there have been two exciting reports indicating the presence of functional domains of synaptotagmin. First, Elferink *et al.* (1993) reported that exocytosis in PC12 cells was suppressed by injection of a long peptide containing the C2A domain of synaptotagmin. Second, Bommert *et al.* (1993) showed that neurotransmitter release in the squid giant synapse is rapidly and reversibly inhibited by injection of a short peptide which has a lysine cluster within the C2A or C2B domain of synaptotagmin. These domains, which are homologous to the C2 region of PKC, are thought to mediate the binding of both phospholipids and Ca^{2+} to synaptotagmin as well as to PKC (Brose *et al.*, 1992; Davletov *et al.*, 1993). Therefore, these domains seems to mediate the binding of synaptotagmin to phospholipids.

The results obtained by DiAntonio *et al.* (1993) and Littleton *et al.* (1993) suggest that synaptotagmin functions as a barrier to spontaneous neurotransmitter release. Sollner *et al.* (1993) have reported that synaptotagmin operates as a "clamp"

to prevent fusion from proceeding in the absence of a signal. Thus the function of synaptotagmin seems to be a regulatory element preventing nonspecific and spontaneous neurotransmitter release.

How can I evaluate involvement of $InsP_4$ in the function of synaptotagmin? Recently, Fukuda *et al.* (1994) (my colleagues) reported that C2B domain was essential for $InsP_4$-binding. It was shown that while C2A domain binds phospholipid in a Ca^{2+}-dependent manner C2B domain binds phospholipids Ca^{2+}-independently. Furthermore, Llinas *et al.*, (1994) (my colleagues) displayed that the inositol high-polyphosphate series ($InsP_4$, $InsP_5$, $InsP_6$) blocked transmission in the squid giant synapse and reduced synaptic noise by spontaneous transmitter release.

These findings speculate the following mechanism for triggering the membrane fusion in vesicular exocytosis. The rise of calcium concentration seems to produce a conformational change in synaptotagmin via its calcium-sensitive C2A domain (Davletov *et al.*, 1993; Chapmann *et al.*, 1994; Fukuda *et al.*, 1994). This conformational change is thought to allow vesicles to fuse with the plasma membrane by direct phospholipid-protein interaction between the membrane and the C2A, C2B domains. Probably, this conformational change is triggered by calcium entry, but only when the C2B domain is free from inositol high-polyphosphate.

IP4BP2a / aldolase A

IP4BP2a have a structural similarities to aldolase A : (i) close identity of their N-terminal amino acid sequences (Table 3); (ii) the molecular weight determined by SDS-PAGE is about 40K similar to aldolase (Fig. 12); (iii) the apparent molecular weight on gel filtration chromatography is estimated to be 160K (Fig. 13); (iv) formation of tetramer by cross-linking (Fig. 14). Based on these results, I concluded

that purified IP4BP2a is mouse aldolase A.

Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) is a ubiquitous glycolytic enzyme that catalyzes the cleavage of fructose-1,6-bisphosphate. In vertebrates, three isozyme forms exist (Penhoet *et al.*, 1966). These three enzymes, aldolase A (muscle type), aldolase B (liver type) and aldolase C (brain type) have been purified from rabbit tissues, and have been well characterized. These isozymes have nearly the same molecular size, but they can be distinguished by their electrophoretic mobilities and substrate specificities as well as their tissue distribution (Penhoet *et al.*, 1966; Penhoet *et al.*, 1967; Horecker *et al.*, 1972). These are expressed in a tissue-specific manner. In the adult mammal aldolase A is found in every tissue except the liver, while aldolase B is found in the liver, kidney and small intestine. Brain is the only adult tissue which contains aldolase C (Lebherts *et al.*, 1969). Amino acid sequence studies indicate they are highly conserved and probably derived by divergent evolution from a common ancestral gene (Kukita *et al.*, 1988).

Koppitz *et al.* (1986) previously demonstrated that aldolase A from rabbit skeletal muscle binds with $InsP_3$ ($[L]_{0.5}$: values substituted for K_D= 0.58 μM) and $InsP_4$-mixture ($[L]_{0.5}$ = 0.83 μM). The inositol polyphosphate $InsP_3$, $InsP_4$-mixture, and Ins 1,4-P_2 are potent competitive inhibitors of the glycolytic activity of aldolase A. They induce a conformational change upon binding to the enzyme.

In skeletal muscle, aldolase A together with glyceraldehyde-3-phosphate dehydrogenase also exist in the triad junction. In this region the foot protein, which connects the terminal cisternae with the T-tubular membrane, might be involved in the binding of aldolase A. Vergara *et al.* (1985) and Volpe *et al.* (1985) suggested that $InsP_3$ in skeletal muscle mobilizes Ca^{2+}, and Varsányi *et al.* (1989) reported that in fast-twitch skeletal muscle inositol-phospholipid-metabolizing enzymes are associated

with triads. Since $InsP_3$ can displace aldolase A from the triad junction, Thieleczek *et al.* (1989) and Heilmeyer Jr. *et al.* (1990) thought that $InsP_3$ may regulate the glycolysis in the triad junction.

The binding mechanism between aldolase A and the triad junction appears to be electrostatic interaction, because it has been reported that aldolase can be solubilized easily from membranes by high salt concentrations and behave as water soluble proteins during purification (Thieleczek *et al.*, 1987; Varsányi *et al.*, 1989). In addition, aldolase A also binds to actin and tubulin. Many of glycolytic enzymes including aldolase A are basic protein with isoelectric point near 8 (Malamud *et al.*, 1978), while most proteins that bind glycolytic enzymes are acidic e.g., actin (Garrels *et al.*, 1976) and tubulin (Kelly *et al.*, 1983). Their binding mechanisms are also electrostatic interaction. Furthermore, proteolytic removal of the acidic C-terminals of tubulin results in loss of interaction between aldolase and tubulin (Carr *et al.*, 1993), which proved that the interaction is electrostatic. Inositol polyphosphates may competitively inhibit protein-protein interactions e.g., interaction between aldolase A and the triad junction (Thieleczek *et al.*, 1989).

Then, in brain, what kind of function does aldolase carry out? The K_D of purified IP4BP2a / aldolase A from mouse cerebella for $InsP_4$ is 1.2 μM. This value is nearly equal to $[L]_{0.5}$ of $InsP_4$-mixture-binding to aldolase A of skeletal muscle (Koppitz *et al.*, 1986). However $InsP_x$-binding-specificity of purified brain aldolase A is different from that of muscle aldolase A e.g., muscle aldolase A has the higher specificity against $InsP_3$. These may be caused by the difference in species or by the following hypothetical reason.

In brain of adult mammal, both A and C forms of aldolase exist. They have the same essential catalytic properties to cleave fructose-1,6-bisphosphate, but they are

different in their kinetic parameters (Penhoet *et al.*, 1969). While the kinetics of $InsP_x$-binding activity of aldolase C have not been reported, aldolase C seems to have distinct $InsP_x$-binding-specificity from aldolase A. In addition, aldolase is composed of four subunits, and in brain three aldolase A-C hybrid set : heterotetramers (A_3C, A_2C_2, and AC_3) are formed. The catalytic activities of their hybrids differ (Penhoet *et al.*, 1971). Because of identical N-terminal amino acid sequence, most of IP4BP2a should be aldolase A, but from the reasons mentioned above IP4BP2a fraction may contain small amount of aldolase C. It is not known at present by which step most of aldolase C had been separated.

Interestingly, Ahn *et al.* (1994) recently reported that the Zebrin *II* is aldolase C. Immunocytochemical staining with monoclonal antibody of Zebrin *II* reveal, as its name suggest, a series of stripes that are continuous across most folia of vermis (the middle third of the cerebellum). Zebrin *II* is a intracellular antigen found throughout the Purkinje cell from dendritic spine to axon tip (Hawkes *et al.*, 1985; Hawkes and Leclerc, 1986, 1987). Considering that in cerebellum (especially in Purkinje cell) the phosphoinositide cascade is operating vigorously, it is highly likely that aldolase A and aldolase C are involved in cerebellar functions. It seems that each distribution of aldolase A and aldolase C in the same cerebellar section needs to be studied.

IP4BP2b / PLP

I showed that IP4BP2b had almost the same structural characteristics as myelin proteolipid protein (PLP). The structural similarities between IP4BP2b and PLP include : (i) identity of their N-terminal amino acid sequences (Table 3); (ii) nearly the same molecular weight of IP4BP2b determined by SDS-PAGE (26K) as that of PLP (Fig. 19); (iii) immunoreactivity of 26K molecules against anti-PLP monoclonal

antibody (AA3 and AH7-2a) (Fig. 21), and immunoabsorption of $InsP_4$-binding activity of IP4BP2b by AA3 antibody (Fig. 22). From these results, I concluded that the purified IP4BP2b is mouse PLP.

Myelin proteolipid protein (PLP) is the most abundant component and the major integral membrane protein of CNS myelin, which is one of the most hydrophobic proteins. PLP can be dissolved in organic solvents (Folch-Pi and Lees, 1951) and thus called "proteolipid protein". Another less abundant proteolipid , DM20 whose mRNA was produced by alternative splicing of the PLP-mRNA precursor (Morello *et al.*, 1986; Hudson *et al.*, 1987), shows immunological cross-reactivity with PLP (Lees *et al.*, 1984). PLP and DM20 are synthesized by oligodendrocytes (Stoffel *et al.*, 1984). The molecular weights of PLP has been estimated to be 24K-26K and that of DM20 to be about 5K less than that of PLP by SDS-PAGE (Lees *et al.*, 1984). Lipids are covalently attached to PLP by thioester linkages (Ross and Braun, 1988; Bizzozero and Good, 1990; Bizzozero *et al.*, 1990; Weimbs and Stoffel, 1992). The amino acid and nucleotide sequences of the PLP of cow, rat, mouse, and human are closely homologous (Milner *et al.*, 1985; Naismith *et al.*, 1985; Diehl *et al.*, 1986; Gardinier *et al.*, 1986; Nave *et al.*, 1986; Hudson *et al.*, 1987; Macklin *et al.*, 1987; Puckett *et al.*, 1987; Ikenaka *et al.*, 1988). Further, the mutation within the PLP gene causes severe dysmyelination (Hogan and Greenfield 1984). Therefore, PLP is thought to play a significant role in myelination in CNS, presumably by promoting the apposition of extracellular surfaces of the myelin lamellae.

Since PLP is one the most hydrophobic proteins in nature and insoluble in aqueous solution, the usual purification of PLP is carried out after solubilization by organic solvents like mixture of chloroform and methanol (Lees and Sakura, 1978). However, in this study, I have succeeded in purifying PLP after solubilization with

Triton X-100. Using nondenaturing detergent, PLP seems to have been purified in closely natural conformation.

Current model (Fig. 29) for topology of PLP in the plasma membrane is proposed by Weimbs and Stoffel (1992). This model shows that PLP has four α-helical transmembrane regions, two pairs of disulfide bonds (Cys^{183}-Cys^{227}, Cys^{200}-Cys^{229}), and six cysteine residues (in positions 5, 6, 9, 108, 138, and 140) acylated with long-chain fatty acids. The N-terminus and the C-terminus of PLP in this model are on cytoplasmic side. In addition, the highly basic residues deleted in DM20 is on cytoplasmic side.

The two monoclonal antibodies against PLP, AH7-2a (epitope is amino acid residue number 209-217) and AA3 (epitope is 264-276), bind with both PLP and DM20, further, N-terminal sequences of PLP and DM20 are identical. Immunoblot analysis of myelin fraction detected two bands of 26K and 20K, corresponding to PLP and DM20, respectively (Fig. 21). Since the final sample only contained 26K protein, I concluded that purified IP4BP2b (26K protein) is PLP itself.

From immunoblot analysis of each purification step with AA3, it became clear that most of DM20 was removed at the Heparin-Agarose step (data not shown). Since PLP is a highly basic protein with an isoelectric point greater than 9 (Draper *et al.*, 1978), PLP binds with heparin, which is an acidic polysaccaride having many sulfate groups. Their binding mechanisms are probably electrostatic interaction. DM20, however, did not bind to heparin , probably because the basic residues 116 to 150 of PLP is deleted in DM20 (Nave *et al.*, 1987) and DM20 is more hydrophobic than PLP (Skalidis *et al.*, 1986). As DM20 has not been purified, it is not known whether DM20 has $InsP_4$-binding activity or not.

How do $InsP_4$ or $InsP_x$ modulate the function of PLP? PLP is thought to

FIG. 29

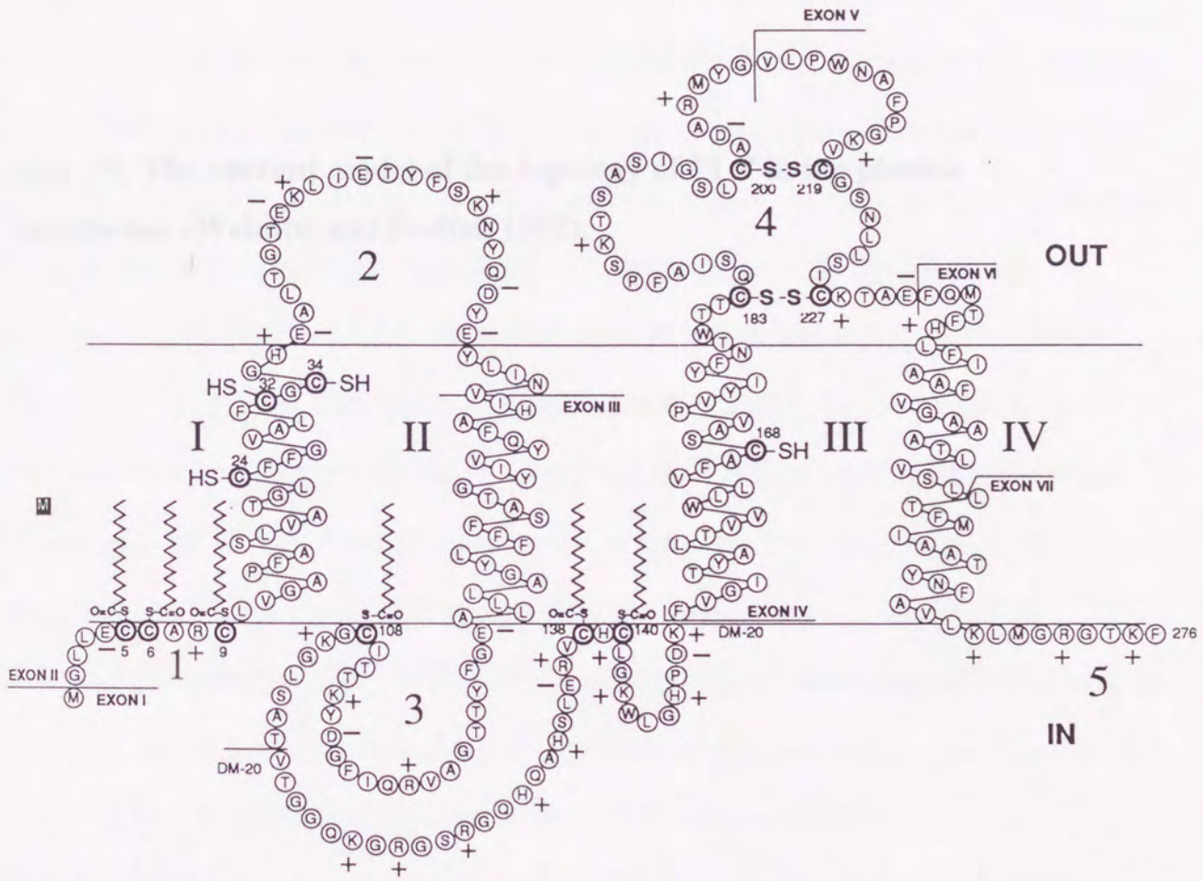

EXON V
OUT
EXON VI
EXON III
EXON VII
EXON IV
DM-20
EXON II
EXON I
DM-20
IN
I
II
III
IV
1
2
3
4
5
HS
SH
HS
SH
M

FIG. 29. The current model of the topology of PLP in the plasma membrane (Weimbs and Stoffel, 1992).

promote close packing of lipid bilayer in intact CNS myelin. Therefore, it is difficult to think that PLP binds with $InsP_x$ in tightly packed myelin.

Generally, integral membrane proteins are translated in rough endoplasmic reticulum (rough ER). The nascent protein is cotranslationally inserted into the ER membrane and passes through the Golgi apparatus en route to the plasma membrane (Sabatini *et al.*, 1982). PLP mRNA is also associated with polysome on rough ER (Colman *et al.*, 1982) and immunoreactive product has been detected in membranous structures interpreted as cisternae of the Golgi apparatus of oligodendrocytes in vivo (Nussbaum and Roussel, 1983; Schwob *et al.*, 1985; Roussel *et al.*, 1987). Sinoway *et al.* (1994) showed that DM20 alone and PLP coexpressed with DM20 assume appropriate conformations for transport to the cell surface using transfected HeLa cells. Gow *et al.* (1994) also reported that PLP is synthesized in the rough ER and passes through the Golgi apparatus to the cell surface in the Cos-7 cells transfected with human PLP cDNA. During this process, a significant lag exists between biosynthesis of PLP on rough ER and its insertion into the expanding myelin membrane (Benjamins *et al.*, 1978; Colman *et al.*, 1982). This delay can be as long as 20 days, in the case of cultured oligodendrocytes from postnatal day 2 rat (Hudson *et al.*, 1989). The delay between biosynthesis and insertion of PLP into plasma membrane of oligodendrocytes suggested that PLP has some relation with integral membrane protein transport system. It is possible that $InsP_4$ or $InsP_x$ may regulate the protein-protein interaction during this process e.g., the interaction between the cytoplasmic basic region of PLP and cytoskeletal protein having acidic region (actin, tubulin, *etc.*).

On the other hand, very interestingly, Kitagawa *et al.* 1993 demonstrated that the proteolipid DM polypeptide found in elasmobranch CNS contain regions having

homology to the pore-forming region of ligand-gated channels (the rat GluR1 and the Torpedo nAcR). In addition, many groups reported that the PLP purified through solubilization in organic solvents have ionophoretic ability (Na^+, K^+, or Ca^{2+} channel activities) within the lipid bilayer (Ting-Beall *et al.*, 1979; Helynck *et al.*, 1983; de Cózar *et al.*, 1987; Díaz *et al.*, 1990). $InsP_4$ or $InsP_x$ may regulate these channel activities. To prove the existence of such a regulation, it is necessary to perform the planar lipid bilayer experiments using the sample in nearly native condition like the PLP purified in this study.

Other $InsP_4$ and $InsP_x$-binding proteins

Recently, Donié and Reiser (1991) reported solubilization and partial purification of a high-affinity $InsP_4$-binding protein (K_D = 5.6 nM) from pig cerebellum. Theibert *et al.* (1991) also isolated two types of proteins (K_D = 3-4 nM) with $InsP_4$-binding activity from rat cerebellum. Although their functions have not yet been elucidated, they are certainly $InsP_4$- binding proteins because they can be labeled with a specific $InsP_4$ photoaffinity probe (Reiser *et al.*, 1991; Theibert *et al.*, 1992).

The same inositol polyphosphate ($InsP_x$) -binding protein has also been isolated by two other groups. Very interestingly, partial amino acid sequencing of this protein has shown that it is clathrin assembly protein (AP-2) which is possibly an essential protein in the endocytotic or recycling pathway of synaptic vesicles (Theibert *et al.*, 1991; Chadwick *et al.*, 1992; Voglemaier *et al.*, 1992; Timerman *et al.*, 1992). The K_D of $InsP_x$-binding protein toward $InsP_6$ reported by Theibert *et al.* (1991) is 12 nM and that of the protein reported by Chadwick *et al.* (1992) is 0.12 μM. Dependence of the $InsP_x$-binding on the salt concentration of assay system seems to cause this difference. In addition, coatomer which is a cytosolic protein complex containing subunits of

non-clathrin-coated Golgi intercisternal transport vesicles, has shown to have the $InsP_x$-binding activity (K_D for $InsP_6$ = 0.2 nM, K_D for $InsP_4$ = 0.1 nM) by Fleischer *et al.* (1994).

In this study, by which purification steps these $InsP_4$-binding proteins were eliminated? As a start material, Donié and Reiser (1991) used pig cerebellum and Theibert *et al.* (1991) used rat cerebellum. Since I used mouse cerebellum, the $InsP_4$-binding proteins found in other materials may not be present in starting material. However, there is a good possibility that at the step of solubilization they were eliminated from IP4BP in this study. The protein of Donié and Reiser (1991) was solubilized by treatment with Brij 58 (1.5%) under hypertonic condition (400mM NaCl). The protein of Theibert *et al.* (1991) was solubilized by treatment with CHAPS (1%) under hypotonic condition (low salt). Because of solubilization with Triton X-100 (1%) under hypotonic condition (low salt), their proteins(especially the protein of Donié and Reiser) are unlikely to have been solubilized. Considering the protein of Theibert *et al.* (1991), it was not described which kind of ion-exchange resin adsorbed this protein. However, since this protein was eluted from Heparin -Agarose column by the solution containing 0.75 M NaCl at pH 7.7, it may have been separated from IP4BP1 at the step of 0.5 M NaCl elution at pH 8.0 on Heparin-Agarose chromatography and from IP4BP2 at the step of adsorption at pH 8.0 to DE-52. Further, most of vesicles containing AP-2 or coatomer may have been contained in the cytosol fraction (the supernatant of centrifuge at 100,000 x g).

From the viewpoint of $InsP_4$ or $InsP_x$-binding activities, IP4BP1/synaptotagmin *II* and IP4BP2b/PLP purified in this study resemble AP-2, whereas IP4BP2a/aldolase A does not fit into any groups because of its comparatively lower affinity and different specificities toward $InsP_4$ or $InsP_x$. The $InsP_x$-binding site of synaptotagmin

II was determined to be C2B region (amino acid residues, 315-346) (Fukuda *et al.*, 1994). Although the C2A domain also contains lysine-rich sequence, $InsP_4$ bound only to the C2B domain. This fact indicated that the C2A and C2B domains of synaptotagmin *II* have different conformation and function. The C2B domain of synaptotagmin is highly conserved from *C. elegans* to human (Perin *et al.*, 1991, b; Wendland *et al.*, 1991; Bommert *et al.*, 1993; Nonet *et al.*, 1993), suggesting that the $InsP_x$-binding ability has also been kept during evolution. However, the consensus sequence of the $InsP_x$-binding site is still unclear, and furthermore the $InsP_x$-binding site of other proteins (PLP, AP-2, and coatomer) have not been determined. Probably, the $InsP_x$-binding site is in the basic region and governed by a certain rule. It is necessary to determine the binding site of other proteins.

The possibility that IP4BP purified in this study regulates intracellular Ca^{2+} concentration as IP4R

Many observations that $InsP_4$ regulates intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) have been reported (described in "Introduction"). Can IP4BP purified in this study play a role as channel proteins? Aldolase A could not be a channel protein because it is a cytosolic and glycolytic enzyme. Although synaptotagmin is an integral membrane protein of synaptic vesicles, it may not make a channel because it only has one single transmembrane region unlike general channel proteins [e.g. ligand-gated channels (nAcR, GluR, etc.) or voltage-gated channels (K^+, Na^+, Ca^{2+}-channel)]. Since PLP has four α-helical transmembrane regions and already have been reported as a channel protein (Ting-Beall *et al.*, 1979; Helynck *et al.*, 1983; de Cózar *et al.*, 1987; Díaz *et al.*, 1990), there is a possibility that PLP regulates $[Ca^{2+}]_i$ as a IP4R.

To prove this, I tried to reconstitute the purified PLP in the planar lipid bilayer,

or to measure $[Ca^{2+}]_i$ with Fura 2-AM in the NIH/3T3 fibroblast forced to produce PLP. Preliminary experiments suggested that PLP can form a channel in the planar lipid bilayer system (data not shown). However, when $InsP_4$ was applied by diffusion from the patch pipette into the cytoplasm of the NIH/3T3 fibroblast forced to produce PLP, change in $[Ca^{2+}]_i$ was similar to that of parent NIH/3T3 fibroblast serving as a control (data not shown). These findings are against the hypothesis that PLP regulates $[Ca^{2+}]_i$ by forming $InsP_4$-sensitive calcium channel. It is also unlikely that AP-2 and coatomer on vesicles to have such roles, and therefore, other $InsP_4$-binding proteins that serve as "IP4R" might exist. On the other hand, since the physiological roles of $InsP_4$ reported so far were mostly observed in cells of peripheral tissues (described in "Discussion"), IP4R playing such a role may not be distributed in brain.

Relationships among $InsP_x$-binding proteins

Only aldolase A is an enzyme. Synaptotagmin *II*, AP-2, and coatomer are well known to participate in vesicle transport system. As stated above, PLP also seems to be involved in this system. Therefore, the following discussion centers the relationship among synaptotagmin *II*, PLP, AP-2, and coatomer.

Interestingly Zhang *et al.* (1994) reported that synaptotagmin has a property as the AP-2 receptor and that AP-2 binds to the C2B domain of the molecule. Because the C2B domain is the $InsP_4$-binding site as discussed above, the protein -protein interaction between synaptotagmin and AP-2 is presumably regulated by $InsP_x$ as that of aldolase and triad junction. In addition, $InsP_3$ and $InsP_x$ affect three activities of AP-2: self-association, clathrin binding, and AP-2-mediated coat assembly (Beck and Keen 1991). These findings suggest strong involvement of $InsP_x$ in the regulation of

the endocytotic pathway of synaptic vesicles.

On the other hand, AP-2 , coatomer, and additionally AP-3 (cardiac clathrin assembly protein) forms potassium-selective ion channels regulated by $InsP_x$ (Chadwick *et al.*, 1992; Timerman *et al.*, 1992; Kijima *et al.*, 1993). PLP also seems to form a cation-selective channel (Ting-Beall *et al.*, 1979; Helynck *et al.*, 1983; de Cózar *et al.*, 1987; Díaz *et al.*, 1990). During the vesicle transport pathway they may control the ionic condition in the vesicles, modulated by $InsP_x$.

In conclusion, I identified synaptotagmin *II* and PLP as $InsP_x$-binding proteins and from the relationship with other $InsP_x$-binding proteins I propose that inositol tetrakisphosphate and other inositol high-polyphosphates may be important as putative second messenger in regulation of exo- or endocytosis.

Acknowledgments

I would like to thank Prof. Katsuhiko Mikoshiba and Prof. Kazuhiro Ikenaka for their continuing support and helpful discussion throughout this study. I express my gratitude to Dr. Niinobe for his helpful assistance and valuable discussions. I appreciate supports of Prof. Fumio Sakiyama. I wish to thank all the members of the Laboratory of Neural Information and the Mikoshiba Laboratory, especially Dr. Shinji Nakade, Mr. Mitsunori Fukuda, and Dr. Tetsushi Kagawa, for their helpful assistance and discussions. I am grateful to Dr. Marjorie B. Lees (E. K. Shriver Center for Mental Retardation) for her gift of anti-PLP monoclonal antibodies, and Dr. Shoichiro Ozaki (Ehime University) and Dr. Masato Hirata (Kyusyu University) for their gift of inositol polyphosphates. My thanks are also due to Mr. Akira Yamamoto and Mrs. Yumi Yoshimura for their excellent technical assistance.

References

Ahn, A. H., Dziennis, S., Hawkes, R. and Herrup, K. (1994).
The cloning of zebrin *II* reveals its identity with aldolase C. *Development* **120**, 2081-2091.

Beck, K. A. and Keen, J. H. (1991).
Interaction of phosphoinositide cycle intermediates with the plasma membrane-associated clathrin assembly protein AP-2. *J Biol Chem* **266**, 4442-7.

Benjamins, J. A., Iwata, R. and Hazlett, J. (1978).
Kinetics of entry of proteins into the myelin membrane. *J Neurochem* **31**, 1077-85.

Berridge, M. J. and Irvine, R. F. (1989).
Inositol phosphates and cell signalling. *Nature* **341**, 197-205.

Bizzozero, O. A. and Good, L. K. (1990).
Myelin proteolipid protein contains thioester-linked fatty acids. *J Neurochem* **55**, 1986-92.

Bizzozero, O. A., Good, L. K. and Evans, J. E. (1990).
Cysteine-108 is an acylation site in myelin proteolipid protein. *Biochem Biophys Res Commun* **170**, 375-82.

Bommert, K., Charlton, M. P., DeBello, W. M., Chin, G. J., Betz, H. and Augustine, G. J. (1993).
Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis. *Nature* **363**, 163-5.

Brose, N., Petrenko, A. G., Südhof, T. C. and Jahn, R. (1992).
Synaptotagmin: a calcium sensor on the synaptic vesicle surface. *Science* **256**, 1021-5.

Carr, D. and Knull, H. (1993).
Aldolase-tubulin interactions: removal of tubulin C-terminals impairs interactions. *Biochem Biophys Res Commun* **195**, 289-93.

Chadwick, C. C., Timerman, A. P., Saito, A., Mayrleitner, M., Schindler, H. and Fleischer, S. (1992).
Structural and functional characterization of an inositol polyphosphate receptor from cerebellum. *J Biol Chem* **267**, 3473-81.

Changya, L., Gallacher, D. V., Irvine, R. F. and Petersen, O. H. (1989).
Inositol 1,3,4,5-tetrakisphosphate and inositol 1,4,5-trisphosphate act by different mechanisms when controlling Ca^{2+} in mouse lacrimal acinar cells. *Febs Lett* **251**, 43-8.

Chapman, E. R. and Jahn, R. (1994).
Calcium-dependent interaction of the cytoplasmic region of synaptotagmin with membranes. Autonomous function of a single C2-homologous domain. *J Biol Chem* **269**, 5735-41.

Colman, D. R., Kreibich, G., Frey, A. B. and Sabatini, D. D. (1982).
Synthesis and incorporation of myelin polypeptides into CNS myelin. *J Cell Biol* **95**, 598-608.

Davletov, B. A. and Südhof, T. C. (1993).
A single C2 domain from synaptotagmin I is sufficient for high affinity Ca^{2+}/phospholipid binding. *J Biol Chem* **268**, 26386-90.

de Cózar. M., Lucas, M. and Monreal, J. (1987).
Ionophoric properties of the proteolipid apoprotein from bovine brain myelin. *Biochem Int* **14**, 833-41.

DiAntonio, A., Parfitt, K. D. and Schwarz, T. L. (1993).
Synaptic transmission persists in synaptotagmin mutants of Drosophila. *Cell* **73**, 1281-90.

Díaz, R. S., Monreal, J. and Lucas, M. (1990).
Calcium movements mediated by proteolipid protein and nucleotides in liposomes prepared with the endogenous lipids from brain white matter. *J Neurochem* **55**, 1304-9.

Diehl, H. J., Schaich, M., Budzinski, R. M. and Stoffel, W. (1986).
Individual exons encode the integral membrane domains of human myelin proteolipid protein [published erratum appears in *Proc Natl Acad Sci U S* 1991 Apr;86(6):617-8]. *Proc Natl Acad Sci U S A* **83**, 9807-11.

Donié, F., Hulser, E. and Reiser, G. (1990).
High-affinity inositol 1,3,4,5-tetrakisphosphate receptor from cerebellum: solubilization, partial purification and characterization. *Febs Lett* **268**, 194-8.

Donié, F. and Reiser, G. (1989).
A novel, specific binding protein assay for quantitation of intracellular inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) using a high-affinity $InsP_4$ receptor from cerebellum. Febs Lett **254**, 155-8.

Donié, F. and Reiser, G. (1991).
Mass measurements of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate in a neuronal cell line stimulated with bradykinin: inositolphosphate response shows desensitization. *Biochem Biophys Res Commun* **181**, 997-1003.

Draper, M., Lees, M. B. and Chan, D. S. (1978).
Isoelectric focusing of proteolipid. *J Neurochem* **31**, 1095-1099.

Elferink, L. A., Peterson, M. R. and Scheller, R. H. (1993).
A role for synaptotagmin (p65) in regulated exocytosis. *Cell* **72**, 153-9.

Ely, J. A., Hunyady, L., Baukal, A. J. and Catt, K. J. (1990).
Inositol 1,3,4,5-tetrakisphosphate stimulates calcium release from bovine adrenal microsomes by a mechanism independent of the inositol 1,4,5-trisphosphate receptor. *Biochem J* **268**, 333-8.

Ferris, C. D., Huganir, R. L., Bredt, D. S., Cameron, A. M. and Snyder, S. H. (1991).
Inositol trisphosphate receptor: phosphorylation by protein kinase C and calcium calmodulin-dependent protein kinases in reconstituted lipid vesicles. *Proc Natl Acad Sci U S A* **88**, 2232-5.

Ferris, C. D., Huganir, R. L., Supattapone, S. and Snyder, S. H. (1989).
Purified inositol 1,4,5-trisphosphate receptor mediates calcium flux in reconstituted lipid vesicles. *Nature* **342**, 87-9.

Fleischer, B., Xie, J., Mayrleitner, M., Shears, S. B., Palmer, D. J. and Fleischer, S. (1994).
Golgi coatomer binds, and forms K^+-selective channels gated by, inositol polyphosphates. *J Biol Chem* **269**, 17826-32.

Folch, J. and Lees, M. (1951).
Ploteolipids, a new type of tissue lipoprotein. *J Biol Chem* **191**, 807-817.

Fukuda, M., Aruga, J., Niinobe, M., Aimoto, S. and Mikoshiba, K. (1994).
Inositol-1,3,4,5-tetrakisphosphate binding to C2B domain of IP4BP/synaptotagmin *II*. *J Biol Chem* **269**, 29206-29211.

Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. and Mikoshiba, K. (1989). Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P_{400}. *Nature* **342**, 32-8.

Gardinier, M. V., Macklin, W. B., Diniak, A. J. and Deininger, P. L. (1986). Characterization of myelin proteolipid mRNAs in normal and jimpy mice. *Mol Cell Biol* **6**, 3755-62.

Garrels, J. I. and Gibson, W. (1976). Identification and characterization of multiple forms of actin. *Cell* **9**, 793-805.

Geppert, M., Archer, B. 3. and Südhof, T. C. (1991). Synaptotagmin *II*. A novel differentially distributed form of synaptotagmin. *J Biol Chem* **266**, 13548-52.

Gow, A., Friedrich, V. J. and Lazzarini, R. A. (1994). Intracellular transport and sorting of the oligodendrocyte transmembrane proteolipid protein. *J Neurosci Res* **37**, 563-73.

Greengard, P., Valtorta, F., Czernik, A. J. and Benfenati, F. (1993). Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* **259**, 780-5.

Hawkes, R., colonnier, M. and Leclerc, N. (1985). Monoclonal antibodies reveal sagittal banding in the rodent cerebellar cortex. *Brain Res* **333**, 359-65.

Hawkes, R. and Leclerc, N. (1986). Immunocytochemical demonstration of topographic ordering of Purkinje cell axon terminals in the fastigial nuclei of the rat. *J Comp Neurol* **244**, 481-91.

Hawkes, R. and Leclerc, N. (1987).
Antigenic map of the rat cerebellar cortex: the distribution of parasagittal bands as revealed by monoclonal anti-Purkinje cell antibody mabQ113. *J Comp Neurol* **256**, 29-41.

Heilmeyer, L. J., Han, J. W., Thieleczek, R., Varsányi, M. and Mayr, G. W. (1990).
Relation of phosphatidylinositol metabolism to glycolytic pathway in skeletal muscle membranes. *Mol Cell Biochem* 99, 111-6.

Helynck, G., Luu, B., Nussbaum, J. L., Picken, D., Skalidis, G., Trifilieff, E., Van, D. A., Seta, P., Sandeaux, R., Gavach, C., Heitz, F., Simon, D. and Spach, G. (1983).
Brain proteolipids. Isolation, purification and effect on ionic permeability of membranes. *Eur J Biochem* **133**, 689-95.

Hill, T. D., Dean, N. M. and Boynton, A. L. (1988).
Inositol 1,3,4,5-tetrakisphosphate induces Ca^{2+} sequestration in rat liver cells. *Science* **242**, 1176-8.

Hogan, E. L. and Greenfield, S. (1994).
Animal models of genetic disorders of myelin. In *Myelin*, (P. Morell, eds.) pp. 489-534. Plenum Press. New York.

Horecker, B. L. (1972).
Aldolase. In *The Enzymes*, (P. D. Boyer, eds.) pp. 213-258. Acasemic Press. New York.

Hudson, L. D., Berndt, J. A., Puckett, C., Kozak, C. A. and Lazzarini, R. A. (1987).
Aberrant splicing of proteolipid protein mRNA in the dysmyelinating jimpy mutant mouse. *Proc Natl Acad Sci U S A* **84**, 1454-8.

Hudson, L. D., Friedrich, V. J., Behar, T., Dubois, D. M.
and Lazzarini, R. A. (1989).
The initial events in myelin synthesis: orientation of proteolipid protein in the plasma membrane of cultured oligodendrocytes. *J Cell Biol* **109**, 717-27.

Ikenaka, K., Furuichi, T., Iwasaki, Y., Moriguchi, A., Okano, H.
and Mikoshiba, K. (1988).
Myelin proteolipid protein gene structure and its regulation of expression in normal and jimpy mutant mice. *J Mol Biol* **199**, 587-96.

Irvine, R. F. and Moor, R. M. (1986).
Micro-injection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca^{2+}. *Biochem J* **240**, 917-20.

Iwamatsu, A. (1991).
Seikagaku (in Japan) **63**, 139-143.

Kelly, W. G., Passaniti, A., Woods, J. W., Daiss, J. L. and Roth, T. F. (1983).
Tubulin as a molecular component of coated vesicles. *J Cell Biol* **97**, 1191-9.

Kijima, Y., Mayrleitner, M., Timerman, A. P., Saito, A., Schindler, H. and Fleischer, S. (1993).
A cardiac clathrin assembly protein forms a potassium channel in planar lipid bilayers. *J Biol Chem* **268**, 16253-8.

Kitagawa, K., Sinoway, M. P., Yang, C., Gould, R. M.
and Colman, D. R. (1993).
A proteolipid protein gene family: expression in sharks and rays and possible evolution from an ancestral gene encoding a pore-forming polypeptide. *Neuron* **11**, 433-48.

Koppitz, B., Vogel, F. and Mayr, G. W. (1986).
Mammalian aldolases are isomer-selective high-affinity inositol polyphosphate binders. *Eur J Biochem* **161**, 421-33.

Kukita, A., Mukai, T., Miyata, T. and Hori, K. (1988).
The structure of brain-specific rat aldolase C mRNA and the evolution of aldolase isozyme genes. *Eur J Biochem* **171**, 471-8.

Laemmli, U. K. (1970).
Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-5.

Lebherz, H. G. and Rutter, W. J. (1969).
Distribution of fructose diphosphate aldolase variants in biological systems. *Biochemistry* **8**, 109-21.

Lees, M. B. and Brostoff, S. W. (1984).
Proteins of myelin. In *Myelin*, (P. Mollel, eds.) pp. 197-224. Prenum Press. New York.

Lees, M. B. and Sakura, J. D. (1978).
Preparation of Proteolipid. In *Research Methods in Neurochemistry*, (N. Markes and R. Rodnight, eds.) pp. 354-370. Plenum Press. New York.

Littleton, J. T., Stern, M., Schulze, K., Perin, M. and Bellen, H. J. (1993).
Mutational analysis of Drosophila synaptotagmin demonstrates its essential role in Ca^{2+}-activated neurotransmitter release. *Cell* **74**, 1125-34.

Llinás, R., Sugimori, M., Lang, E. J., Morita, M., Fukuda, M., Niinobe, M., Mikoshiba, K. (1994).
The inositol high-polyphosphate series blocks synaptic transmission by preventing vesicular fusion: A squid giant synapse study. *Proc Natl Acad Sci U S A* **91**, 12990-12993.

Lucas, A. H., Madison, D. L., Simons, K. and Pfeiffer, S. E. (1994).
Myelin membrane biogenesis by oligdendrocytes / Developmental reguration of low molecular weight GTP-binding proteins. *Febs Lett* **347**, 273-278.

Lückhoff, A. and Clapham, D. E. (1992).
Inositol 1,3,4,5-tetrakisphosphate activates an endothelial Ca^{2+}-permeable channel [see comments]. *Nature* **355**, 356-8.

Macklin, W. B., Campagnoni, C. W., Deininger, P. L.
and Gardinier, M. V. (1987).
Structure and expression of the mouse myelin proteolipid protein gene. *J Neurosci Res* **18**, 383-394.

Maeda, N., Kawasaki, T., Nakade, S., Yokota, N., Taguchi, T., Kasai, M. and Mikoshiba, K. (1991).
Structural and functional characterization of inositol 1,4,5-trisphosphate receptor channel from mouse cerebellum. *J Biol Chem* **266**, 1109-16.

Maeda, N., Niinobe, M. and Mikoshiba, K. (1990).
A cerebellar Purkinje cell marker P_{400} protein is an inositol 1,4,5-trisphosphate ($InsP_3$) receptor protein. Purification and characterization of $InsP_3$ receptor complex. *EMBO J* **9**, 61-7.

Mailleux, P., Takazawa, K., Erneux, C. and Vanderhaeghen, J. J. (1991).
Inositol 1,4,5-trisphosphate 3-kinase distribution in the rat brain. High levels in the hippocampal CA1 pyramidal and cerebellar Purkinje cells suggest its involvement in some memory processes. *Brain Res* **539**, 203-10.Malamud, D. and Drysdale, J. W. (1978).
Isoelectric points of proteins: a table. *Anal Biochem* **86**, 620-47.

Matthew, W. D., Tsavaler, L. and Reichardt, L. F. (1981).
Identification of a synaptic vesicle-specific membrane protein with a wide distribution in neuronal and neurosecretory tissue. *J Cell Biol* **91**, 257-69.

McPherson, M. J., Quirke, P. and Taylor, G. R. (1991).
PCR: Polymerase chain reaction. In *A Practical Approach*, (Rickwood, D. and Hames, B.D. eds.) pp. IRL Press. Oxford.

Milner, R. J., Lai, C., Nave, K. A., Lenoir, D., Ogata, J. and Sutcliffe, J. G. (1985).
Nucleotide sequences of two mRNAs for rat brain myelin proteolipid protein. *Cell* **42**, 931-9.

Morello, D., Dautigny, A., Pham, D. D. and Jolles, P. (1986).
Myelin proteolipid protein (PLP and DM-20) transcripts are deleted in impy mutant mice. *EMBO J* **5**, 3489-93.

Morris, A. P., Gallacher, D. V., Irvine, R. F. and Petersen, O. H. (1987).
Synergism of inositol trisphosphate and tetrakisphosphate in activating Ca^{2+}-dependent K^+ channels. *Nature* **330**, 653-5.

Naismith, A. L., Hoffman, C. E., Tsui, L. C. and Riordan, J. R. (1985).
Study of the expression of myelin proteolipid protein (lipophilin) using a cloned complementary DNA. *Nucleic Acids Res* **13**, 7413-25.

Nakade, S., Maeda, N. and Mikoshiba, K. (1991).
Involvement of the C-terminus of the iositol 1,4,5-trisphosphate receptor in Ca^{2+} release analysed using region-specific monoclonal antibodies. *Biochem J* **277**, 125-131.

Nakade, S., Rhee, S. K., Hamanaka, H. and Mikoshiba, K. (1994).
Cyclic AMP-dependent phosphorylation of an immunoaffinity-purified homotetrameric inositol 1,4,5-trisphosphate receptor (type I) increases Ca^{2+} flux in reconstituted lipid vesicles. *J Biol Chem* **269**, 6735-42.

Nave, K. A., Lai, C., Bloom, F. E. and Milner, R. J. (1986).
Jimpy mutant mouse: a 74-base deletion in the mRNA for myelin proteolipid protein and evidence for a primary defect in RNA splicing. *Proc Natl Acad Sci U S A* **83**, 9264-8.

Nave, K. A., Lai, C., Bloom, F. E. and Milner, R. J. (1987).
Splice site selection in the proteolipid protein (PLP) gene transcript and primary structure of the DM-20 protein of central nervous system myelin. *Proc Natl Acad Sci U S A* **84**, 5665-9.

Nonet, M. L., Grundahl, K., Meyer, B. J. and Rand, J. B. (1993).
Synaptic function is impaired but not eliminated in *C. elegans* mutants lacking synaptotagmin. *Cell* **73**, 1291-305.

Nussbaum, J. L. and Roussel, G. (1983).
Immunocytochemical demonstration of the transport of myelin proteolipids through the Golgi apparatus. *Cell Tissue Res* **234**, 547-59.

Penhoet, E., Kochman, M., Valentine, R. and Rutter, W. J. (1967).
The subunit structure of mammalian fructose diphosphate aldolase. *Biochemistry* **6**, 2940-9.

Penhoet, E., Rajkumar, T. and Rutter, W. J. (1966).
Multiple forms of fructose diphosphate aldolase in mammalian tissues. *Proc Natl Acad Sci U S A* **56**, 1275-82.

Penhoet, E. E., Kochman, M. and Rutter, W. J. (1969).
Molecular and catalytic properties of aldolase C. *Biochemistry* **8**, 4396-402.

Penhoet, E. E. and Rutter, W. J. (1971).
Catalytic and immunochemical properties of homomeric and heteromeric combinations of aldolase subunits. *J Biol Chem* **246**, 318-23.

Perin, M. S., Brose, N., Jahn, R. and Südhof, T. C. (1991, a).
Domain structure of synaptotagmin (p65) [published erratum appears in *J Biol Chem* 1991 May 25;**266**(15):10018]. *J Biol Chem* **266**, 623-9.

Perin, M. S., Johnston, P. A., Ozcelik, T., Jahn, R., Francke, U. and Südhof, T. C. (1991, b).
Structural and functional conservation of synaptotagmin (p65) in Drosophila and humans. *J Biol Chem* **266**, 615-22.

Puckett, C., Hudson, L., Ono, K., Friedrich, V., Benecke, J., Dubois, D. M. and Lazzarini, R. A. (1987).
Myelin-specific proteolipid protein is expressed in myelinating Schwann cells but is not incorporated into myelin sheaths. *J Neurosci Res* **18**, 511-8.

Reiser, G., Schäfer, R., Donié, F., Hülser, E., Nehls, S. M. and Mayr, G. W. (1991).
A high-affinity inositol 1,3,4,5-tetrakisphosphate receptor protein from brain is specifically labelled by a newly synthesized photoaffinity analogue, N-(4-azidosalicyl)aminoethanol(1)-1-phospho-D-myo-inositol 3,4,5-trisphosphate. *Biochem J* **280**, 533-9.

Ross, N. W. and Braun, P. E. (1988).
Acylation in vitro of the myelin proteolipid protein and comparison with acylation in vivo: acylation of a cysteine occurs nonenzymatically. *J Neurosci Res* **21**, 35-44.

Roussel, G., Neskovic, N. M., Trifilieff, E., Artault, J. C. and Nussbaum, J. L. (1987).
Arrest of proteolipid transport through the Golgi apparatus in Jimpy brain. *J Neurocytol* **16**, 195-204.

Sabatini, D. D., Kreibich, G., Morimoto, T. and Adesnik, M. (1982).
Mechanisms for the imcorporation of proteins into membranes and organelles. *J Cell Biol* **92**, 1-22.

Schwob, V. S., Clark, H. B., Agrawal, D. and Agrawal, H. C. (1985).
Electron microscopic immunocytochemical localization of myelin proteolipid protein and myelin basic protein to oligodendrocytes in rat brain during myelination. *J Neurochem* **45**, 559-71.

Sinoway, M. P., Kitagawa, K., Timsit, S., Hashim, G. A. and Colman, D. R. (1994).
Proteolipid protein interactions in transfectants: implications for myelin assembly. *J Neurosci Res* **37**, 551-62.

Skalidis, G., Trifilieff, E. and Luu, B. (1986).
Selective extraction of the DM-20 brain proteolipid. J Neurochem **46**, 297-9.

Smith, D. B. and Johnson, K. S. (1988).
Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**, 31-40.

Sollner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H. and Rothman, J. E. (1993).
A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* **75**, 409-18.

Stoffel, W., Hillen, H. and Giersiefen, H. (1984).
Structure and molecular arrangement of proteolipid protein of central nervous system myelin. *Proc Natl Acad Sci U S A* **81**, 5012-6.

Südhof, T. C. and Jahn, R. (1991).
Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron* **6**, 665-77.

Supattapone, S., Worley, P. F., Baraban, J. M. and Snyder, S. H. (1988).
Solubilization, purification, and characterization of an inositol trisphosphate receptor. *J Biol Chem* **263**, 1530-4.

Theibert, A. B., Estevez, V. A., Ferris, C. D., Danoff, S. K., Barrow, R. K., Prestwich, G. D. and Snyder, S. H. (1991).
Inositol 1,3,4,5-tetrakisphosphate and inositol hexakisphosphate receptor proteins: isolation and characterization from rat brain. *Proc Natl Acad Sci U S A* **88**, 3165-9.

Theibert, A. B., Estevez, V. A., Mourey, R. J., Marecek, J. F., Barrow, R. K., Prestwich, G. D. and Snyder, S. H. (1992).
Photoaffinity labeling and characterization of isolated inositol 1,3,4,5-tetrakisphosphate- and inositol hexakisphosphate-binding proteins. *J Biol Chem* **267**, 9071-9.

Theibert, A. B., Supattapone, S., Worley, P. F., Baraban, J. M., Meek, J. L. and Snyder, S. H. (1987).
Demonstration of inositol 1,3,4,5-tetrakisphosphate receptor binding. *Biochem Biophys Res Commun* **148**, 1283-9.

Thieleczek, R., Behle, G., Messer, A., Varsányi, M., Heilmeyer, L. J. and Drenckhahn, D. (1987).
Localization of phosphorylase kinase subunits at the sarcoplasmic reticulum of rabbit skeletal muscle by monoclonal and polyclonal antibodies. *Eur J Cell Biol* **44**, 333-40.

Thieleczek, R., Mayr, G. W. and Brandt, N. R. (1989).
Inositol polyphosphate-mediated repartitioning of aldolase in skeletal muscle triads and myofibrils. *J Biol Chem* **264**, 7349-56.

Timerman, A. P., Mayrleitner, M. M., Lukas, T. J., Chadwick, C. C., Saito, A., Watterson, D. M., Schindler, H. and Fleischer, S. (1992).
Inositol polyphosphate receptor and clathrin assembly protein AP-2 are related proteins that form potassium-selective ion channels in planar lipid bilayers. *Proc Natl Acad Sci U S A* **89**, 8976-80.

Ting-Beall, H., P., Lees, M. B. and Robertson, J. D. (1979).
Interactions of Folch-Lees proteolipid apoprotein with planar lipid bilayers. *J Membr Biol* **51**, 33-46.

Varsányi, M., Messer, M. and Brandt, N. R. (1989).
Intracellular localization of inositol-phospholipid-metabolizing enzymes in rabbit fast-twitch skeletal muscle. Can D-myo-inositol 1,4,5-trisphosphate play a role in excitation-contraction coupling? *Eur J Biochem* **179**, 473-9.

Vergara, J., Tsien, R. Y. and Delay, M. (1985).
Inositol 1,4,5-trisphosphate: a possible chemical link in excitation-contraction coupling in muscle. *Proc Natl Acad Sci U S A* **82**, 6352-6.

Voglmaier, S. M., Keen, J. H., Murphy, J. E., Ferris, C. D., Prestwich, G. D., Snyder, S. H. and Theibert, A. B. (1992).
Inositol hexakisphosphate receptor identified as the clathrin assembly protein AP-2. *Biochem Biophys Res Commun* **187**, 158-63.

Volpe, P., Salviati, G., Di, V. F. and Pozzan, T. (1985).
Inositol 1,4,5-trisphosphate induces calcium release from sarcoplasmic reticulum of skeletal muscle. *Nature* **316**, 347-9.

Weber, K. and Osborn, M. (1969).
The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J Biol Chem* **244**, 4406-12.

Weimbs, T. and Stoffel, W. (1992).
Proteolipid protein (PLP) of CNS myelin: positions of free, disulfide-bonded, and fatty acid thioester-linked cysteine residues and implications for the membrane topology of PLP. *Biochemistry* **31**, 12289-96.

Wendland, B., Miller, K. G., Schilling, J. and Scheller, R. H. (1991).
Differential expression of the p65 gene family. Neuron **6**, 993-1007.

Worley, P. F., Baraban, J. M., Colvin, J. S. and Snyder, S. H. (1987).
Inositol trisphosphate receptor localization in brain: variable stoichiometry with protein kinase C. *Nature* **325**, 159-61.

Yamada, M., Kakita, A., Mizuguchi, M., Rhee, S. G., Kim, S. U. and Ikuta, F. (1993).
Developmental profile of inositol 1,4,5-trisphosphate 3-kinase in rat cerebellar cortex: light and electron microscopic immunohistchemical studies. *Dev Brain Res* **71**, 137-145.

Yamamura, T., Konola, J. T., Wekerle, H. and Lees, M. B. (1991).
Monoclonal antibodies against Myelin Proteolipid protein: identification and characterization of two major determinants. *J Neurochem* **57**, 1671-1680.

Yoo, S. H. (1991).
Inositol 1,3,4,5-tetrakisphosphate-induced Ca^{2+} sequestration into bovine adrenal-medullary secretory vesicles. *Biochem J* **278**, 381-5.

Zhang, J. Z., Davletov, B. A., Südhof, T. C. and Anderson, R. G. (1994).
Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling. *Cell* **78**, 751-60.

Hollycorn