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Biochemical and immunological properties of NP185: A brain clathrin-coated vesicle-associated protein

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BIOCHEMICAL AND IMMUNOLOGICAL PROPERTIES OF NP185: A BRAIN CLATHRIN COATED VESICLE ASSOCIATED PROTEIN

by

BORCHERNG SU

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

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ABSTRACT

BIOCHEMICAL AND IMMUNOLOGICAL PROPERTIES OF NP185:
A BRAIN CLATHRIN COATED VESICLE ASSOCIATED PROTEIN

by

BORCHERNG SU

Advisor: Professor Saul Puszkin

A neuronal protein, NP185, associated with clathrin coated vesicles (CCVs), was recently identified and partially characterized with two monoclonal antibodies (mAbs): 8G8 & 6G7 (Kohtz & Puszkin, 1988), In this thesis I describe a method I developed to extract NP185 from brain CCVs, present a procedure for its purification by affinity chromatography and describe new binding properties for other coat proteins. After elution from a mAb 8G8 conjugated affinity column and from a hydroxylapatite

column, the NP185 was found associated with clathrin light chains (LCs). When phosphorylated and non-phosphorylated clathrin LCs were crosslinked to Sepharose 4B beads, NP185 molecules did not bind to the phosphorylated LCs. The NP185 however, became tightly bound to the non-phosphorylated LCs and was eluted by 50% ethylene glycol pH 11.5, which weakens binding through hydrophobic domains. Similarly, NP185 retained on the mAb 8G8 affinity column did not bind the phosphorylated LCs from solution, while the non-phosphorylated LCs were bound by NP185 and were eluted as a complex with 0.15 M glycine buffer pH 2.5. The complex of NP185 with clathrin LCs had casein kinase II activity which phosphorylated the clathrin LCs in the presence of polylysine. The data suggest that in brain the functions of NP185 may in part depend on their association to clathrin LCs in CCVs. When CCVs were partially proteolyzed, the clathrin LCs were degraded but the smallest NP185 fragment (65 kD), recognized by mAb 8G8, remained bound to the vesicle membrane. Nerve endings CCVs contained in addition to clathrin molecule, NP185, assembly polypeptides (APs), synaptophysin and two protein kinases, entities similar to those of CCVs from whole brain. The NP185 molecules, linked to an 8G8 affinity column, retained salt pre-treated synaptics vesicles, decoated CCVs, synaptosomal membrane, tubulin and clathrin molecules. NP185 induced polymerization of clathrin cages. The resulting pellet which passed through a 10% sucrose barrier contained NP185. These results suggest that NP185 is involved in membrane homeostasis in nerve endings.

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INTRODUCTION

One of the most important physiological systems in the human body is the nervous system, whose cells, neurons, provide the receiving sites for the conduction and transmission of electrochemical signals (Erulkar, 1989). These signals are transmitted from one cell to another at synapses (Smith and Augustine, 1988). The chemical compounds which sustain nerve transmissions, the neurotransmitter molecules, are stored in small synaptic vesicles (SVs) concentrated near an active zone (Ceccarelli & Hurlbut, 1980) beneath the synaptosomal plasma membrane (SPM) at the nerve terminal. The SVs contain several proteins bound on their membrane surface which are believed to mediate their physical movement toward the active zone (Trimble & Scheller, 1988) where they fuse and secrete their contents. This movement depends on the interaction of the vesicle bound proteins with the actin cytoskeleton (Hirokawa et al., 1989). The rise in Ca2+ concentration in the nerve terminal causes the SVs to fuse with the SPM, discharging their contents into the synaptic cleft (Heuser & Reese, 1981). After the fusion of SVs with SPM, the excess of SPM is retrieved by a process of endocytosis (Lentz, 1983).

Endocytosis produces the uptake of macromolecular material into a living cell's cytoplasm (Goldstein et al., 1985). Three different types of processes involving three different types of organelles carry out endocytic events. The engulfment of large

particles is termed phagocytosis (Silverstein et al., 1977), and the organelle formed is termed a phagosome. The uptake of large bubbles of extracellular medium is termed macropinocytosis (Lewis, 1931), and the organelle formed is a macropinosome. The uptake of receptor-bound molecules and small collections of extracellular fluid is mediated by coated pits (Roth & Porter, 1964) and from these, receptosomes (endosomes) are formed (Pastan & Willingham, 1985).

With the advent and refinement of the electron microscope in the 1950s and 1960s, the uptake of signal molecules was observed, including the uptake of ferritin (Bessis et al., 1958). The entry of ferritin was observed in erythroblastic cells through small indentations in the plasma membrane, which later were identified as coated pits (Fawcett, 1964). The uptake of yolk protein in oocytes (Roth & Porter, 1964) by similar coated pits resulted in the postulate that these structures represent a specialized form of receptor-mediated endocytosis, although the receptor and the specific ligand had not been biochemically identified. In 1961, Gray recognized coated pits in neuronal tissue. Several investigators now believe that membrane retrieval from the SPM takes place through coated vesicles (Heuser & Reese, 1981), although uncoated vesicles may also play an important role (Ceccarelli & Hurlbut, 1980).

The concept of receptor-mediated endocytosis was formulated in 1974 to explain the observation that regulation of cellular

cholesterol metabolism depended on the sequential cell surface binding, internalization, and intracellular degradation of plasma low density lipoprotein (LDL) (Goldstein et al., 1985). A wide variety of molecules have been observed to enter cells by receptor-mediated endocytosis. These include hormones (Marshall, 1985), growth factors (Deuel & Huang, 1984), transport proteins that carry cholesterol (LDL) (Goldstein et al., 1985) or iron (transferrin) (Ward et al., 1984), proteins modified for degradation such as protease-alpha-2-macroglobulin complexes (Kaplan & Nielsen, 1979) or asialoglycoproteins (Schwartz, 1984) and some antibodies (Kuhn & Kraenbuhl, 1982; Abrahamson & Rodewald, 1981). In addition to these physiologically important molecules, two groups of foreign substances often enter cells by this pathway: toxins (Olsnes & Phil, 1982) and viruses (Marsh, 1984). Materials present in the extracellular fluid for which the cell lacks specific receptors also enter cells by the same pathway, but in an unconcentrated form (Pastan & Willingham, 1985).

Receptor-mediated endocytosis occurs at specific regions of the plasma membrane. These regions, which account for up to 2% of the cell surface, were first observed by Roth and Porter (1964) in mosquito oocytes. They are distinguished from the rest of the plasma membrane by the presence of small invaginations which are coated on the inner, cytoplasmic face by clathrin (Pearse, 1976). Receptors with or without ligand must migrate to these invaginated areas to be efficiently endocytosed and most

endocytosed receptors migrate to coated pits without ligand (Goldstein et al., 1985). Since membrane lipids are apparently continuously flowing towards coated pits (Pugsley, 1989), it may be that endocytic receptors remain trapped in them, probably by interactions between their cytoplasmic tails with clathrin or clathrin-associated proteins (Glickman et al., 1989; Pearse, 1988 & 1985), whereas all other proteins diffuse away. Brown and Goldstein in 1976 demonstrated in studies of receptor from patients with hypercholesterolemia that there are specific interactions between the cytoplasmic domains of endocytic receptors and components of the coated pits.

The first stage of the endocytic pathway involves the further internalization of the coated pits and the formation of clathrin-coated vesicles (CCVs). After a short while, CCVs lose their protein coats, probably through the action of an ATP-dependent uncoating protein (Schmid, 1984). The resultant uncoated vesicles together with their contents of ligands and receptors are delivered to the primary sorting endosomal compartment and are shunted into different pathways (Goldstein et al., 1985).

The CCVs are directly implicated as the organelle involved in membrane recycling at the nerve terminal by the studies of Heuser and Reese (1973 & 1977). These vesicles are thought to carry membrane, extracellular proteins, and lipoprotein to the inside of the cell (Pearse, 1976). Subsequent observations in

various tissues have confirmed that endocytosis of macromolecules involves coated pit formation, coated vesicle budding, coat disassembly, and component recycling (Pearse & Crowther, 1987). A characteristic polygonal or bristle coating on the surface of vesicles was revealed by electron micrographs of thin sections of bovine brain CCVs (Kaneseki & Kadota, 1969).

Pearse (1975) reported the first biochemical purification of coated vesicles from porcine brain and named the major protein clathrin (Pearse, 1976). Clathrin forms the lattice on the vesicle's cytoplasmic surface. The CCVs are quite small and relatively heterogeneous in size, ranging between 60 and 250 nm in diameter (Pearse, 1975). Also she estimated that 75% of the CCV vesicle mass was proteins and 25% was membrane lipid, but this ratio might have overestimated the protein content since there was a large number of empty cages (Fine & Ockleford, 1984). The CCVs are quite stable as isolated by the procedures described elsewhere (Schook & Puszkin, 1985). They remain sedimentable and the coats remain intact by both morphological structure and biochemical protein function criteria for hours at room temperature or for weeks at 4 oC (Keen, 1985).

In contrast to this apparent stability, various compounds are released from the vesicle. The results (Blitz et al., 1977; Woodward & Roth, 1978; Schook et al., 1979; Keen et al., 1979) indicate that the lattice-work structure of CCVs is held together by non-covalent bonds. Most of the coat proteins are readily

released from CCVs by relatively small changes in pH or ionic strength (Keen, 1985). Thus, they are not tightly embedded in the vesicle bilayer and may be more appropriately refered to as peripheral membrane proteins (Keen, 1985).

The entire molecular sequence of clathrin was reported recently, and found to be conserved throughout species (Kirchhausen et al., 1987). The calculated molecular weight of clathrin was 192 kD, and migrated to an 180 kD area on SDS-PAGE. The clathrin molecule is approximately 45nm long, consists of a proximal arm, a distal arm, and a terminal domain (Pearse & Crowther, 1984). Three clathrin molecules (Appendix A) linked with its carboxyl terminus form a trimer in a native state (Brodsky et al., 1987). The triskelion (Appendix B) (Ungewickell & Branton, 1981) is formed by three clathrin molecules containing three clathrin heavy chains and three clathrin light chains (LCs) in the proximal arms of the heavy chains. The terminal domain of clathrin has been proposed to interact with assembly polypeptides (APs) in forming the coat (Vigers et al., 1986).

The molecular sequences of several clathrin LCs were also reported (Brodsky et al., 1987; Jackson et al., 1987; Kirchhausen et al., 1987; Jackson & Parham, 1988) with a predicted actual molecular weight of 23 to 27 kD (Jackson et al., 1987); however, the bovine brain clathrin LCs migrate as 33 and 36 kD in SDS-PAGE (Schook et al., 1987). There are several domains present in brain clathrin LCs: a phosphorylation domain, a conserved domain,

a variable domain, a heavy chain binding domain, an exposed domain where tissue-specific sequences can be inserted, and a domain containing cysteine residues (Brodsky, 1988). These domains indicate that clathrin LCs may regulate several different aspects of clathrin function. It was also reported that the clathrin LCs provide the binding sites for an uncoating ATPase (Schmid et al., 1984), for Ca+2 (Mooibroek et al., 1987), and for calmodulin (Moskowitz et al., 1982a & 1982b).

The clathrin LCs reportedly modulate the AP50 phosphorylation (Pauloin & Jolles, 1984; Hanson et al., in press). Brain CCVs contain two protein kinases, a pp50 kinase and a casein kinase II, both with different substrate specificities (Kadota et al., 1982; Pauloin et al., 1982; Pfeffer et al., 1983; Usami et al., 1984; Schook & Puszkin, 1985; Usami et al., 1985; Bar-Zvi & Branton, 1986) resulting in the phosphorylation of the 33 kD clathrin LC and AP50 as shown in recent studies (Pauloin & Jolles, 1986; Keen et al., 1987; Schook et al., 1987; Hill et al., 1988; Pauloin et al., 1988; Hanson et al., in press).

The pp50 kinase, which phosphorylated AP50 at threonyl residues, was a Ca+2-Calmodulin and c-AMP independent protein kinase (Pauloin, 1982). ATP and Mg+2 were required for the pp50 kinase to phosphorylate AP50 (Pauloin et al., 1982). The real function of pp50 in the CCV is still unknown; however, this kinase was suggested to participate in a ATP/Mg+2 regulated

phosphorylation/dephosphorylation cascade (Pauloin et al., 1988). A Casein kinase II has been identified in both the nucleus and cytoplasm of a wide variety of eukaryote cells (Hathaway & Traugh, 1981). Casein kinase II phosphorylated both at seryl and threonyl residues (Usami et al., 1984); the recognition sequence for the enzyme requires two acidic residues following the phosphorylatable residue and has been identified as Ser(Thr)-Glu(Ser-P)-Asp(Glu) (Tuazon et al., 1979). Casein kinase II utilized both ATP and GTP as phosphoryl donors in the phosphotransferase reaction (Usami et al., 1985). Polyamines could cause activation of the enzyme under some conditions (Schook et al., 1985 & 1987). Phosphatase 2A was reported to dephosphorylate the casein kinase II-phosphorylated LCb in vitro (Pauloin et al., 1988).

In addition to the clathrin molecule, a large group of associated proteins has been identified. These associated proteins are called assembly polypeptides (APs), because they modulate the assembly of clathrin coat (Keen, 1987). There are two major categories of APs (Keen, 1987; Glickman et al., 1989). The AP1 or HAI consists of AP100 (Beta' & Gamma adaptins), AP47, and AP19, while AP2 or HAII consists of AP100 (Alpha & Beta adaptins), AP50, AP16. It has been postulated that the APs provide t e binding affinity for cytoplasmic domains of certain receptors (Pearse, 1985 & 1988; Glickman et al., 1989). Recently, Ahle and co-workers (1988) demonstrated that HAI (AP1) is present in the Golgi area but HAII (AP2) is mainly close to

the plasma membrane. Taken together, APs may play a large role in the sorting of proteins for cellular transportation. Other APs (AP180, Ahle & Ungewickell, 1986; AP155, Keen & Black, 1986; AP3, Keen, 1987) have also been mentioned; however, the particular roles of these APs are still under investigation.

Tubulin has been found to be a component of brain CCVs (Stephens, 1986; Weidenmann & Mimms, 1983; Zisapel et al., 1980; Kadoka et al., 1976), and binds to AP50 (Pfeffer et al., 1983; Weidenmann & Mimms, 1983) and NP185 (Kohtz & Puszkin, 1989). Although tubulin molecules in the cytoplasm of cells serve as the pool for microtubules. the function of tubulin in the vesicle membrane has not been clarified (Stephens, 1986). Tubulin is the precursor of microtubules, a cytoskeletal structure which participates as the tracts for the transport of cytoplasmic organelles and for cell motility (Stephens, 1986). Individual tubulin molecules inserted in the membrane of cells and vesicles may function as nucleation sites or as anchoring sites for other structural proteins or their associated polypetides (Pfeffer et al., 1983; Weidenmann & Mimms, 1983).

In contrast to brain CCVs and SVs, CCVs from nerve endings have not been studied biochemically and their putative functions have been extrapolated from studies with whole brain CCVs (Pfeffer et al., 1985; Kadota et al., 1984 & 1985; Weidenmann et al., 1985). Recently, a brain CCVs associated protein NP185 was identified and found to bind to tubulin through a mechanism

regulated by protein phosphorylation (Kohtz & Puszkin, 1989). I was wandoring if NP185 had other binding sites available for CCV coat proteins and if the interaction was also regulated by a protein phosphorylation mechanism. Besides, I also wanted to know if Np185 could be the bridge factor between clathrin coat and vesicle membrane in the CCV, since NP185 is a large molecule associated with CCV. In my work, I describe studies showing new properties of NP185, its purification, its interaction with clathrin LCs and provide evidence that this interaction may be regulated by the casein kinase II phosphorylation of clathrin LCs. Furthermore, the evidence for a larger role of NP185 and its capability to bind to SVs, decoated CCVs and SPM, and to induce the polymerization of clathrin into cages is also presented. All these events could be explained by their binding affinity to membrane bound tubulin and I postulate that in brain tissue, NP185 is a candidate molecule to act as a bridge between the membrane of these vesicles and protein of the clathrin coat.

Materials and Methods

Materials: Standard buffers and salts were purchased from Sigma. Tissue culture reagents were purchased from Flow laboratories and from Gibco. [Gamma-32P]-ATP (specific activity: 3000 mCi/mM) was purchased from New England Nuclear.

Cells: All hybridoma cell lines were carried in RPMI (Roswell Park Memorial Institute) medium 1640 supplemented with 15% fetal calf serum, 1 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM MEM (Minimum Essential Medium) with non-essential amino acids, MEM vitamins, 15 ug/ml hypoxanthine, 7.6 ug/ml thyidine.

Generation of hybridomas and characterization of monoclonal antibodies: Female balb/C mice (6 to 8 weeks of age) were initially immunized intraperitoneally with 100 ug of heat denatured coated vesicle proteins (8G8 and 4E5) or chymotryptically digested clathrin cages (2D9) emulsified in complete Freund's adjuvant (day 74). Mice were boosted intraperitoneally on day 32 with 100 ug of antigen emulsified in incomplete Freund's adjuvant. Mice were boosted a final time with intraperitoneal and intravenous injections of 50 ug of antigen (in normal saline) on days four and three before fusion on day one. SP 2/0-ag-14 myeloma cells (azaguanine resistant) were cultured in RPMI 1640 supplemented with 15% fetal calf

serum, 1 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM minimal essential medium (MEM) non-essential amino acids, MEM vitamin solution, 0.1 mM 8-azaguanine, and antibiotics. Spleen cells from immunized animals were fused at a 5:1 ratio to SP 2/0-ag-14 myeloma cells using de-ionized 44% polyethylene glycol 1000 in RPMI 1640. Cells were plated into 96 well plates 24 hours after fusion at a density of 10000 myeloma cells per well (0.2 ml) and selected by addining 15 ug/ml hypoxanthine, 7.6 ug/ml thymidine, and 0.18 ug/ml aminopterin. This procedure usually yields at least one clone per well by the end of ten days. Clones were passed to 1 ml wells prior to screening. Hybridoma supernatants were screen with an enzyme-linked immunoabsorbant assay (ELISA). Briefly, polyvinyl chloride plates (Linbro) were coated with antigen at concentrations varying from 5 ug/ml (purified proteins) to 50 ug/ml (whole coated vesicles) in 5 mM tris pH 8.0. Binding was performed at 37 oC for one hour followed by a 4 oC incubation overnight. Free binding sites were saturated using 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) pH 7.5. Wells were incubated with culture supernatants for two hours at 37 oC washed three times with TBS containing either 0.5% Tween 20 (purified antigens) or 0.05% Tween 20 (whole coated vesicles). Wells were incubated with goat anti-mouse IgG/IgM conjugated to alkaline phosphatase (BOEhringer) for one hour at 37 oC, washed three times, then incubated for one hour at 37 oC with para-nitrophenyl (Sigma) in 100 mM glycine, 1mM magnessium chloride, 0.1 mM zinc chloride, pH 10.4. Absorbance (405 nm) was quantitated with a Titertek Multiskan microelisa reader.

Positive hybridomas were subcloned by limiting dilution. Ascites fluid was generated in pristane (2,6,10,14-Tetramethylpentadecane) (Aldrich) primed Balb/c mice. Where indicated antibodies were purified from ascites fluid by Affi-gel (Bruck et al., 1982) or by high pressure liquid chromatography (Bio-Rad).

Preparation of clathrin-coated vesicles: Bovine brain CCVs were prepared by a modification of a method described by Schook and Puszkin (1985). Brains were obtained from a slaughterhouse, the meninges removed, and the gray matter separated from the white by suction. All procedures were performed at 4 oC in the presence of several proteolytic inhibitors (1 mM benzadine, 0.005 mM leupeptin, and 2 ug/ml soybean trypsin inhibitor). The gray matter was resuspended in 0.1 M MES (2-[-N-Morphonlino]ethanesulfonic acid), pH 6.5, 1 mM EGTA (Ethyleneglycol-bis-(B-Aminoethyl ether) N,N,N',N'Tetraacetic acid), 0.5 mM MgCl2, 0.02% sodium azide plus 0.3 mM PMSF (phenymethylsulfonylfluoride), 1mM benzamidine, 5 uM leupeptin, and 2 ug/ml soybean trypsin inhibitor. Tissue was homogenized in a Waring Blender and the homogenate spun down at 17,000 rpm for 15 min in a Sorvall RC2B centrifuge with a SS34 rotor. This pellet was discarded and the supernatant spun down at 33,000 rpm in a Beckman ultracentrifuge for 1 h with a 45Ti rotor (Beckman). The pellet was resuspended in MES buffer and mixed with an equal volume of 12.5% Ficoll and 12.5% sucrose in MES buffer, and spun

at 40,000 xg for one hour. The supernatant was recovered and diluted five times in MES buffer. The vesicle fraction was recovered from this solution as a pellet after centrifugation in a 45Ti rotor at 33,000 rpm for one hour. The pellet was suspended in MES buffer and run twice over a 100 cm x 2.5 cm S-1000 (Pharmacia) column. Five ml fractions were collected, and protein peaks were monitored by absorbance at 280 nm. This procedure yields approximately 10 mg of highly purified (two cycles of S-1000 chromatograph) CCVs per 500 grams starting material.

Purification of NP185: The CCVs were treated briefly and with gentle mixing with 0.5 M Tris-HCl, pH 7.0 (or 0.01 M MES pH 6.5), 0.9 M NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid), 0.1% 2-mercaptoethanol, 0.02% NaN3 and 0.2 mM PMSF at 4 oC and then spun at 100,000 xg to remove the extracted vesicles. The extract was loaded onto a 100 cm x 2.5 cm Sepharose 4B column. The NP185 positive (detected by ELISA or immunoblotting using mAbs 8G8 & 6G7) fractions were pooled and dialyzed overnight against 20x volume of 10 mM phosphate buffer, pH 7.0, 0.15 M NaCl, 0.1% 2-mercaptoethanol, 0.02% NaN3, and 0.02 mM PMSF. The dialyzed solution was then applied to a 5 ml hydroxylapatite column. Proteins were eluted first with 50 mM phosphate buffer, followed with 150 mM phosphate buffer. NP185 was found to co-elute with AP molecules. For further purification, the salt-extracted NP185 molecules were applied to an affinity column

made by covalently coupling mAb 8G8 to CNBr-activated Sepharose 4B resin beads. The bound proteins eluted with a 0.15 M glycine buffer, pH 2.5, recovered by dialysis and concentrated by Amicon filters.

Purification of Clathrin Light Chains: Clathrin LCs were obtained from CCVs as described Lisanti et al. (1982). Approximately 5 ml of CCVs suspended in 0.01 M MES buffer containing 0.15 M Nacl, 0.2 mM EGTA, 0.5 mM MgCl2, 0.1% 2-mercaptoethanol, 0.02% NaN3, and 0.02 mM PMSF, were heated in a boiling water bath at 100 oC for 5 min and spun at 100,000 xg for 1 h to remove large aggregates of denatured material. The supernatant was dialyzed against 20 vol. of 0.01 M MES buffer overnight and concentrated to approximately 1 mg/ml with Amicon filters.

Conjugation of proteins and antibodies to Sepharose 4B: The covalent conjugation were performed as follows: 1 gram of dried powder (beads) was suspended in 1 mM HCl and washed in a sintered glass filter for 15 min. The proteins were dissolved in coupling buffer: 0.1 M NaHCO3, pH 8.3, 0.5 M NaCl and mixed with the gel in a stoppered vessel. The coupling was performed at room temperature for two hours. The unbound and weakly bound ligands were washed away with coupling buffer. The remaining active sites on the Sepharose 4B were blocked with 0.1 M

Tris-HCl, pH 8.0 for two hours at room temperature. The product was washed with three cycles of 0.1 M acetate buffer, pH 4.0, 0.5 M NaCl followed by 0.1 M Tris buffer, pH 8.0, 0.5 M NaCl.

Affinity Chromatography: The 0.9 M NaCl extracts from CCVs described above were first applied to the given columns: 8G8, clathrin LCs and albumin. The unbound and weakly bound proteins were removed by one cycle wash with a 20x column volume of 0.01 M MES, pH 6.5, 0.15 M NaCl, 0.2 mM EGTA, 0.5 mM MgCl2, 0.02% NaN3, 0.1 mM PMSF followed by 0.01 M MES, pH 6.5, 0.5 M NaCl, 0.2 mM EGTA, 0.5 mM MgCl2, 0.02% NaN3, 0.1 mM PMSF. The bound proteins were eluted with either 0.15 M glycine buffer, pH 2.5, 0.15 M NaCl, or 50% ethylene glycol, pH 11.5. The eluted proteins were immediately neutralized and dialyzed against a 20x volume of MES buffer. In determinations where a second ligand protein was used (clathrin LCs, albumin), these were applied after the MES-NaCl buffer washes and incubated in the columns for 30 min. After one cycle wash the retained protein complexes were eluted from the columns as described above, neutralized and concentrated.

Preparation of Synaptic Vesicles: Enriched synaptic vesicles were prepared from bovine brain gray matter, as described by DeLorenzo and Freedman (1978). Further purification was obtained by passage through a 100 cm x 1.0 cm column of Sephacryl S-1000. Proteins in the fraction were determined by

absorbance at 280 nm, fractions were pooled, purity was established by negative staining. Three drops of each purified samples were placed on a wax plate. The formvar-coated grids were covered onto each sample drops for 15 second and dotted off excess liquid. The grids were then transferred onto 2% uranyl acetate drops for 20 min. The sample grids were allowed to air dry for one hour and viewed in a JOEL 100-B electron microscope at 80 kV.

Preparation of decoated vesicles and washed synaptic vesicles: Coated vesicles from the second Sephacryl S-1000 column chromatography were treated with 0.5 M Tris-HCl buffer, pH 7.8, for 3 h at 4 oC under gentle shaking conditions. The clathrin coat was recovered in the supernatant after centrifugation at 100,000 xg. The last step was repeated twice using the pellet containing the decoated vesicles. The final pellet was resuspended in 0.01 M MES, pH 6.5, with 0.15 M NaCl, and used for all the studies. Synaptic vesicles were treated briefly with a 1.0 M NaCl in MES buffer, the vesicles pelleted, washed rapidly twice with 0.5 M Tris-HCl, pH 7.0 and the washed synaptic vesicle pellet finally resuspended in 0.15 M NaCl, 0.01 M MES, pH 6.5.

Extraction of Clathrin: Clathrin was extracted from purified bovine CCVs by treatment with a 0.5 M Tris buffer

following a procedure described previously (Schook et al., 1979). Uncoated membranes were removed by centrifugation at 100,000 xg for 1 h, and the clathrin in solution was concentrated either by ammonium sulfate precipitation or by Amicon "centricon" dialysis. Clathrin was purified by Sepharose 4B chromatography as described (Schook et al., op cit.). Two cycles of assembly/disassembly were used to further purify clathrin (Keen et al., 1979). The isolated coated proteins in Tris-HCl buffer, pH 7.0 were transferred into a dialyzing bag and placed into a 0.1 M MES buffer, pH 6.5 overnight. The dialyzed sample were collected and centrifuged at 100,000 xg for 60 min. The pellet was collected and dissolved in a 0.1 M Tris-HCl buffer, pH 7.0.

Chymotryptic digestion: Chymotrypsin was dissolved first in 50 mM Tris, 10 mM MgCl2, pH 7.5, at 1 mg/ml, then diluted to 0.5 ug/ml in a 1 mg/ml CCVs preparation in 0.1 M MES buffer, pH 6.5. Digestions were performed for 30 seconds to 90 min at room temperature and stopped with 1 mM PMSF. Samples were then spun at 100,000 xg in a Beckman airfuge and pellets and supernatants were dissolved in Laemmli sample buffer.

Electron microscopy: Approximately 10 ug of polymerized clathrin molecules sedimented by high speed centrifugation were suspended in 0.1 M Tris buffer pH 7.0 and used for negative staining. The sample was placed on formvar-coated grids, stained

with 1% uranyl acetate, air dried, and examined in a JOEL 100-B electron microscope at 80 kV (Lisanti et al, 1982).

Binding of NP185 to tubulin and treated vesicles by affinity chromatography: For these determinations the mAb 8G8 was conjugated to CNBr-Sepharose 4B beads. The 0.9 M NaCl extract containing the NP185 molecules was passed first through the column, allowed to bind to the mAb, washed with several column volumes of low and high NaCl buffers and the tubulin or suspensions of treated vesicles loaded onto the column, incubated for 30 min, washed and finally eluted with 0.15 M glycine buffer pH 2.5 containing NaCl. Eluted samples were analyzed by SDS-PAGE.

Phosphorylation and autoradiography: Phosphorylation of CCVs was conducted in 100 mM Tris, 10 mM MgCl2, 100 mM MES buffer, pH 7.5. Reaction mixture (0.1 ml) contained 0.1 mg of CCVs, 20 uM ATP, and 1 uCi [gamma32P]-ATP. Incubations were performed for 20 min at room temperature. Reactions were stopped by adding SDS-PAGE sample buffer. A standard phosphorylation of membrane decoated vesicle and washed synaptic vesicle proteins (50 ug) by casein kinase II was performed in a 0.1 ml reaction volume. The reaction buffer consists of 100 mM Tris, 10 mM MgCl2. A proper amount (5 to 1 w/w) of polylysine was also added. Incubations were performed at room temperature for 20 min and stopped by adding of sample buffer: 1% SDS, 15%

2-mercaptoethanol, 20% glycerol, and 0.2% phenol red. The samples were loaded and run through 5-15% gradient polyacrylamide slab gels. Gels were stained and dried. An autoradiograph was obtained by overlaying Kodak X-Omat R film onto the dry gel and exposing the film for 15 h at 4 oC.

Gel Electrophoresis and Immunoblotting: SDS-PAGE was performed following the method of Laemmli (1970). One-dimensional polyacrylamide gels consisted of a continuous gradient of acrylamide from 5% to 15%. Protein samples were heated to 100 oC prior to electrophoresis. Gels were stained with Coomassie Brilliant Blue and/or dried under vaccum and autoradiographed. Proteins were transferred to nitrocellulose (Bio-Rad) electrophoretically using a Bio-Rad transfer apparatus using the procedure described by manufacturer. The protein samples on Nitrocellulose filters were fixed, then the filters were saturated with 0.1% BSA and 0.05% Tween 20 in Tris-buffered saline (TBS), pH 7.5. Filters were incubated at 4 oC overnight with primary antibodies in TBS, pH 7.5, containing 0.1% BSA and 0.05% Tween 20. Filters were washed three times with TBS containing 0.05% Tween 20, then incubated 1 h at 4 oC with peroxidase-conjugated goat anti-mouse IgG/IgM (1:600) (Boehringer) diluted in TBS containing 0.1% BSA and 0.05% Tween 20. After three washes with TBS containing 0.05% Tween 20 and one wash with 20 mM Tris, 0.5 M NaCl, pH 7.5, reactive bands were

visualized with 4-chlorol-naphthol (Bio-Rad) and 0.015% hydrogen peroxide.

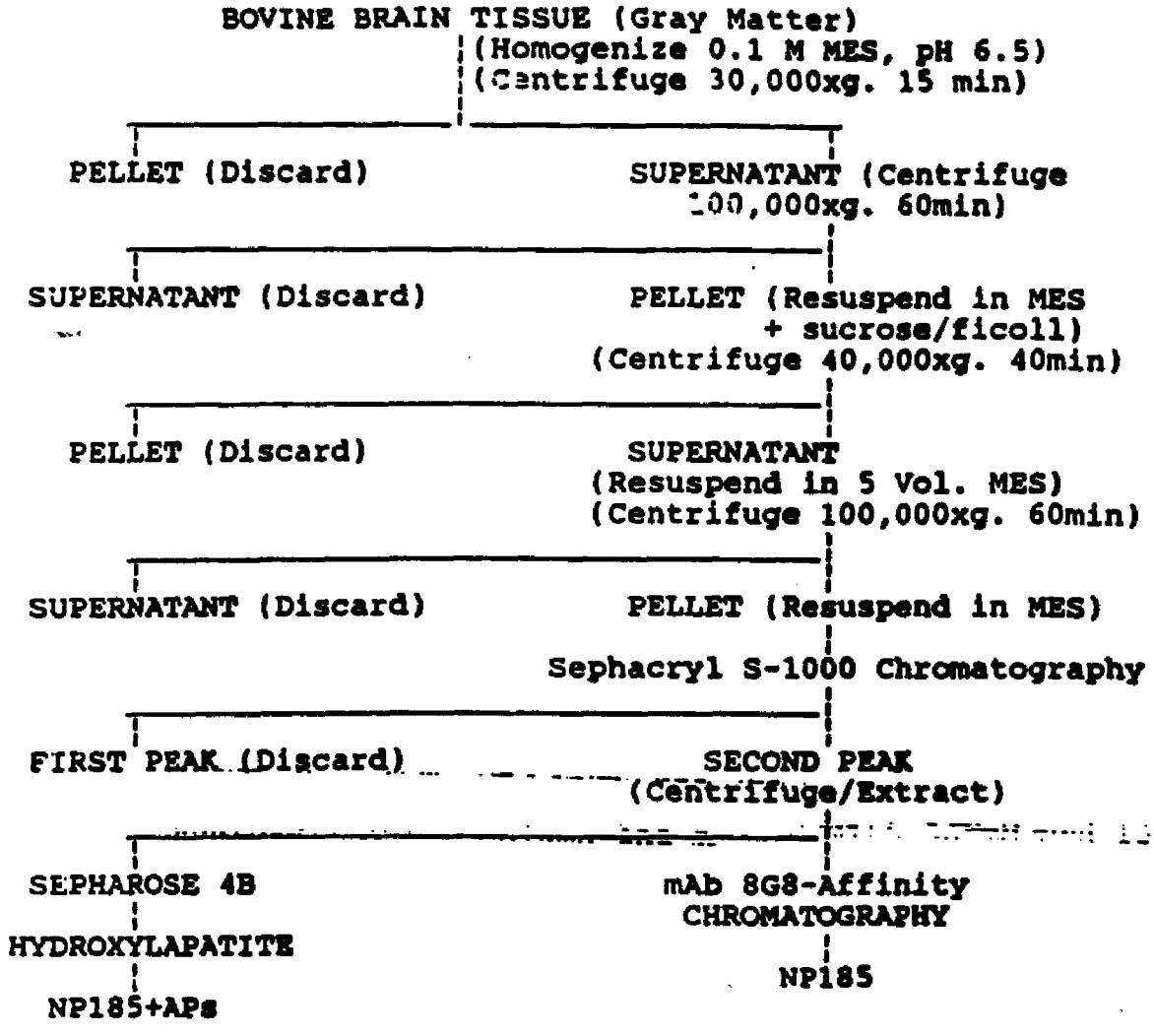
RESULTS:

Column Chromatography of NP185.-

An important step in the separation of the NP185 from other accompanying proteins was the passage of the 0.9 M NaCl Tris buffer extract obtained from purified CCVs through a gel filtration Sepharose 4B column. Screening of the Sepharose 4B eluted samples by SDS-PAGE revealed clathrin and the clathrin LCs in the early fractions. No other major proteins emerged in these fractions. The fraction corresponding to the clathrin peak is shown in figure 1a, lane 2. Proteins eluting afterwards (lanes 6-10) showed bands in the molecular range of 100 and 50 kD while the concentration of the 180 kD and 33-36 kD bands diminished steadily. Screening of the eluates with mAb 8G8 and western blotting revealed NP185 molecules present in fractions 3,4,5 (Fig. 1b). There was NP185 in fractions 9-10. The elution profile showed NP185 molecules partially overlapping clathrin and the 100 kD-50 kD APs molecules.

Because of the presence of additional proteins with the NP185 molecules, attempts were made to separate them by hydroxylapatite chromatography. In figure 2, lane 3 of panel A

SUMMARY OF THE NP185 PURIFICATION PROCEDURE



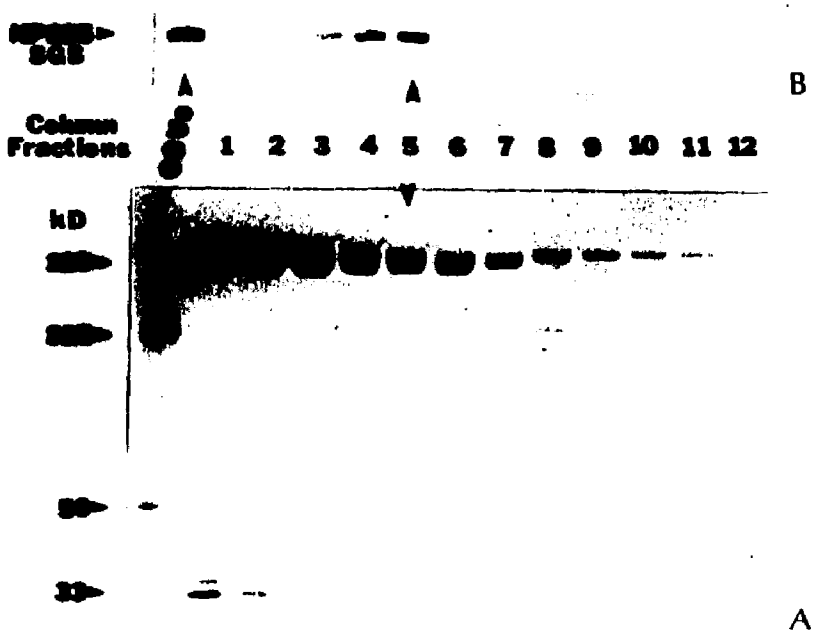


FIGURE 1. The NP185 molecules are present in the fractions from Sepharose 4B chromatography. The coat proteins of CCVs were dissociated with 0.9 M NaCl in 0.5 M Tris buffer, pH 7.0 and chromatographed through a Sepharose 4B column. Every other column fraction starting from the clathrin peak (Lane 1) was resolved by SDS-PAGE and either stained with Coomassie Blue (Panel A) or immunoblotted with mAb 8G8 (Panel B). The arrowheads indicate the peak fraction of NP185 (Lane 5).

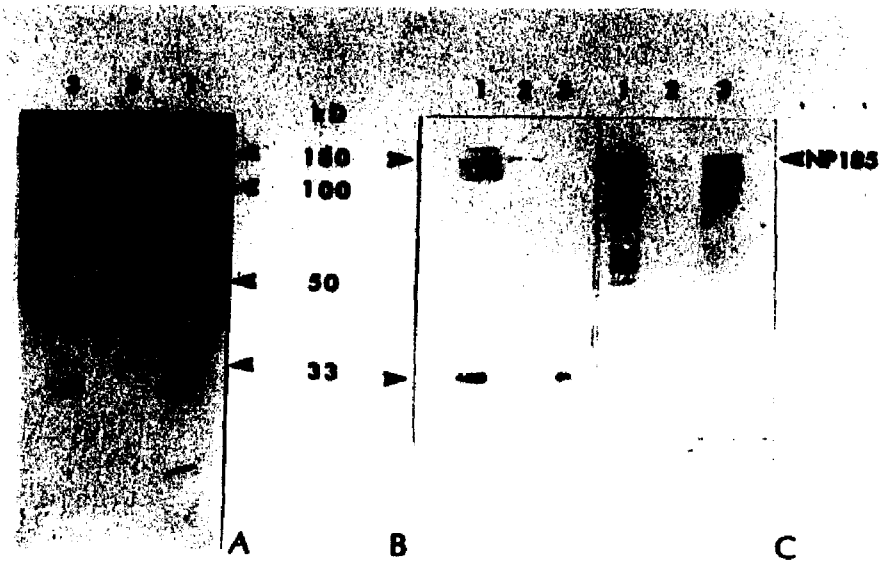


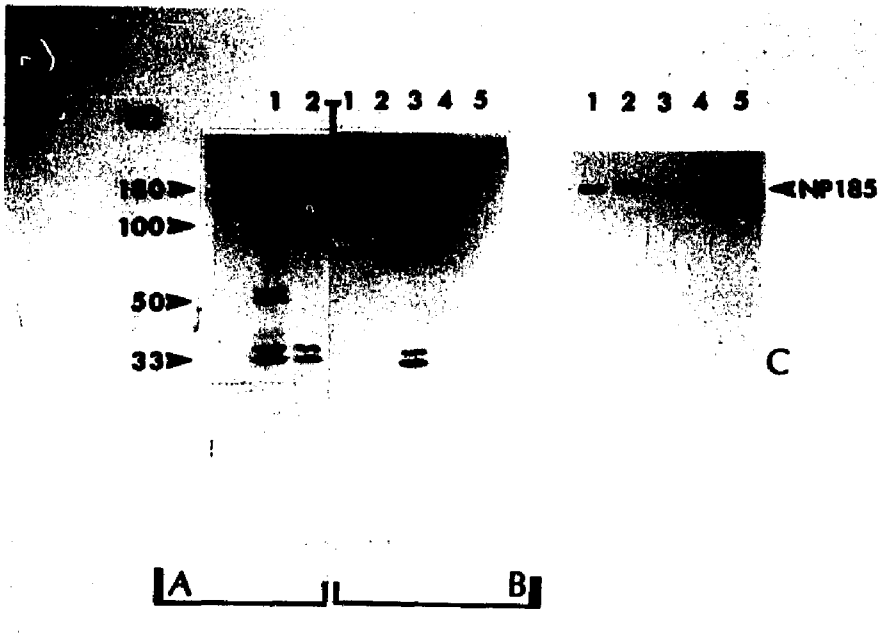
FIGURE 2. The NP185 coeluted with AP100 and clathrin LCb by hydroxylapatite chromatography. A pool of NP185 containing fractions from the Sepharose 4B column was loaded on a hydroxylapatite column and eluted with 50 mM and 150 mM phosphate buffer, pH 7.0. The eluted proteins were concentrated and resolved by SDS-PAGE. The protein profile of each phosphate buffer eluted fractions was revealed by Coomassie Blue (Panel A) and immunoblot with mAb 2D9-mAb 4E5 (Panel B) and mAb 8G8 (Panel C). Lane 1, 50 ug of CCVs; Lane 2, 20 ug of 150 mM phosphate buffer eluted proteins; Lanes 3, 20 ug of 50 mM phosphate buffer eluted protein.

shows the profile of proteins eluted with 50 mM phosphate buffer. There were protein bands in the 100 kD and 33 kD regions. The presence of NP185 in this fraction was detected by immunoblotting with mAb 8G8 and is shown in lane 3 of panel B (Figure 2). The proteins eluted with the 150 mM phosphate buffer were of 180 kD, 100 kD and 50 kD (lane 2 of panel A). The corresponding immunoblot with mAb 2D9, reactive against the clathrin heavy chain, revealed the presence of clathrin. These results showed that NP185 separated from the 50 kD, and from clathrin. A flow chart of the NP185 purification summarizes the different steps described.

Protein binding properties of NP185.-

Affinity chromatography using mAb 8G8 conjugated to CNBr-Sepharose 4B provided further evidence for the interacting affinity between the NP185 and the clathrin LCs. Figure 3 (lane 2 of panel A), shows a 0.9 M NaCl, 10 mM MES buffer pH 6.5 extract obtained from purified bovine brain CCVs clarified after high speed centrifugation. This supernatant was diluted, loaded onto the column, washed with MES buffer containing 0.15 M NaCl, followed by MES buffer containing 0.5 M NaCl, and finally eluted with a 0.15 M glycine buffer pH 2.5. The protein profile of the eluted fractions is illustrated in panel B. A band of 185 kD accompanied by a pair of bands 33-36 kD was detected. Panel C

FIGURE 3. The NP185 and clathrin LCs are coeluted from a mAb 8G8 affinity column. A 0.9 M NaCl, 10 mM MES buffer, pH 6.5 CCVs extract was loaded onto a mAb 8G8 affinity column, and the bound proteins were eluted with 0.15 M glycine buffer, pH 2.5 and 0.15 M NaCl. Panel A: lane 1, 50 ug of CCVs; lane 2, the 0.9 M NaCl/MES extract of lane 1. Panel B: fractions eluted from the column stained with Coomassie Blue. Panel C: the mAb 8G8 immunoreplica of Panel B.



shows the 8G8 immunoblot of the gel shown in panel B. The NP185 was the protein detected in the eluted fractions. An immunoblot performed with 2D9, an antibody against clathrin heavy chain, was negative. Results from these determinations suggest that the NP185 and the clathrin LCs exhibit binding affinity for each other.

When extraction of bovine brain CCVs was performed with 0.9 M NaCl, 0.1 M Tris buffer pH 7.0, the protein profile obtained revealed the presence of a major band in the 185 kD region (figure 4, lane 1). There were small amounts of other clathrin coat proteins. When this extract was loaded onto the 8G8 affinity column and eluted as described above, only one band of 185 kD was detected. Thus, short extraction time using highly purified CCVs with low Tris, high NaCl and neutral pH, favors the extraction of NP185 dissociated from other accompanying CCVs proteins.

Further studies on the interaction of NP185 with clathrin LCs were performed by affinity chromatography. In figure 5, the extract from CCVs prepared with 0.9 M NaCl, 0.1 M Tris buffer was loaded onto the 8G8-conjugated affinity column. The protein eluted was the NP185 devoid of other bands. The profile of the eluted fraction is shown on lane 1. When purified clathrin LCs (lane 2) were passed through the affinity column after the protein in the 0.9 M NaCl extract had been allowed to bind to the

FIGURE 4. The NP185 is purified from the mAb 8G8 affinity chromatography column. The protein was extracted with a 0.9 M NaCl, 100 mM Tris buffer, pH 7.0. The extract was loaded on the column and eluted with the 0.15 M glycine buffer, pH 2.5. Lane 1, the 0.9 M NaCl/100 mM Tris extract from CCVs shown in lane 2. Lane 2, 50 ug of CCVs. Lanes 3 to 5, glycine buffer eluted fractions. Stained with Coomassie Blue.

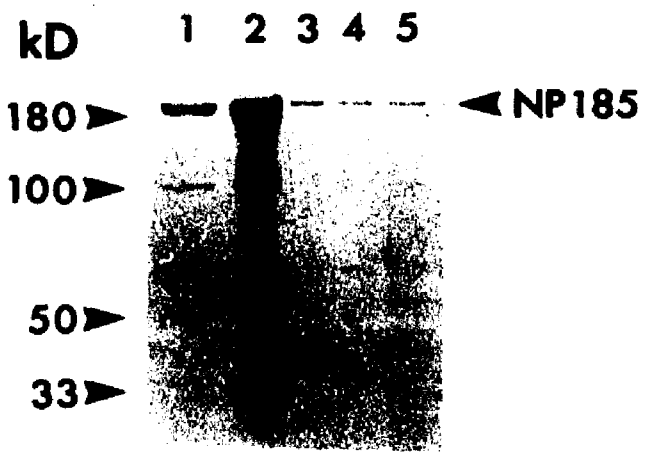
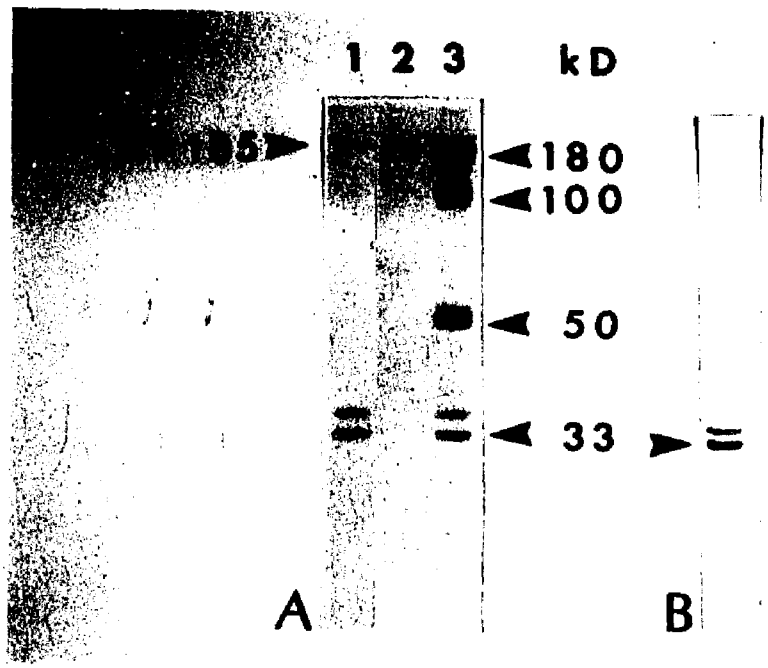


FIGURE 5. Purified clathrin LCs are retained in the mAb 8G8 affinity column containing previously absorbed NP185 molecules. Purified clathrin LCs were loaded onto the NP185 saturated mAb 8G8 affinity column and eluted with glycine buffer. Panel A: lane 1, eluted proteins from the column; lane 2, NP185 bound to the mAb in the column; lane 3, 50 ug of CCVs. Panel B: 20 ug of purified clathrin LCs used for binding to NP185. Stained with Coomassie Blue.

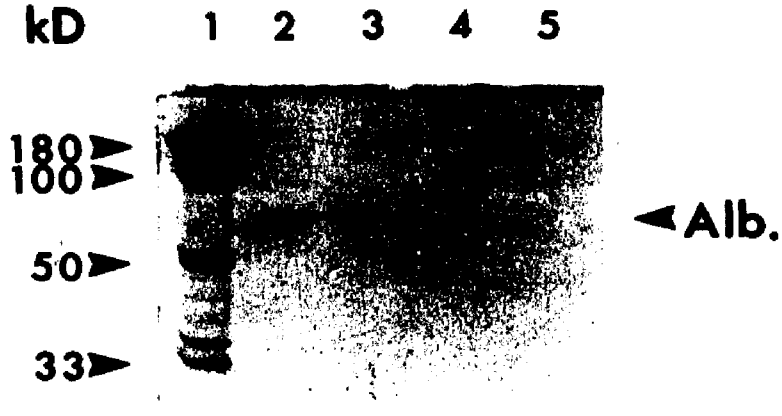


mAb 8G8-conjugated Sepharose beads, the protein profile of the eluted fractions revealed the presence of NP185 and the clathrin LCs (lane 4). In a separate experiment when depolymerized clathrin molecules (lane 3) were passed through the column after the 0.9 M NaCl extract with the NP185 molecules had been adsorbed, the fraction eluted afterwards showed the presence of NP185 and clathrin molecules (lane 5). These data indicate that the adsorbed NP185 has available sites for binding clathrin LCs free in solution or when LCs are part of the clathrin molecule. In support of this, clathrin devoid of LCs by partial proteolysis was not retained in the column (not shown).

The control experiments for NP185 binding were performed with the mAb 8G8-conjugated Sepharose 4B column using albumin instead of the CCVs-NaCl extract and conversely with albumin-conjugated Sepharose 4B using the CCVs salt extract. Figure 6 shows that albumin molecules in solution were not retained by the mAb 8G8-conjugated Sepharose 4B column and emerged with the buffer wash (lanes 2 and 3). Likewise, NP185 in the salt extract was not retained by the albumin-conjugated Sepharose 4B beads (lanes 4 and 5).

The NP185 binding of clathrin LCs was examined further using phosphorylated and non-phosphorylated LCs covalently linked to Sepharose 4B beads. The applied proteins consisted of a 0.9 M

FIGURE 6. Two control experiments for the binding of NP185. The first control experiment was to pass albumin through the mAb 8G8 affinity column followed by buffer washes. The second control experiment was to load a 0.9 M NaCl-CCVs extract through an albumin conjugated affinity column, wash buffers and elute with glycine buffer. Lane 1, 50 ug of CCVs; lanes 2 & 3, proteins detected in the wash solution from a mAb 8G8 affinity column; lane 4 & 5, eluted fraction from the albumin affinity column revealing the absence of NP185. Stained with Coomassie Blue.



NaCl-Tris extract of brain CCVs containing NP185. After washing, the retained proteins eluted only with a 50% ethylene glycol solution, pH 11.5. Figure 7 (lanes 2 of panels A and B) shows that NP185 was the only protein eluted from the non-phosphorylated clathrin LCs column. The column with the phosphorylated LCs did not retain NP185 (lanes 3, panels A and B). These results indicated that NP185 binds tightly to clathrin LCs conjugated to Sepharose 4B beads when the LCs are unphosphorylated.

To begin an analysis of the role of NP185 in CCVs, the clathrin LCs were phosphorylated on purified CCVs. The phosphorylated CCVs were sedimented by high speed centrifugation. Pellet and supernatant were analyzed by SDS-PAGE and immunoblot. Figure 8, panel A shows a large amount of coat protein released in the supernatant (lanes 2 & 3). Lesser amounts of coat proteins were released in the non-phosphorylated CCVs (lanes 4 & 5). The corresponding 8G8-immunoblot is shown in panel B. Lane 3 reveals a strong signal for NP185 molecules bound to the vesicle pellet after a large portion of clathrin molecules had been released from the coat lattice. The results suggest that clathrin LCs phosphorylation may interfer the interaction of NP185 with coat proteins through the LCs.

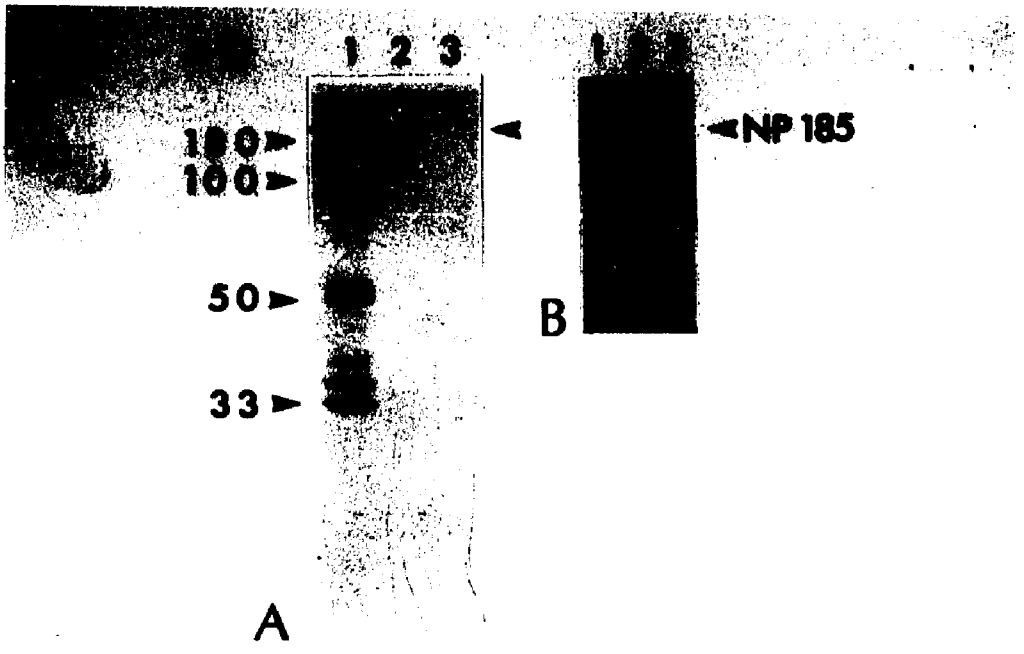
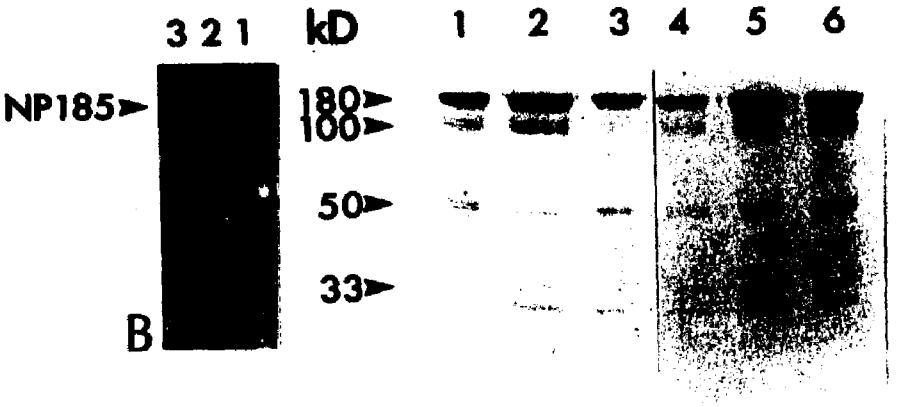


FIGURE 7. NP185 is retained by the clathrin LCs conjugated affinity column. The 0.9 M NaCl-CCVs extract was loaded onto either non-phosphorylated or phosphorylated clathrin LCs affinity columns and eluted with a 50% ethylene glycol solution. Panel A: lane 1, 50 ug of CCVs; lane 2, the eluted protein from the non-phosphorylated clathrin LCs affinity column; lane 3, eluted fraction from the phosphorylated clathrin LCs column. Panel B: a mAb 8G8 immunoreplica of panel A.

Casein kinase II was reportedly to associated with clathrin LCs and to phosphorylate LCs in vitro when LCs were dissociated from CCVs (Bar-Zvi & Branton, 1986). To examine the possibility that casein kinase II activity is present in an NP185-LCs complex and able to phosphorylate clathrin LCs bound to NP185, the eluate from a mAb 8G8-conjugated affinity column containing the NP185-LCs complex was tested for kinase activity. Figure 9, lanes 1 of panels A and B show that casein kinase II was present in the complex and capable of phosphorylating the LCs . When NP185, was eluted from an affinity column without LCs (lanes 2 of panels C and D), casein kinase II activity which phosphorylated clathrin LCb was not detected even in the presence of exogenously added clathrin-free LCs (lanes 3 of panels C and D).

Having we characterized the binding properties of NP185 for the clathrin coat proteins. Here we present evidence for the binding of NP185 molecules to the membrane of synaptic vesicles and CCVs. The CCVs after incubation with 0.5 ug/ml of chymotrypsin showed the formation of fragments of NP185 after 5 min of proteolysis (panels A, figures 10 and 11). These fragments were degraded further until a polypeptide of 65 kD remained attached to the CCVs pellet after 90 minutes of proteolysis (panels B, figures 10 and 11). Initially, weak mAb 8G8 blotted signals for NP185 and later for the 65 kD fragment were detected in the supernatant. The clathrin heavy chains were resistant to proteolysis while the clathrin LCs were easily degraded (Kohtz et

FIGURE 8. The NP185 molecules remained bound to the vesicle membrane after clathrin LCs were phosphorylated. The CCVs were treated to phosphorylate clathrin LCs and the released proteins were analyzed in the supernatant after high speed centrifigation. Panel A: lane 1, 25 ug of phosphorylated CCVs; lane 2, the supernatant of lane 1; lane 3, the pellet of lane 1; lane 4, the supernatant of lane 6; lane 5, the pellet of lane 6; lane 6, 25 ug of non-phosphorylated CCVs. Panel B: the mAb 8G8 immunoreplica of panel A, lanes 1 to 3.



A

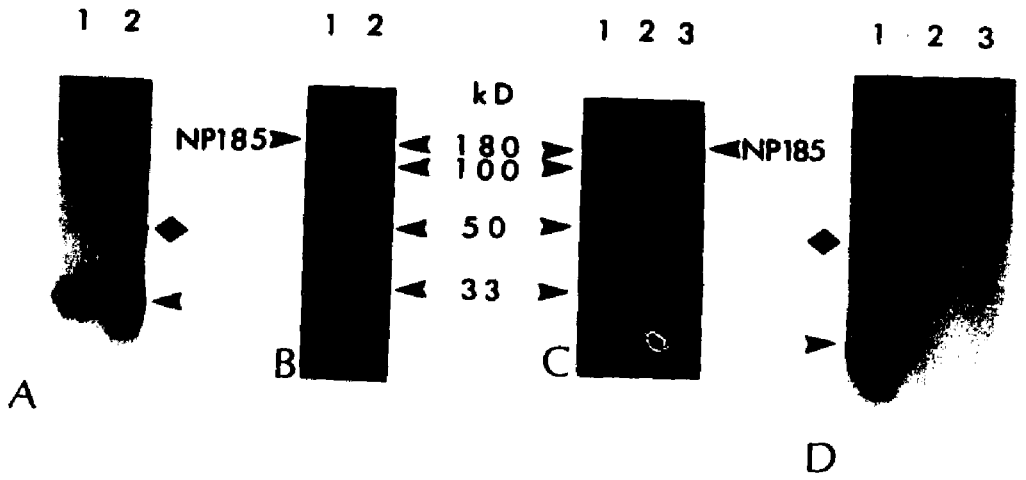
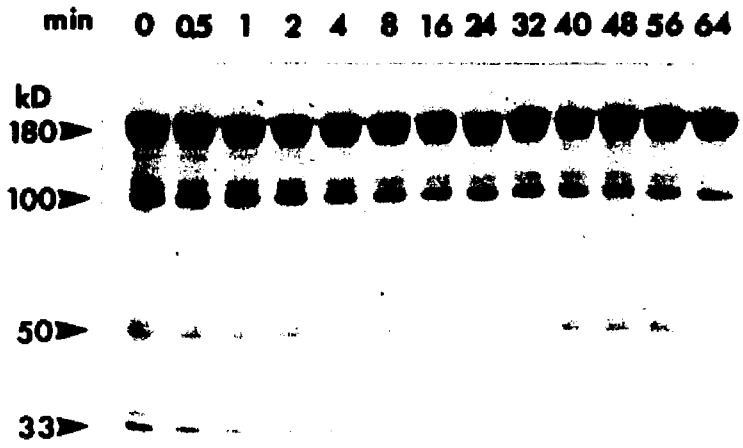
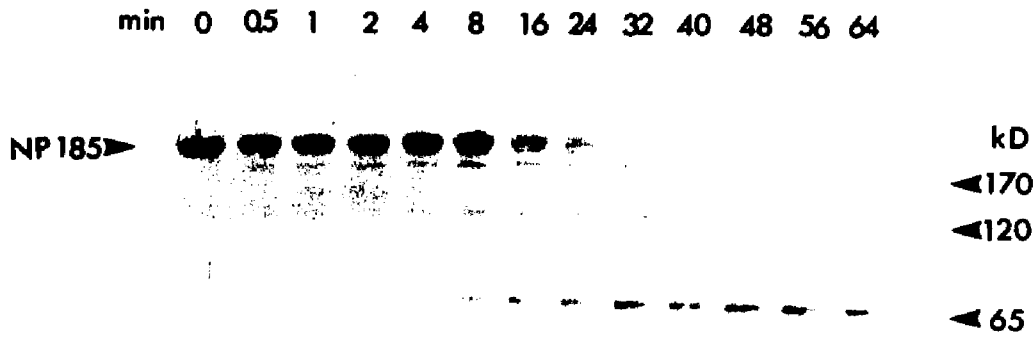


FIGURE 9. NP185-clathrin LCs complex displays casein kinase II activity. The NP185 purified from mAb 8G8 affinity column either with clathrin LCs (Figure 3, lane 3) or without clathrin LCs (Figure 4, lanes 3-5) were incubated with ATP, Mg and polylysine. Panel A: autoradiography of panel B. Panel B: lane 1, 20 ug of NP185-clathrin LCs complex; lane 2, 50 ug of CCVs. Panel C: lane 1, 50 ug of CCVs; lane 2, 10 ug of NP185 without clathrin LCs; lane 3, 10 ug of NP185 plus 2 ug of exogenous clathrin LCs (circle). Panel D: autoradiography of panel C. In panel A and D the diamonds denote pp50, and the arrowheads denote the phosphorylated clathrin LCs.

FIGURE 10. NP185 is sensitive to proteolysis. Brain CCVs (1 mg/ml) in MES were incubated with alpha-chymotrypsin (0.25 ug/ml) from 0 to 64 min. Panel A is a Coomassie Blue stained gel and shows that clathrin LCs and some APs were rapidly degraded (0-15 min), The clathrin heavy chain band did not degradation within 0-64 min. Panel B is the mAb 8G8 immunoreplica of panel A and shows that the NP185 molecule forms 170 & 120 kD fragments and a smaller 65 kD fragment stable up to 64 min enzyme incubation.


min 0 0.5 1 2 4 8 16 24 32 40 48 56 64
kD
180
100
50
33

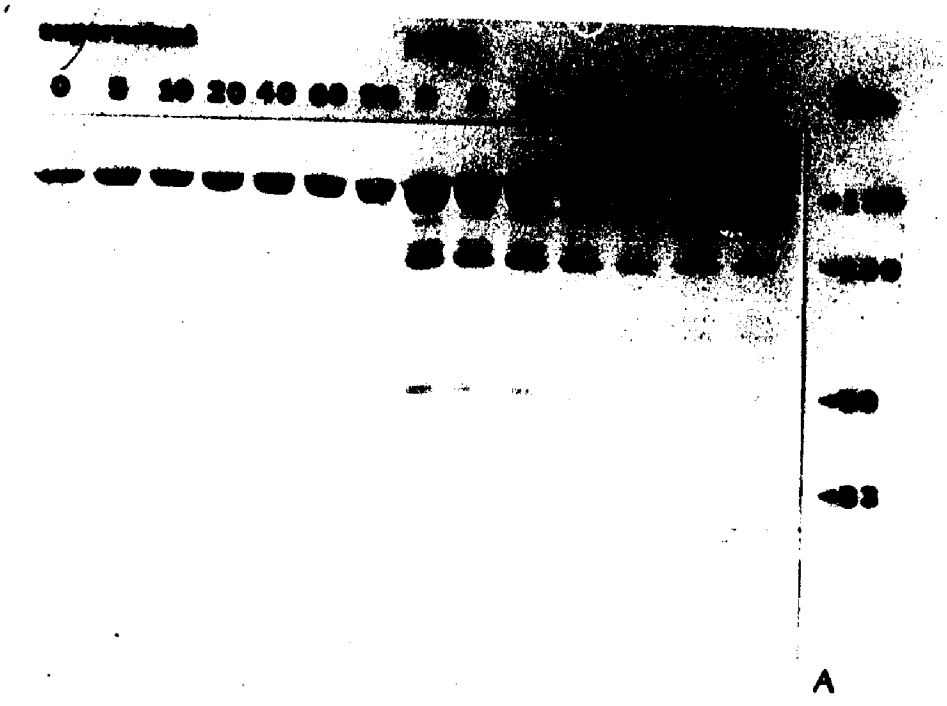
A

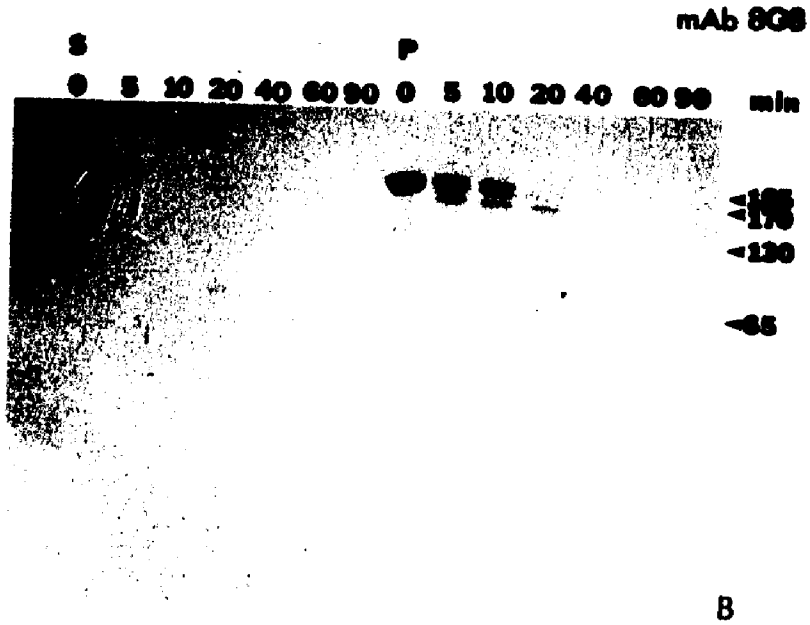

min 0 0.5 1 2 4 8 16 24 32 40 48 56 64
NP 185
kD
170
120
65

mAb 8G8

B

FIGURE 11. The smallest 65 kD NP185 fragment stays bound to the vesicle membrane pellet. CCVs (1mg/ml) in MES were incubated with alpha-chymotrypsin (0.25 ug/ml) from 0 to 90 min, supernatants and pellets were separated by high speed centrifugation. Panel A is a Coomassie Blue stained gel and shows that clathrin LCs are degraded. Panel B is the mAb 8G8 immunoreplica of panel A and indicates that the smaller 65 kD NP185 fragment detected remained bound to the vesicle membrane pellet.


0 5 10 20 40 60
A


mAb 8G8
S
P
0 5 10 20 40 60 90 0 5 10 20 40 60 90 min
170
130
B

al., 1987; Figure 10 & 11). These results indicate that in CCVs the NP185 molecules and its 65 kD fragment display binding affinity for elements of the vesicle membrane.

To determine if binding sites for NP185 were present on vesicles, CCVs were treated to remove clathrin and NP185. The decoated vesicles (figure 12, lane 2, panel A) were loaded onto a 8G8-affinity column saturated with NP185-LCs complex (lane 3, panel A). The eluate, after phosphorylation in the presence of polylysine, is shown in lane 2 of panel B. The phosphorylation pattern detected indicated that decoated CCVs had been retained by the NP185.

A similar approach was used with synaptic vesicles and synaptosomal plasma membrane. The protein profile of synaptic vesicles before and after washing to remove membrane bound proteins is shown in figure 13, lane 1, panel A and lane 1, panel B respectively. The eluate obtained is illustrated in lanes 4 and 5. The results indicated that these vesicles had been retained by the NP185 molecules in the column. Similar results were obtained with preparations of synaptosomal membrane (not shown).

Previous reports from the laboratory suggested that tubulin could be the possible binding site for NP185 to the vesicle

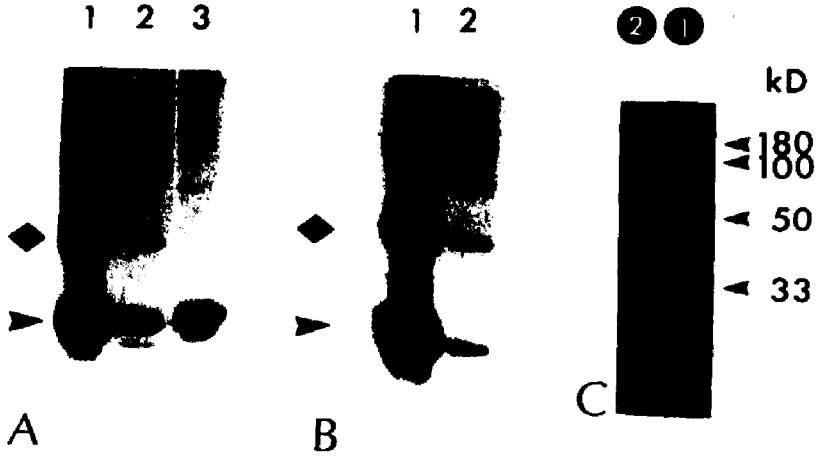


FIGURE 12. The decoated CCVs are retained by the NP185 pre-bound mAb 8G8 affinity column. The mAb 8G8 affinity column was loaded with the 0.9 M NaCl CCVs extract containing NP185, washed, andthe decoated vesicles were then incubated with the NP185 pre-bound mAb 8G8 affinity column. The eluted proteins were treated with ATP, Mg+2, and polylysine, resolved and visualized by SDS-PAGE and autoradiography. Panel A is an autoradiograph; lane 1, 50 ug of CCVs; lane 2, 25 ug of decoated CCVs; lane 3, 25 ug of the 0.9 M NaCl/10 mM MES CCVs extract. Panel B is an autoradiograph of panel C. Panel C is the Coomassie Blue stained gel; lane 1, 50 ug of CCVs; lane 2, 25 ug of eluted protein. The diamond points to pp50 and the arrowhead to the clathrin LCs.

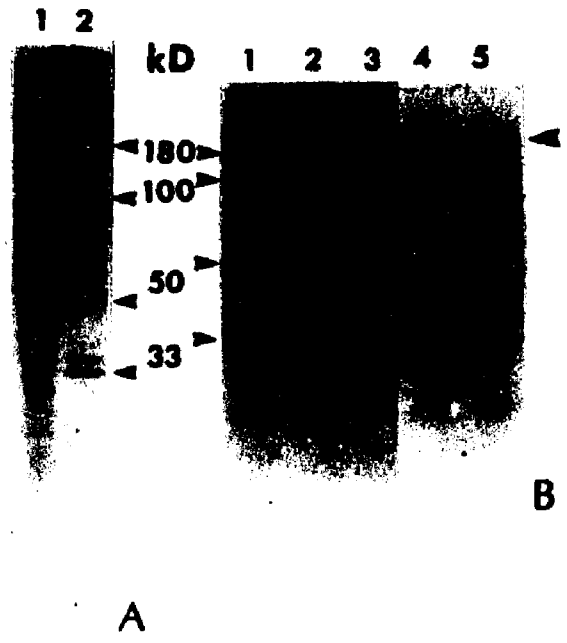


FIGURE 13. The washed synaptic vesicles are retained by the NP185 pre-bound mAb 8G8 affinity column. NP185 from a NaCl extract was allowed first to bind to mAb 8G8 on the matrix, washed, and then incubated with pre-washed synaptic vesicles. Proteins were resolved by SDS-PAGE. Panel A: lane 1, 50 ug of synaptic vesicle; lane 2, 50 ug of CCVs. Panel B: lane 1, 25 ug of pre-washed synaptic vesicles; lane 2, 50 ug of CCVs; lane 3, 10 ug of the extract; lanes 4 & 5, the eluted vesicle protein profiles. The arrowhead in panel B indicates the presence of NP185.

membrane. To examine this possibility we used the 8G8-affinity column with bound NP185 and purified tubulin in solution. Figure 14, panel A, lane 4 shows that NP185 and tubulin were present in the eluate. Panel B illustrates the identity of tubulin in the immunoblot.

To determine if CCVs from nerve endings were similar in their polypeptide composition to CCVs from whole brain, CCVs were obtained from enriched preparations of synaptosomes. Figure 15, panels A and B show that nerve endings CCVs contain clathrin heavy chains, clahtrin LCs, NP185, APs100, AP50 and also synaptophysin. In addition, the nerve ending CCVs displayed pp50 and casein kinase II activities. Thus, it can be postulated that properties determined in CCVs from whole brain also apply to CCVs of nerve endings (Kadota et al., 1983 & 1984; Pfeffer et al., 1985; Weidenmann et al., 1985; Figure 15).

The ability of NP185 to bind clathrin molecules from solution was tested with the 8G8-affinity column saturated with bound NP185 devoid of clathrin LCs (figure 16, panel A, lane 1). Purified clathrin (panel B) was then loaded onto the column. The protein profile of the eluted complex revealed the presence of clathrin and NP185. That NP185 bound clathrin molecules through the LCs was inferred from experiments using an NP185-LCs complex

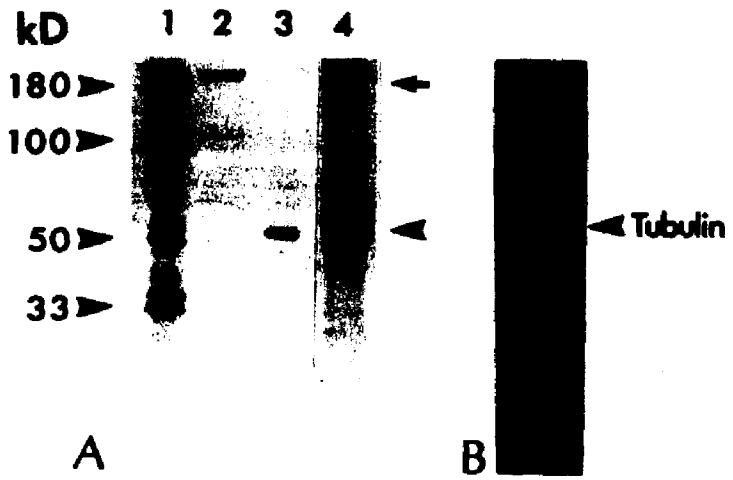


FIGURE 14. Tubulin is retained by the NP185 pre-bound mAb 8G8 affinity column. Tubulin was loaded onto the NP185 pre-bound mAb 8G8 affinity column, washed, and the eluted proteins were resolved and visualized by SDS-PAGE (panel A) and anti-tubulin immunoblot (panel B). Lane 1, 50 ug of CCVs; lane 2, 20 ug of the extract; lane 3, 10 ug of tubulin; lane 4, the eluted proteins. The arrow indicates in panel A the presence of NP185 and the arrowhead in both panels A and B points to tubulin.

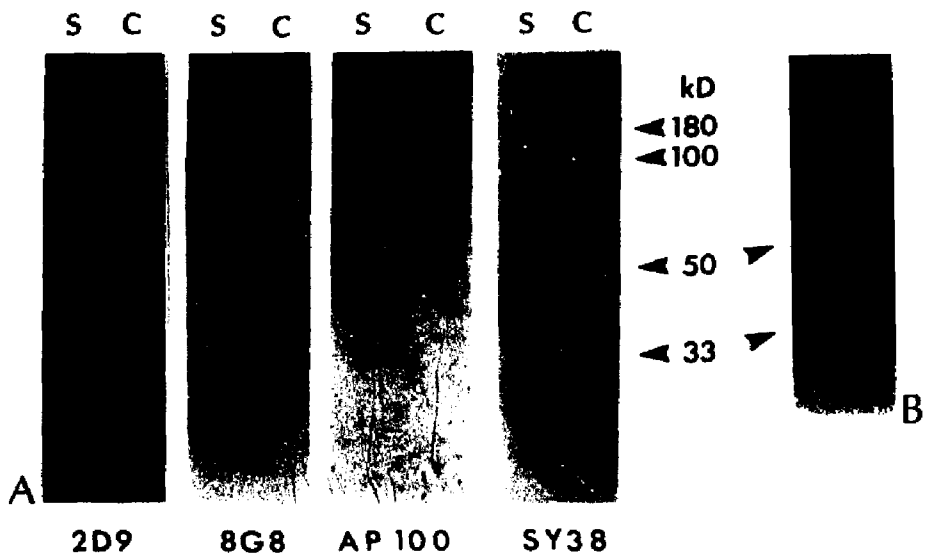


FIGURE 15. The major proteins of brain CCVs (C) and nerve ending CCVs (S). Panel A: immunoblots of the two different types of CCVs (C & S) with anti-clathrin (2D9), anti-NP185 (8G8), anti-AP100 (AP100), anti-synaptophysin (SY38). Panel B shows a protein phosphorylation profile of nerve ending CCVs kinases activated with exogenously added polylysine (left) and without polylysine (right).

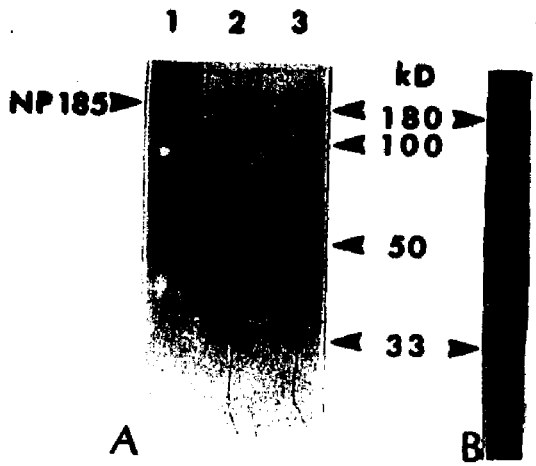


FIGURE 16. Clathrin molecules are retained by the NP185 pre-bound mAb 8G8 affinity column. Clathrin molecule from Sepharose 4B chromatography were passed through the NP185 pre-bound mAb 8G8 affinity column and the eluted proteins were resolved by SDS-PAGE. Panel A: lane 1, 10 ug of pre-bound NP185; lane 2, 50 ug of CCVs; lane 3, the eluted proteins. Panel B. 50 ug of clathrin obtained from the Sepharose 4B chromatography.

on the 8G8-affinity column. Under these conditions clathrin was not retained by NP185 and passed unretarded (not shown).

Experiments designed to show interaction of NP185 with clathrin molecules using a different experimental approach consisted of incubating clathrin molecules with NP185. The protein mixture was then placed on top of a 10 % sucrose barrier (Virshup & Bennet, 1988) and centrifuged at high speed. The pellet formed, which sedimented below the sucrose layer, and the top supernatant were analyzed by ELISA and by negative staining electron microscopy. Results obtained by ELISA revealed that in the pellet, the NP185 signal produced by 8G8 monoclonal antibodies had increased approximately by a factor of 10. The pellet also contained clathrin (table I). Controls consisting of each protein alone failed to detect the formation of a pellet below the sucrose layer. These results suggested that NP185 induced clathrin polymerization. To evaluate morphologically the type of clathrin polymer formed the pellet was examined under the electron microscope. Figure 17 shows that a large number of clathrin cages had been assembled.

TABLE 1

ELISA determinations of NP185/Clathrin interaction

mAb		8G8(NP185)	2D9(Clathrin)
BSA control		0.002	0.001
NP185		1.125	0.004
Clathrin(CLA)		0.110	1.553
Mix	Supernat	0.121	0.215
	Pellet	0.917	1.275
NP-185	Supernat	0.844	0.009
	Pellet	0.141	0.006
CLA	Supernat	0.097	0.893
	Pellet	0.106	0.186

BSA: Bovine serum albumin (100ug).

MIX: NP185, clathrin reaction mixture (100ug/100ug).

Reported values are from a typical experiment performed with five different protein preparations.

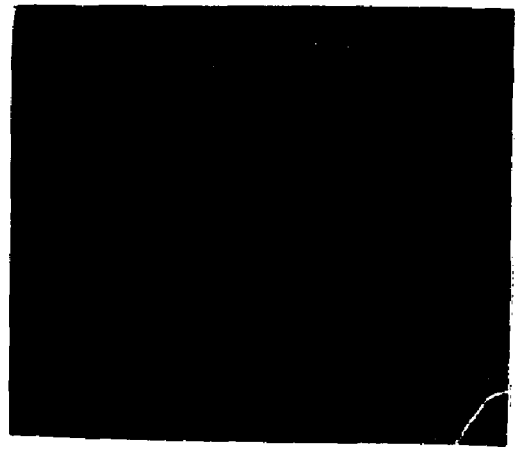


FIGURE 17. Electron microscopy of negatively stained clathrin cages. The NP185/clathrin pellet formed through a sucrose barrier as described in the legend for figure 16 was resuspended and stained. The structures formed consisted of clathrin cages. Bar indicates 0.1 um.

DISCUSSION

NP185 was identified as one of the components of brain CCVs through a series of biochemical and immunological determinations (Kohtz & Puszkin, 1988 & 1989). This molecule was found only in the brain tissue and in the neurite growth factor (NGF) differentiated PC12 cells. In the studies of brain CCVs, NP185 migrated slightly slower and was overlapped by the clathrin heavy chain on a 5-15% SDS-PAGE. It was visualized by the reactivity of mAbs 8G8 and 6G7 as a band positioned above the mAb 2D9 which identified clathrin heavy chain on the immunoblot. These results brought to this newly found protein an estimated molecular weight of 185 kD and a name of neuronal protein 185 (NP185). Since the CCVs coat was formed by non-covalent interactions of several molecules, there were various methods described to isolate CCVs proteins (Keen, 1985). The most efficient extraction for NP185 from CCVs was to use high salt buffers (Kohtz & Puszkin, 1988), which left behind the majority of clathrin and APs remain bound on vesicle membrane.

The salt extract CCVs proteins containing NP185 were subjected to a gel filtration chromatography by using a Sepharose 4B column. The result indicated that NP185 may associate with other coat proteins in the native state, because the molecule, identified by mAbs, eluted between the clathrin peak and the APs peak. The approximate molecular weights of clathrin molecule

complex and APs complex were 600 and 300 kD respectively; therefore, the NP185 complex should had a molecular weight between the range of 300 to 600 kD. However, in the non-reduced PAGE (5-15%) the protein remained in the level of 185 kD (unpublished data). These results suggested that NP185 did not polymerize with itself ionically or through disulfide bonds, but probably interacted with other coat proteins in the CCVs and can be extracted together by high salt buffers.

To understand what proteins could possibly associate with NP185 in CCVs, the NP185 containing fractions of Sepharose 4B chromatography were pooled and loaded onto a hydroxylapatite adsorption column. Two types of eluted protein profiles were generated from this matrix by using a discontinuous phosphate buffer (0-50-150 mM) (Pearse & Robinson, 1984; Virshup & Bennett, 1988). The NP185 was found co-eluted with 100 kD APs and clathrin LCb in the 50 mM phosphate buffer, while clathrin was found co-eluted with the 100 kD and 50 kD APs in the 150 mM phosphate buffer. These results suggested that NP185 may interact with either AP100 or clathrin LCs or both, and the complex, which probably is also a suitable substrate for CCVs kinases, yields a molecular mass smaller than the clathrin molecule and larger than APs complex, when subjected to molecular sieve filtration on Sepharose 4B chromatography.

Although previous preparation of NP185 obtained by immunoprecipitation were accompanied with AP50 and AP100 (Kohtz &

Puszkin, 1988), recent studies of NP185 obtained by chromatography indicated that NP185 was separated from other accompaning contaminated APs. The NP185 molecules were successfully isolated from the 0.9 M NaCl/100 mM Tris buffer CCVs extract by the mAb 8G8 affinity column without APs; however, when the 0.9 M NaCl/10 mM MES CCVs extract was loaded onto the column, the presence of NP185 and clathrin LCs was detected in the eluate. The possible explanation for this discrepancy was that the MES buffer favored an intact clathrin cage but the Tris buffer favored its dissociation (Schook et al., 1979). It was not clear initially how the mAb 8G8 affinity column retained clathrin LCs. Three possibilities were raised: non-specific interaction with either (a) mAb 8G8, (b) Sepharose 4B beads, (c) NP185 in the column. Except the NP185 molecule, mAb 8G8 did not recognize and did not bind other coat proteins (Kohtz & Puszkin, 1988) (a). The possibility of non-specific binding of clathrin LCs to the Sepharose 4B beads was also ruled out, since high salt solutions should have removed any proteins retained by non-specific interactions (Goding, 1986) and the albumin affinity column did not retain any 0.9 M NaCl CCVs extract proteins containing NP185 (b).

The interaction of NP185 with clathrin LCs was tested by the following set of experiments (c). First, it was found that the exogenously added clathrin LCs co-eluted with NP185 from a NP185 pre-bound mAb 8G8 affinity column. Second, the reverse approach showed that NP185 was retained by the clathrin LCs affinity

column and the elution was achieved by using a 50% ethylene glycol solution (Goding, 1986) instead of Tris buffer or a glycine solution. The results obtained re affirmed the possibility that the NP185-clathrin LCs interaction was real and was most likely mediated through the hydrophobic domains of each molecule. The recently reported molecular sequences of clathrin LCs (Jackson et al., 1987) revealed that brain clathrin LCs contained cytoplasmic exposed hydrophobic (brain specific) insertion sequence domains which possibly interacts with other cytoplasmic proteins and fulfills the necessity of membrane recycling which take place in the nerve ending (Brodsky, 1988).

Protein phosphorylation was one of the major regulatory mechanisms for neuronal functions (Browning et al., 1985). The phosphorylation of synapsin I on SVs induced the fusion of SVs to SPM (Hemmings et al., 1989). This event apparently could also apply to the coat proteins of CCVs (Schmid et al., 1984). Clathrin LCb was the substrate of a CCVs associated casein kinase II (Schook et al., 1987), and was functionally required to recognize an uncoating ATPase in the cytoplasm (Schmid et al., 1984) which was related to the heat shock protein family (Ungewickell, 1985). It was reported that a 1 M salt/10 mM MES buffer CCVs extract expressed the casein kinase II activity (Bar-Zvi & Branton, 1986). Furthermore, the kinase activity was also identified in the affinity eluted NP185-clathrin LCs complex. Therefore, it was reasonable to postulate that the

NP185-clathrin LCs interaction may be regulated by the mechanism of protein phosphorylation.

The result from the phosphorylated clathrin LCs affinity column showed that the NP185 molecules were not retained by the phosphorylated clathrin LCs molecules. It indicated that the phosphorylated clathrin LCs did not provide binding sites to NP185. To understand the role of NP185 in intact CCVs, CCVs were subjected to an in vitro phosphorylation under conditions suitable for activation of the CCVs associated casein kinase II (Schook et al., 1987), the clathrin LCs isolated in the supernatant which was clarified by high speed centrifugation. The results showed that NP185 remained attached to vesicle membrane. It indicated that the phosphorylation of clathrin LCs weakened the interaction of NP185-clathrin LCs in CCVs. Although the NP185-clathrin LCs complex carried the kinase, it was not identified in the clathrin LCs-free NP185 samples. These findings provided further evidence for the interaction of NP185 with clathrin LCs in CCVs and suggested a possible steric conformation site in the structure of NP185-clathrin LCs complex suitable for casein kinase II regulation of the binding in CCVs. The clathrin LCs was phosphorylated at the sites next to the brain insertion sequence (Brodsky, 1988) which probably is the binding site for NP185. The phosphorylated seryl residues on LCs possibly displaced and interferes the NP185-LCs interaction, because of the conformational change of the phosphorylated LCs. The overall results also supported the finding that the

NP185-clathrin LCs complex was expectable to elute out between clathrin molecule and APs complex peaks from Sepharose 4B chromatography.

The casein kinase II was identified in both the nucleus and cytoplasm of a wide variety of eukaryotes (Hathaway & Traugh, 1981). It was also reported as one of the endogenous CCV kinases (Schook et al., 1985). The kinase required polyamines as the activators (Schook et al., 1987). Polylysine was found in the tail region of the c-Ki-ras protein and a synthtic peptide corresponding to that region was capable of inducing phosphorylation of proteins in the Xenopus laevis oocyte membrane (Gatica et al., 1987). Clathrin LCs were demonstrated to be phosphorylated in vivo (Bar-Zvi et al., 1988). The phosphatase 2A was reportedly to dephosphorylate the casein kinase II-phosphorylated clathrin LCb (Pauloin et al., 1988).

Both NP185 and clathrin LCs were highly sensitive to proteolytic enzymes (Kohtz & Puszkin, 1988; Kohtz et al., 1987). The partial proteolytic degradation of NP185 in CCVs showed that this molecule generated three major polypeptides (170, 120 & 65 kD) and the smallest 65 kD fragment detected by the mAb as stating on the CCVs membrane while the clathrin LCs were rapidly degraded. It was possible that this small NP185 fragment remained bound to vesicle membrane tubulin, since NP185 bound tubulin (Kohtz & Puszkin, 1989) and tubulin was one of the major CCVs proteins (Pfeffer et al., 1983).

The NP185 was first identified in CCVs and studies in subcellular distribution of NP185 indicated that NP185 was mainly associated with membrane organelles, eg. vesicles and plasma membrane (data not shown). In addition, brain CCVs shared similar elements to SVs (Pfeffer et al., 1985; Weidenmann & Mimms, 1985; Kadota et al., 1984 & 1985), and CCVs from nerve endings also showed a similar major protein profile and kinases activities. This indicated that a large part of brain CCVs were formed as the result of synaptic membrane recycling in the nerve endings. Since the NP185 was present on the membrane of CCVs, SVs, and SPM, the NP185 binding sites on the membrane ought to be un-occupied. My results showing that decoated CCVs, pre-treated SVs and SPM were retained by NP185 pre-bound to the mAb 8G8 affinity column seemed to confirm this possibility. It also indicated that NP185 can re-attach to vesicle membrane probably through the membrane tubulin molecule to produce membrane recycling. In addition, NP185 was also capable of inducing the clathrin polymerization possibly through its interaction with clathrin LCs.

Taken together, NP185 interacted with clathrin LCs in CCVs coat and with tubulin on vesicle membrane, both interactions were shown regulated by mechanisms of protein phosphorylation. Casein kinase II was demonstrated associated with CCVs and was the candidate involved in the regulation of two interactions. In addition, nerve endings CCVs also shared similar entities with brain CCVs. Furthermore, NP185 was identified as a component

present in the nerve endings organelles and shown to participate in the re-constitution of the coat of CCVs from decoated CCVs, pre-treated SVs and SPM. It is possible that NP185 is an important link in the mediating of the recycling of CCVs through SVs and SPM, then back to CCVs in the nerve endings.

APPENDIX A

Diagram of clathrin molecule

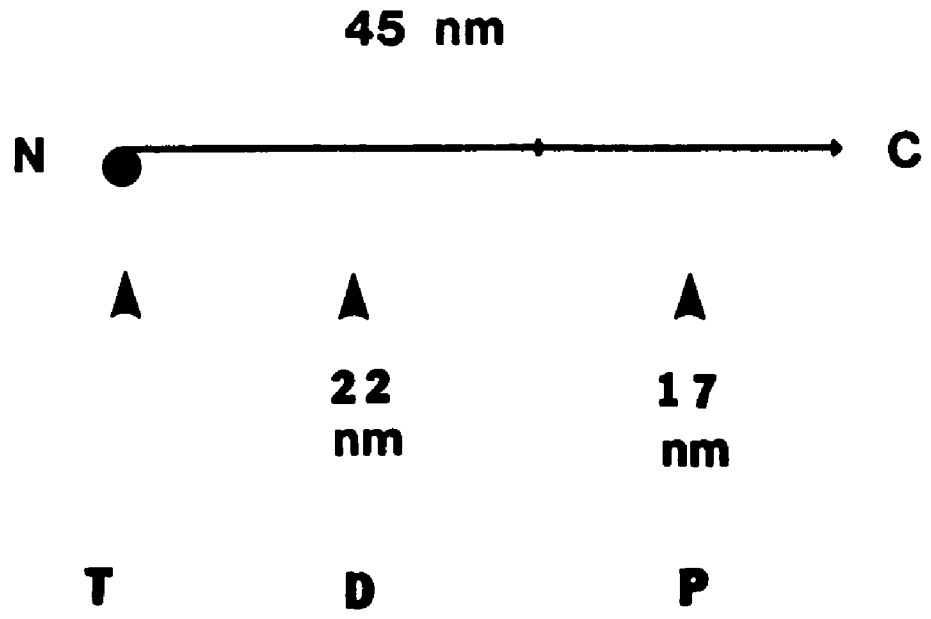
C: C-terminus of clathrin

D: Distal arm of clathrin

N: N-terminus of clathrin

P: Proximal arm of clathrin

T: Terminal domain of clathrin



APPENDIX B

Diagram of clathrin triskelion

C: C-terminus of clathrin

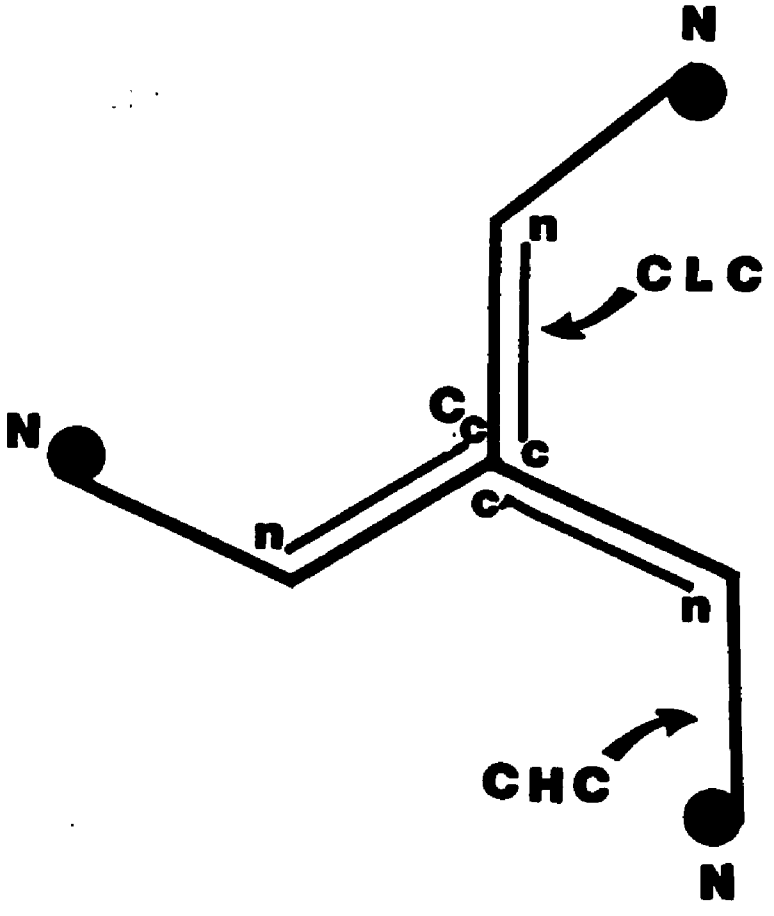
c: c-terminus of clathrin light chain

CHC: Clathrin Heavy Chain

CLC: Clathrin Light Chain

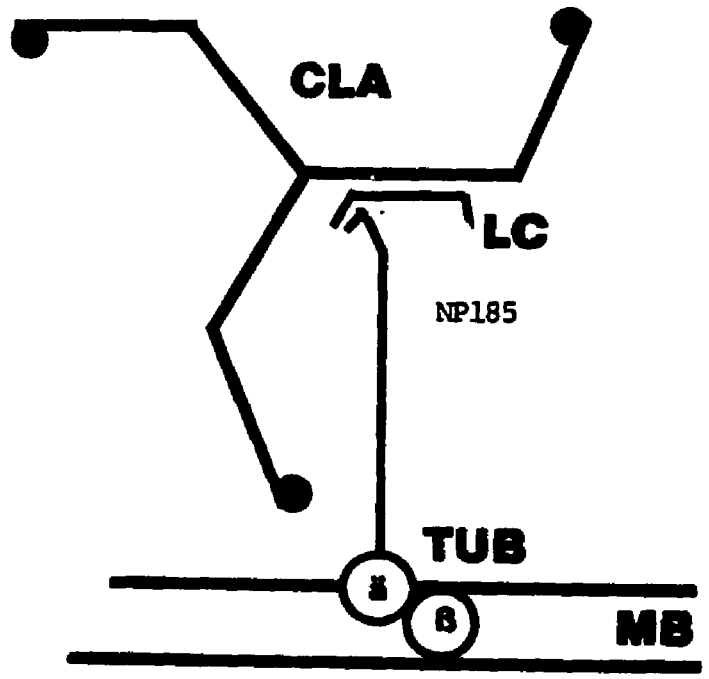
N: N-terminus of clathrin

n: n-terminus of clathrin light chain



APPENDIX C

Diagram of the interaction of NP185 to tubulin and LCs



CLA: Clathrin Triskelion

LC: clathrin Light Chain

MB: plasma membrane of CCV

TUB: Tubulin molecules

APPENDIX D

Diagram of the hypothesized role of NP185 in the nerve ending.

1. The retrieval of synaptosomal plasma membrane (SPM) is illustrated by the attachment of clathrin triskelions (C) through their clathrin light chains to a site on the NP185 molecule. The NP185 is shown bound to the SPM.

2. Partially formed clathrin lattice causes the indentation of SPM through a coated pit (CP) structure.

3. The formation of a clathrin coated vesicle (CCV) is the result of the internalization of SPM.

4. The clathrin triskelion is released from the CCV by the endogenous casein kinase II phosphorylation of the clathrin light chains. The NP185 molecules remain bound to the vesicle (V').

5. The NP185 molecules (N) are released from the decoated vesicle (V') probably through the phosphorylation of tubulin by tubulin kinase which weakens the interaction of NP185 with tubulin.

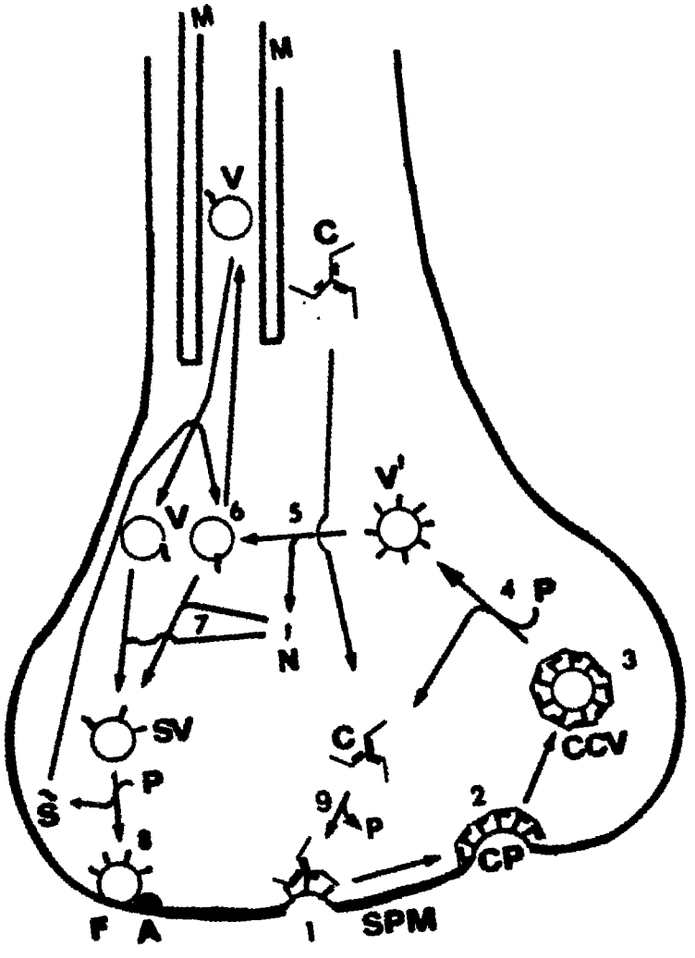
6. The vesicle (V) is either reloaded with neurotransmitters for the next cycle of signal transduction or sent back to the cell body by retrograde axonal transport.

7. The NP185 molecules become attached to vesicles (V), to participate in the recycling mechanism. Synapsin I (S) is known to play a role in the fusion of synaptic vesicles (SV) to the SPM.

8. Synapsin I is released from SV through a mechanism regulated by phosphorylation and the SV then fuses with SPM near the active zone (A).

9. The phosphorylated LCs are dephosphorylated by phosphatase 2A. The dephosphorylated LCs then have the binding site available again for NP185.

Diagram of the hypothesized role of NP185 in the nerve ending.



KEY

A: Active Zone

C: Clathrin Triskelion

CCV: Clathrin Coated Vesicle

CP: Coated Pit

F: Fusion

M: Microtubule

N: NP185

P: Phosphorylation

S: Synapsin I

SPM: Synaptosomal Plasma Membrane

SV: Synaptic Vesicle

V': Decoated CCV

V: Vesicle

ABBREVIATION	
AP1	Assembly Polypeptide Complex 1
AP2	Assembly Polypeptide Complex 2
AP3	Assembly Polypeptide Complex 3
AP16	Assembly Polypeptide 16 kD
AP19	Assembly Polypeptide 19 kD
AP47	Assembly Polypeptide 47 kD
AP50	Assembly Polypeptide 50 kD
AP100	Assembly Polypeptide 100 kD
AP155	Assembly Polypeptide 155 kD
AP180	Assembly Polypeptide 180 kD
APs	Assembly Polypeptides
ATP	Adenosine Tri-Phosphate
BSA	Bovine Serum Albumin
CCV	Clathrin Coated Vesicle
CCVs	Clathrin Coated Vesicles
dCCV	decoated CCV
dCCVs	decoated CCVs
EDTA	EthyleneDiamineTetraacetic Acid
EGTA	EthyleneGlycol-bis-(B-aminoethyl ether) N,N,N',N' Tetraacetic Acid
EXT	CCVs 0.9 M NaCl extract (MES or TRIS)
HA I	HydroxylApatite complex I
HA II	HydroxylApatite complex II
HA	HydroxylApatite
kD	kilo-Dolton

LC	clathrin Light Chain
LCa	clathrin Light Chain 36 kD
LCb	clathrin Light Chain 33 kD
LCs	clathrin Light Chains
MES	2-[-N-Morpholino] ethanesulfonic acid
PMSF	PhenylMethylSulfonylFluoride
RPMI 1640	Roswell Park Memorial Institute medium 1640
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
SPM	Synaptosomal Plasma Membrane
SV	Synaptic Vesicle
SVs	Synaptic Vesicles
TBS	Tris-Buffered Saline
TRIS	Tris(hydroxymethyl)aminomethane
TRIS-HCl	Tris(hydroxymethyl)aminomethane-HCl

BIBLIOGRAPHY

Abrahamson, D. R., & Rodewald, R. (1981) Evidence for the sorting of sorting of endocytic vesicle contents during the receptor-mediated transport of IgG across the newborn rat intestine. J. Cell Biol. 91, 270.

Ahle, S., & Ungewickell, E. (1986) Purification and properties of a new clathrin assembly protein. EMBO J., 5, 3143.

Ahle, S., Mann, A., Eichelsberger, U., & Ungewickell, E. (1988) Structural relationships between clathrin assembly protein from the golgi and plasma membrane. EMBO. 7, 919.

Bar-Zvi, D., & Branton, D. (1986) Clathrin-coated vesicles contain two protein kinase activities. J. Biol. Chem., 261, 9614.

Bar-Zvi, D., Mosley, S. T., & Branton, D. (1988) J. Biol. Chem. 263, 4408.

Bessis, M. (1963) Cytologic aspects of hemoglobin production. The Harvey Lecture Series, 58, 125.

Blitz, A. L., Fine, R. E., & Toselli, P. A. (1977) Evidence that coated vesicles isolated from brain are calcium-sequestering organelles resembling sarcoplasmic reticulum. J. Cell Biol. 75, 135.

Brodsky, F. M. (1988) Living with clathrin: its role in intracellular membrane traffic. Science, 242, 1396.

Browning, M. D., Huganir, R., & Greengard, P. (1985) Protein phosphorylation and neuronal function. J. Neurochem., 45, 11.

Bruck, C., Portetelle, D., Glineur, C., Bollen, A. (1982) One step purification of mouse monoclonal antibodies from ascites fluid by DEAE Affi-Gel Blue chromatography. J. Immunol. Methods, 53, 313.

Ceccarelli, B. & Hurlbut, W. P. (1980) Vesicle hypothesis of the release of quanta of acetylcholine. Physiol. Rev. 60, 396.

Cheng, T. P.-O., & Wood, J. G. (1987) Recycling of plasmalemma in chick tectal growth cones. J. Neurosci., 7, 1752.

Crowther, R. A., Finch, J. T., & Pearse, B. M. F. (1976) On the structure of coated vesicles. J. Mol. Biol. 103, 785.

DeLorenzo, R. J., & Freedman, S. D. (1978) Calcium dependent neurotransmitter release and protein phosphorylation in synaptic vesicles. Biochem. Biophys. Res. Comm., 80, 183.

Deuel, T. F., & Huang, J. S. (1984) Platelet-derived growth factor. Structure, function, and roles in normal and transformed cells. J. Clin. Invest. 74, 669.

Erulkar, S. D. (1989) Chemically mediated synaptic transmission: an overview. Basic Neurochemistry. Raven, N.Y.

Fawcett, D. W. (1964) Local specialization of the plasmalemma in micropinocytosis vesicles of erythroblasts. Anat. Rec. 148, 370 (Abstr.).

Fine, R. E., & Ockleford, C. D. (1984) Supramolecular cytology of coated vesicles. Itnl. Rev. Cyto. 91, 1.

Gatica, M., Allende, C. C., Antonelli, M., & Allende, J. E. (1987) Polylysine-containing peptides, including the carboxyl-terminal segment of the human c-Ki-2 protein, affect the activity of some key membrane enzymes. Proc. Natl. Acad. Sci. USA., 84, 324.

Glickman, J. N., Conibear, E., & Pearse, B. M. F. (1989) Specific of binding of clathrin adaptors to signals on the mannose-6-phosphate insulin-like growth factor II receptor. EMBO. 8, 104.

Goding, J. W. (1986) Monoclonal Antibodies:principle and practice. 2nd ed. Academic (N.Y.)

Goldstein, J. L., Brown, M.S., Anderson, R. G. W., Russel, D.W., Schneider, W.J. (1985) Receptor mediated endocytosis: concepts emerging from the LDL receptor system. Ann. Rev. Cell Biol., 1, 1.

Gray, E. G. (1961) The granule cells, mossy synapesa, and Purkinji spine synapses of the cerebellum: light and electron microscopic observations. J. Anat. 95, 345.

Hanson, V. G., Schook, W. J., & Puszkin, S. (in press) Novel regulatory role of phosphorylated clathrin light chain B in bovine brain coated vesicles. J. Neurochem. 53, 000.

Hathaway, G. M. and Traugh, J. A. (1979) J. Biol. Chem. 256, 11442.

Hathaway, G. M. and Traugh, J. A. (1981) Curr. Top. Cell. Regul. 21, 101.

Hemmings, H. C. Jr., Nairn, A.C., McGuinness, T. L., Huganir, R. L., & Greengard, P. (1989) Role of protein phosphorylation in neuronal signal transduction. FASEB. 3, 1583.

Heuser, J. E., & Reese, T. S. (1973) Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J. Cell Biol. 57, 315.

Heuser, J. E., & Reese, T. S. (1977) Structure of the synapse. Handbook of Physiology, The Nervous System I. Bethesda: American Physiological Society, pp. 261.

Heuser, J. E., & Reese, T. S. (1981) Structural changes after transmitter release at the frog neuromuscular junction. J. Cell Biol., 88, 564.

Hill, B. L., Drickamer, K., Brodsky, F. M., & Parham, P. (1988) Identification of the phosphorylation sites of clathrin light chain LCb. J. Biol. Chem., 263, 5499.

Hirokawa, N., Sobue, K., Harada, A., & Yorifuji, H. (1989) The cytoskeletal architecture of the presynaptic terminal and molecular structure of synapsin 1. J. Cell Biol., 108, 111.

Jackson, A. P., Seow, H.-F., Holmes, N., Drickamer, K., & Parham, P. (1987) Clathrin light chains contain brain-specific insertion sequences and a region of homology with intermediate filaments. Nature, 326, 154.

Jackson, A. P., & Parham, P. (1988) Structure of human clathrin light chains. J. Biol. Chem., 263, 16688.

Kadota, T., Kadota, K., & Gray, E. G. (1976) Coated-vesicle shells, particle/chain material, and tubulin in brain synaptosomes. J Cell Biol., 69, 608.

Kadota, K., Usami, M., & Takahashi, A. (1982) A protein kinase and its substrate associated with the outer coat and the inner core of coated vesicles from bovine brain. Biomed. Res., 3, 575.

Kadota, K., Toyota, N., Miyazaki, N., & Kadota, T. (1983) Purification of coated vesicles from synaptosomes from bovine brain. Biomed. Res., 4, 421.

Kadota, K., Toyota, N., Miyazaki, N., & Kadota, T. (1984) Casein kinase II activity in coated vesicles prepared from synaptosomes from the bovine cerebral cortex. Biomed. res., 5, 507.

Kaneseki, T., & Kadota, K. (1969) The "vesicle in a basket". J. Cell Biol. 42, 202.

Kaplan, J., & Nielsen, M. L. (1979) Analysis of macrophage surface receptors. 1. Binding of alpha-macroglobulin-protease complexes to rabbit alveolar macrophages. J. Biol. Chem. 254, 7323.

Keen, J. H., Willingham, M. C., & Pastan, I. (1979) Clathrin-coated vesicles: isolation, dissociation and factor-dependent reassociation of clathrin baskets. Cell, 16, 303.

Keen, J. H. (1985) The structure of clathrin coated membranes: assembly and disassembly. Endocytosis. Plenum, N.Y.

Keen, J. H., & Black. M. M. (1986) The phosphorylation of coated membrane proteins in intact neurons. J. Cell Biol., 102, 1325.

Keen, J. H. (1987) Clathrin assembly protein: affinity purification and a model for coat assembly. J. Cell Biol., 105, 1989.

Keen, J. H., Chesnut, M. H., & Beck, K. A. (1987) The clathrin coat assembly polypeptide complex. J. Biol. Chem., 262, 3864.

Kirchhausen, T., Harrison, S. C., Ping Chow. E., Mattaliano. R. J., Ramachandran, K. C., Smart, J., & Brosius, J. (1987a) Clathrin heavy chain: molecular cloning and complete primary structure. Proc. Natl. Acad. Sci. U.S.A., 84, 8805.

Kirchhausen, T., Scarmato, P., Harrison, S. C., Monroe. J. J., Chow, E. P., Mattaliano, R. J., Ramachandran, K. L., Smart, J. E., Ahn, A. H., & Brosius, J. (1987b) Clathrin light chains LCA and LCB are similar, polymorphic, and share repeated heptad motifs. Science, 236, 320.

Kohtz. D. S., Hanson, V., Kohtz, J. D., Schook, W. J., & Puszkin, S. (1987) Mapping two functional domains of clathrin light chains with monoclonal antibodies. J. Cell Biol., 104, 897.

Kohtz, D. S., & Puszkin, S. (1988) A neuronal protein (NP185) associated with Clathrin-coated vesicles. J. Biol. Chem., 263, 7418.

Kohtz, D. S., & Puszkin, S. (1989) Phosphorylation of tubulin by casein kinase II regulates its binding to a neuronal protein (NP185) associated with brain coated vesicles. J. Neurochem., 52, 285.

Kuhn, L. C., & Kraenbuhl, J. P. (1982) The sacrificial receptor-translocation of ploymeric IgA across epithelia. TIBS. 7, 299.

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

Lentz, T. L. (1983) Cellular membrane reutilization and synaptic vesicle recycling. Trends Neurosci., Feb, 48.

Lewis, W. H. (1931) Pinocytosis. Bull, Johns Hopkins Hosp., 49, 17.

Lisanti, M. P., Shapiro, L. S., Moskowitz, N., Hua, E. L.,

Puszkin, S., & Schook, W. J. (1982) Isolation and preliminary characterization of clathrin-associated proteins. Eur. J. Biochem. 125, 463.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem., 193, 265.

Marsh, M. (1984) The entry of enveloped viruses into cells by endocytosis. Biochem J. 218, 1.

Marshall, S. (1985) Kinetics of insulin receptor internation and recycling in adipocytes. Shunting of receptors to a degradative pathway by inhibitors of recycling. J. Biol. Chem. 260, 4136.

Miller, T. M., & Heuser, J. E. (1984) Endocytosis of synaptic vesicle membrane at the frog neuromuscular junction. J. Cell Biol., 98, 685.

Mooibroek, M. J., Michiel, D. F., & Wang, J. H. (1987) Clathrin light chains are calcium-binding proteins. J. Biol. Chem., 262, 25.

Moskowitz, N., Schook, W. J., Lisanti, M., Hua, E., Puszkin, S. (1982a) Calmodulin affinity for brain coated vesicle proteins, J. Neurochem., 38, 1742.

Moskowitz, N., Schook, W. J., Puszkin, S. (1982b) Comparison of calmodulin binding to brain synaptic and coated vesicles, Biochim. Biophys. Acta, 689, 523.

Olsnes, S., & Phil, A. (1982) Chimeric toxins. Pharmacol. Ther. 15, 355.

Pastan, I., & Willingham, M. C. (1985) The pathway of endocytosis. Endocytosis. Plenum, N.Y.

Pauloin, A., Bernier, I., & Jolles, P. (1982) Presence of cyclic nucleotide-Ca+ independent protein kinase in bovine brain coated vesicles. Nature, 298, 574.

Pauloin, A., & Jolles, P. (1984) Internal control of the coated vesicle pp50-specific kinase complex. Nature, 311, 265.

Pauloin, A., & Jolles, P. (1986) Presence of a MgATP/ADP-dependent pp50 phosphatase in Bovine brain Coated vesicles. J. Biol. Chem., 261, 12568.

Pauloin, A., Thurieau, C., & Jolles, P. (1988) Cyclic phosphorylation/dephosphorylation cascade in bovine brain coated vesicles. Biochim. Biophys. Acta, 968, 91.

Pearse, B. M. F. (1975) Coated vesicles from pig brain:

purification and biochemical characterization. J. Mol. Biol. 97, 93.

Pearse, B. M. F. (1976) Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. Proc. Natl. Acad. Sci. U. S. A. 73, 1255.

Pearse, B. M. F., & Robinson, M. S. (1984) Purification and properties of 100-kD proteins from coated vesicles and their reconstitution with clathrin. EMBO, 3, 1951.

Pearse, B. M. F. (1985) Assembly of the Mannose-6-phosphate receptor into reconstituted clathrin coats. EMBO. 4, 2457.

Pearse, B. M. F., & Crowther, R. A. (1987) Structure and assembly of coated vesicles. Ann. Rev. Biophys. Biochem. 16, 49.

Pearse, B. M. F. (1988) Receptors compete for adaptors found in plasma membrane coated pits. EMBO. 7, 3331.

Pfeffer, S. R., Drubin, D. G., & Kelly, R. B. (1983) Identification of three coated vesicle components as alpha- and beta-tubulin linked to a phosphorylated 50,000-dalton polypeptide. J. Cell Biol., 97, 40.

Pfeffer, S. R., & Kelly, R. B. (1985) The subpopulation of brain coated vesicles that carries synaptic vesicle proteins contains two unique polypeptides. Cell, 40, 949.

Prasad, K., & Lippoldt, R. E. (1988) Molecular characterization of the AP180 coated vesicle assembly protein. Biochemistry, 27, 6098.

Pugsley, A. P. (1989) Endocytosis. Protein targeting., 211.

Roth, T. F., & Porter, K. R. (1964) Yolk protein uptake in the oocyte of the mosquito Aedes aegypti L. J. Cell Biol. 20, 313.

Schmid, S. L., Braell, W. A., Schlossmann, D. M., Rothman, J. E. (1984) A role for clathrin light chains in the recognition of clathrin by "uncoating ATPase". Nature (London), 311, 228.

Schook, W. J., Puszkin, S., Bloom, W., Ores, C., Kochwa, S. (1979) Mechanochemical properties of brain clathrin: interactions with actin and alpha-actinin and polymerization into basketlike structures or filaments. Proc. Natl. Acad. Sci. U. S. A. 76, 116.

Schook, W. J., & Puszkin, S. (1985) brain clathrin light chain 2 can be phosphorylated by a coated vesicle kinase. Proc. Natl. Acad. Sci. U.S.A., 82, 8039.

Schook, W. J., Parker, C., Silva, W. I., Kohtz, D. S., & Puszkin, S. (1987) phosphorylation characteristics of brain clathrin-coated vesicle endogenous proteins. J. Neurochem., 49, 434.

Schwartz, A. L. (1984) The hepatic asialoglycoprotein receptor. C. R. C. Crit. Rev. Biochem. 16, 207.

Silverstein, S. C., Steinman, R. M., & Cohn, Z. A. (1977) Endocytosis. Ann. Rev. Biochem. 46. 669.

Smith, S. J., & Augustine, G. J. (1988) Calcium ions, active zones and synaptic transmitter release. TINS, 11, 458.

Stephens. R. E. (1986) Membrane tubulin. Biol. Cell., 57, 95.

Tuazon, P. T., Bringham, E. W. and Traugh, J. A. (1979) Eur. J. Biochem. 94, 497.

Trimble, W. S., & Scheller, R. H. (1988) Molecular biology of synaptic vesicle-associated proteins. TIBS, 11, 241.

Ungewickell, E., & Branton, D. (1981) Assembly units of clathrin coats. Nature (London), 289, 420.

Ungewickell, E. (1985) The 70-kD mammalian heat shock proteins are structurally and functionally related to the uncoating protein that releases clathrin triskelia from coated vesicles. EMBO. 4, 3385.

Ungewickell, E., & Oestergarrd, L. (1989) Identification of the clathrin assembly protein AP180 in crude calf brain extracts by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Anali. Biochem., 179, 352.

Usami, M., Takahashi, A., & Kadota, K. (1984) Protein kinase and its endogenous substrates in coated vesicles. Biochim. Biophys. Acta, 798, 306.

Usami, M., Takahashi, A., Kadota, T., & Kadota, K. (1985) Phosphorylation of a clathrin light chain of coated vesicles in presence of Histones. J. Biochem. (Tokyo), 97, 1819.

Vigers, G. P. A., Crowther, R. A., & Pearse, B. M. F. (1986a) Three-dimensional structure of clathrin cages in ice. EMBO. 5, 529.

Vigers, G. P. A., Crowther, R. A., & Pearse, B. M. F. (1986b) Location of the 100 kd-50 kD accessory proteins in clathrin coats. EMBO. 5, 2079.

Virshup, D. M., & Bennett, V. (1988) Clathrin-coated vesicle assembly polypeptides: physical properties and reconstitution

studies with brain membranes. J. Cell Biol., 106, 39.

Ward, J. H., Jordan, I., Kushner, J. P., & Kaplan, J. (1984) Heme regulation of Hela cell transferrin receptor number. J. Biol. Chem. 259, 13235.

Weidenmann, B., & Mimms, L. T. (1983) Tubulin is a major protein constituent of bovine brain coated vesicles. Biochem. Biophys. Res. Commun., 115, 303.

Weidenmann, B., Lawley, K., Grund, C., & Branton, D. (1985) Solubilization of proteins from bovine coated vesicles by protein perturbants and Triton X-100. J. Cell Biol., 101, 12.

Wileman, T., Harding, c., & Stahl, P. (1985) Receptor-mediated endocytosis. Biochem. J., 232, 1.

Woodward, M. P., & Roth, T. F. (1978) Coated vesicles: characterization, selective dissociation, and reassembly. Proc. Natl. Acad. Sci. U. S. A. 75, 4394.

Zisapel, N., Levi, M., & Gozes, I. (1980) Tubulin: an integral protein of mammalian synaptic vesicle membranes. J. Neurochem., 34, 26.