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POLYPEPTIDES THAT CONFER FUNCTIONAL DIVERSITY TO CLATHRIN-COATED VESICLES

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POLYPEPTIDES THAT CONFER FUNCTIONAL DIVERSITY TO CLATHRIN-COATED VESICLES

by

D. Stave Kohtz II

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

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ABSTRACT

POLYPEPTIDES THAT CONFER FUNCTIONAL DIVERSITY TO CLATHRIN COATED VESICLES

by D. Stave Kohtz II

Advisor: Saul Puszkin, Ph.D.

Four new anti-clathrin light chain mAbs are reported here: C-7H12 and C-6C1 react with both forms; C-10B2 and C-4E5 react only with the lower molecular mass form. Characterization of these mAbs indicated that the clathrin light chains contain two structural domains: a non-conserved, accessible domain that is relevant to the phosphorylation of CAP_2 and a conserved inaccessible domain that binds clathrin.

Analysis of brain clathrin-coated vesicles (CCVs) by immunoprecipitation with anti-actin (A-7C11) or anti-tubulin (T-2H9) mAbs coupled to Sepharose 4B revealed that while all brain CCVs contain tubulin, only a distinct subpopulation is enriched in actin. Monoclonal antibodies were developed against clathrin-coated vesicle cargo

molecules that are sorted into either the actin$^+$ or actin$^-$ subpopulations. The distribution of the two forms of clathrin light chains among brain clathrin-coated vesicle was also examined, using monoclonal antibodies specific for each form. Subpopulations enriched in either the higher or lower molecular mass form of clathrin light chain were immunoprecipitated with these monoclonal antibodies.

Two monoclonal antibodies are characterized that react with a previously unidentified membrane-bound polypeptide (SA). Different forms of SA protein are distinguished by one-dimensional gel electrophoresis and immunoblotting. One form (SA_1) is found exclusively in plasma membrane preparations, another (SA_2) is found in clathrin-coated vesicles and synaptic vesicles, while another (SA_3) is found exclusively in Golgi fractions. Analysis by in vitro translation indicates that cells producing all forms of SA protein produce only a single transcript encoding a precursor of SA_1. Successive post-translational modifications appear to target SA protein to specific intracellular membranes.

Finally, two monoclonal antibodies (S-8G8 and S-6G7) are characterized that react with an abundant component of only neuronal clathrin-coated vesicles. This 185 kD polypeptide (NP185) binds the CCV assembly polypeptides and is biochemically, immunochemically, and functionally distinguishable from clathrin. Immunoprecipitation

experiments using radioiodinated CCVs and S-8G8 revealed that NP185 can be competed off the CCV by soluble tubulin. NP185 is apparently bound to neuronal CCVs by its interaction with vesicle membrane-bound tubulin. Further analysis indicated that phosphorylation of tubulin affects its affinity for NP185.

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LIST OF ABBREVIATIONS

actin$^+$	actin enriched
actin$^-$	actin depleted
ATP	adenosine triphosphate
Cal	Ca^{2+}/calmodulin-dependent kinase
Cas	casein kinase II
cAMP	cyclic adenosine monophosphate
CAPs	clathrin associated proteins
CAP_1	clathrin-associated protein (36 kD)
CAP_2	clathrin-associated protein (33 kD)
CAP_x	32 kD CAP cross-reactive phosphoprotein
CCGs	clathrin coated granules
CCVs	clathrin coated vesicles
CCV-NP185	coated vesicle-derived NP185
CFs	chymotryptic CAP fragments
CF_1	chymotryptic CAP fragment (30 kD)
CF_2	chymotryptic CAP fragment (18 kD)
CF_3	chymotryptic CAP fragment (15 kD)
C_2M	C_2 mouse myoblasts
CURL	compartment of uncoupling receptor and ligand
CV	coated vesicle

DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
ELISA	enzyme-linked immunoadsorbent assay
GERL	golgi endoplasmic reticulum lysosome
HAT	hypoxanthine-aminopterin-thymidine
HT	hypoxanthine-thymidine
kD	kilodalton
mAbs	monoclonal antibodies
mcg	microgram (also ug)
MEM	minimum non-essential amino acids
NGF	nerve growth factor
PVC	polyvinylchloride
RER	rough endoplasmic reticulum
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPM	synaptic plasma membrane
SV	synaptic vesicle
SV-185	synaptic vesicle-derived NP185
UFs	unique chymotryptic CAP fragments

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INTRODUCTION

The vesicles and cisternae mediating the intracellular movement of transmembrane and secretory molecules are in a continuous flux. Vesicles bud off larger membranous structures, move through intracellular space, fuse to form larger cisternae in which cargo molecules are rearranged and perhaps modified before entering new vesicles bound for another destination. The process can be frozen, either by chemical fixation or subcellular fractionation, and analyzed in a stepwise fashion. This type of analysis conceptually transforms cellular processes comprised of evanescent intermediates into several series of only apparently distinct organelles. One such organelle is the clathrin-coated vesicle (CCV).

The first images of CCVs were derived from electron microscopic observation of thin sectioned erythroblasts (Bessis and Breton Gorius, 1957). When erythroblasts were pre-incubated with ferritin for 10 minutes, the electron dense marker appeared in the lumen of the vesicles (Bessis, 1963). Several investigators (reviewed by Bowers, 1964) described and occasionally renamed coated vesicles in a variety of animal cells and tissues before Roth and Porter (1964), observing these structures in liver and insect

oocytes, speculated that CCVs are derived from coated pits that "pinch off" the plasma membrane. This hypothesis has been widely accepted for over twenty years. Ironically, however, Roth and Porter were probably observing only coated pits, since nearly all the CCV profiles adjacent to the plasma membrane have been shown by other investigators (Willingham et al., 1981; Wehland et al., 1981; Willingham and Pastan, 1983) to be coated pits still attached to the cell surface. Some investigators (Willingham and Pastan, 1983) have even speculated that CCVs never exist within the cell. Serial section studies comparing 80 nm and 20 nm serial sections of 3T3 cells (van Deurs et al., 1984) revealed that while the thicker sections do give the impression of a greater number of CCVs (25%), free CCVs are also apparent in the thinner sections (4%). Rather than press the vesicular ontological question, perhaps it is best to surmise that CCVs spend most of their time assembling, less time disassembling, and very little (albeit important) time being vesicles.

Morphological criteria alone are not sufficient to identify a CCV. Recent immunoelectron microscopic investigations have revealed vesicular structures on the fringes of the Golgi cisternae that resemble CCVs but are not clathrin-coated (Orci et al., 1986). This study indicates that the identification of CCVs by the mere

presence of a protein coat may be inaccurate, and thereby marks the end an era of usage for the cryptic terminology "coated vesicle." Subsequently, "Golgi coated vesicles" have been distinguished from "clathrin-coated vesicles," which were previously referred to as just "coated vesicles." This recent observation raises the possibility that other "dead ringers" for CCVs may exist that are actually coated by other proteins. Hence, morphological studies done in the absence of specific immunological probes for clathrin should be tempered by this possibility. On the other hand, investigations by Orci et al. (1984) have revealed a large granule in insulin secreting cells that is coated by a diffuse clathrin coat (not in lattice form). This clathrin coat can only be detected immunochemically. Clathrin-coated granules are a unique class of secretory organelles that are related to CCVs of the trans aspect of the Golgi cisternae. Thus, immunochemical studies can not only point out the imposters, they can also reveal the less obvious relatives of CCVs.

1. Clathrin-coated vesicles and endocytosis

Endocytosis consists of three catagories, all of which can involve CCVs or coated pits. The first, fluid-

Figure 1. An individual clathrin-coated vesicle as visualized by negative staining. This clathrin-coated vesicle was purified from bovine brain. The clathrin coat is visible as a network surrounding the membrane vesicle, which appears translucent. Total magnification 320,000 X.

phase endocytosis, involves the internalization of molecules which are not attached to the plasma membrane. A marker commonly used for fluid-phase endocytosis is soluble horseradish peroxidase (HRP). This marker is incubated with live cells or tissues prior to electron microscopy. Incubation of the sections with a substrate for the enzyme produces an electron dense precipitate in the various cellular compartments through which it travels. The second, absorptive endocytosis, involves the interalization of molecules non-specifically associated with the plasma membrane. Plant lectins or polylysine coupled to HRP can be used as markers of absorptive endocytosis. Absorptive endocytosis implies that a particle is adsorbed to the plasma membrane and consequently the efficiency of its internalization is increased. For example, the uptake of HRP in immune complexes is 100 to 1000 times the rate of uptake of the enzyme in fluid-phase (Steinman and Cohn, 1972). This process was originally studied during amoebic pinocytosis (Schumaker, 1958).

An interesting system for distinguishing fluid-phase endocytosis from absorptive endocytosis was devised by Schneider et al. (1979). Using cultured rat embryo fibroblasts, the uptake of control rabbit immunoglobulin and anti-plasma membrane rabbit immunoglobulin coupled to either fluorescein or radiolabelled acetate was compared.

Surprisingly, acetate-coupled control antibodies were taken up at a slow rate proportional to concentration (fluid-phase endocytosis), while fluorescein-coupled control antibodies were internalized at a much faster rate (absorptive endocytosis). Control rabbit immunoglobulin coupled to either label end up in the lysosome. Anti-plasma membrane antibodies coupled to either label recycled on the cell surface and never ended up in the lysosome (to the extent of time observed in this study). These results were consistent even when the control and anti-plasma membrane immunoglobulins were used simultaneously with different labels. In this experiment, the labelling moieties determined whether the molecules were internalized by fluid phase or absorptive endocytosis (barring the remote possibility of a fluorescein receptor), while the specificity of the antibody moiety determined the final subcellular destination.

The third category of endocytosis is receptor-mediated endocytosis. This process is preceded by the binding of a ligand to its receptor in a highly specific manner. The receptor-ligand complex, in some instances (for example, Goldstein et al., 1979), appears to cluster prior to endocytosis, implying that the formation of the receptor-ligand complex may signal its internalization. Unoccupied alpha-2-macroglobulin receptors, for example,

are distributed in a diffuse manner over the cell surface. When alpha-2-macroglobulin receptor complexes are formed, they rapidly cluster in coated regions or pits and are subsequently internalized (Willingham et al., 1979). The existence of internalization-defective low-density-lipoprotein (LDL) receptors produced by nonsense or frameshift mutations that truncate their cytoplasmic domain (Lehrman et al., 1985) indicates that allosteric transmembrane signaling by the receptor-ligand complex may induce clustering and internalization. Mutations in the cytoplasmic domain of a transfected EGF receptor gene also appear to affect internalization (Prywes et al., 1986). Interestingly, the clustering of HLA antigen artificially induced by anti-beta-2-microglobulin antibodies results in its endocytosis; however, the complexes are internalized by smooth vesicles (Huet et al., 1980).

Receptor-mediated endocytosis may be distinguished from absortive endocytosis by the presence of ligand-receptor complex signals that induce selective endocytosis. Prior to endocytosis, transferrin receptors cluster, although this clustering is ligand-independent and occurs during ligand-independent receptor recycling (Hopkins et al., 1985). On the same cells, EGF receptor remains monodisperse until ligand-bound, then aggregates prior to endocytosis (Hopkins op cit.). Thus, in some

receptor systems clustering may be ligand-dependent, while in other it may be ligand-independent (ocurring during constitutive recycling).

Although the amino acid sequences of at least six receptors internalized by the CCV-mediated pathway have been determined, no substantial homology has been observed between their cytoplasmic domains (Stahl and Schwartz, 1986). On the other hand, while some plasma membrane proteins may possess transmembrane signalling mechanisms to facilitate endocytosis, perhaps others have evolved mechanisms to either avoid endocytosis or to be endocytosed by non-clathrin-coated vesicles. Thy-1, a brain and thymic lymphocyte antigen, is covalently linked to the lipid moieties of the plasma membrane, and threrfore lacks a cytoplasmic domain (Low and Kincade, 1985). Thy-1 is excluded from CCVs and coated pits (Bretscher et al., 1980). Carney and Bergmann (1982) have shown that clusters of thrombin-thrombin receptor complexes are not associated with CCVs or coated pits. Interestingly, thrombin receptors apparently cluster in the absence of ligand (Carney and Bergmann, 1982). Non-clathrin-coated membranes internalize cholera and tetanus toxins (Montesano et al., 1982). These toxins may bind surface receptor molecules differently from their normal ligands and consequently alter their routes of internalization.

Interestingly, the receptor for cholera toxin is a glycolipid (Schlegel et al., 1983). This may reflect a mechanism of evading CCV or coated pit endocytosis that is similar to Thy-1. Finally, the clustering process can be inhibited by dansylcadaverine, which also prevents transfer of ligands to the lysosome (Davies et al., 1980). Dansylcadaverine is a potent inhibitor of transglutaminases, although it may have other effects on cell metabolism (Davies et al., op cit.).

Receptor clustering events involve perhaps 10 to 20 receptors (Willingham and Pastan, 1983), and are distinguishable from the so-called capping phenomena by their relative insensitivity to cytoskeleton-binding drugs (for example, MacLean and Sanders, 1983). Receptor-mediated endocytosis is generally unaffected by cytoskeleton-binding drugs whereas "zipper-like" phagocytotic processes tend to be inhibited (Ward and Murray, 1984).

Receptors and endocytosis: Summarizing the receptor systems which participate in pathways mediated by CCVs and coated pits raises some seemingly obvious questions: What simple purpose, if any, does endocytosis serve? Is endocytosis important to the signaling aspect of a receptor, or is it merely a maintenance mechanism? Of

course, these questions do not apply to receptors whose purpose is the internalization of ligand for further processing (LDL, transferrin, poly-Ig), but in the case of growth factor receptors, which induce the expression of specific genes, changes in cell morphology, and DNA synthesis, the role of receptor-mediated endocytosis as a second messenger may be considered. This problem was addressed by Maxfield et al. (1979) by using chemical inhibitors to prevent clustering and endocytosis. These inhibitors enhanced epidermal growth factor (EGF) stimulation of DNA synthesis in cultured cells. This result indicates that one effect of endocytosis is the attenuation of receptor signaling by eventual dissociation of the receptor-ligand complex. Expression of the EGF receptor on the cell surface is also modulated by "down regulation" (Carpenter and Cohen, 1976). This process occurs because the EGF receptor accompanies its ligand to the lysosome, where both are degraded (Haigler et al., 1979). Until newly synthesized EGF receptor reaches the plasma membrane, the surface binding capacity of the cell for EGF is significantly reduced. These results indicate that the endocytosis of growth factor receptors serves to attenuate their potency rather than act as a second messenger system. It can be extrapolated that some components of CCVs, such as clathrin, may be recessively-

acting proto-oncogenes. Mutations adversely affecting the efficiency of receptor-mediated endocytosis may over-potentiate growth factor receptors and thus promote oncogenesis. Mutations such as these would not necessarily be lethal, as has been shown by site-directed mutagenesis of the clathrin gene in yeast (Payne and Schekman, 1985). In addition, some tumors appear to be induced by the loss of certain genes, such as Wilm's tumor (Koufos et al., 1985).

Endocytic cargo of clathrin-coated vesicles: The term "cargo" is frequently used to refer to the passengers of a vesicular transport vehicle (Pearse and Bretscher, 1981). The endocytic cargo of CCVs and coated pits includes a variety of receptors and cell surface molecules. The subsequent pathways through the cell followed by these molecules can vary greatly, and this will be discussed in a later section.

Three experimental approaches have been used to demonstrate that a particular membrane or secretory molecule is a CCV cargo molecule. The first approach is most common, and in many ways the most informative. Immunoelectron microscopy can be performed on cultured cells or tissues. Specific ligands, non-specific ligands, or anti-receptor antibodies are coupled to markers such as

colloidal gold or HRP, and incubated with live cells or detergent treated sections. The former reveals the pathway of a membrane polypeptide in a time-dependent manner, thus yielding information about the processes of internalization and transport. The latter reveals the distribution of the membrane bound polypeptide throughout the entire cell. This occasionally includes secretory elements which are not detected using the endocytic route and live cells. As indicated earlier, labelled anti-clathrin antibodies should be used simultaneously to determine if the ligand is following a pathway involving CCVs or coated pits.

The second method requires the purification of CCVs from the tissue of interest and a subsequent analysis of their contents. This methodology has been used to biochemically characterize CCVs, and identify some previously unknown cargo molecules. These include the muscarinic acetylcholine (Silva et al., 1986) and beta-adrenergic (Chuang et al., 1986) receptors, in CCVs derived from brain tissue. One difficulty with this technique is that the biochemical parameters for assessing CCV purity are not reliable, and the contributions of contaminating smooth vesicles can never be adequately evaluated. In addition, this methodology does not reveal whether the isolated CCVs have been derived from endocytic or secretory pathways, although this problem has been recently addressed

(Hemly et al., 1986).

The third method is essentially a modification of the second. If antibodies are available to the cytoplasmic domain of the putative CCV cargo molecule, purified CCV preparations can be analyzed by immunoprecipitation for the presence of the cargo molecule. If the anti-cargo molecule antibodies precipitate CCVs, then the antigen is a CCV cargo molecule. The presence of CCVs in the precipitates can be evaluated biochemically or by electron microscopy. This method was first used in a reliable manner by Pfeffer and Kelly (1985). Although this method eliminates the possibility of false positives generated by contaminating smooth membranes, the intracellular origin of the vesicles still remains in question. The generation of monoclonal antibodies that can be used as markers of specific CCV subpopulations (discussed in the RESULTS section) or coupling this methodology to that discussed by Hemly et al. (1986) should address this problem.

The phylogenetic limits of CCV distribution have been addressed only morphologically or immunochemically (for review and discussion, Fine et al., 1984), and until nucleic acid probes to CCV structural proteins are available to assess the distribution and divergence of clathrin, these studies cannot be considered complete. Therefore, a detailed discussion of the endocytic cargo of

CCVs will be restricted to the cargo of cells of higher animals. Clathrin-coated vesicles and coated pits have been observed, however, in unicellular Tetrahymena (Nilsson and van Deurs, 1983), unicellular Crithidia, and in Hydra (Csaba et al., 1984). In unicellulars CCVs congregate around cilia and flagella, and are also observed near the Golgi apparatus. In Hydra, their distribution appears random. Since membrane receptors of unicellulars are mainly food receptors, CCVs probably mediate the intake and processing of smaller elements of food in these cells (Csaba et al., 1984). Plant cells also contain an abundance of CCVs (Mersey et al., 1982).

Perhaps the only unifying feature of the CCV cargo molecules of higher animals is the apparent abundance of molecules which modulate the physiology of the organism and the relative paucity of cargo relevant to the metabolic survival of the cell. This generalization, although made in the absence of numerical analysis, is supported by site-directed mutagenesis experiments with yeast (Payne and Schekman, 1985), which indicate that the clathrin gene is unessential to the survival of yeast cells. More detailed studies of diverse organisms may reveal some instances where CCVs mediate more fundamental membrane processes; until then, it appears that this vehicle has been adapted by most cells to highly specialized activites. Possible

exceptions to this generalization may be transferrin (Willingham and Pastan, 1985), and transcobalamin (Takahashi et al., 1980), which mediate transport of nutrients used by all cells. Apparently yeast, which can survive minus their clathrin gene, have devised alternate transport routes or synthetic pathways for these nutrients. Examples of redundant transport routes are present in the cells of higher organisms. Low-density-lipoprotein is taken up and internalized through two parallel routes (Vasile et al., 1983). Both pathways regulate plasma LDL concentrations (although their relative significance is unclear from this paper). One, by transcytosis, supplies cholesterol to other cells, while the other is the well known receptor-mediated pathway (Anderson et al., 1977). Both pathways supply cholesterol to the cell; however, the receptor-mediated pathway supplies significantly less (Vasile et al., op cit.). Similar cases of redundant transport have been observed involving the uptake of cell nutrients (Watanabe et al., 1984; Kolb et al., 1983).

Several polypeptide growth factors and hormones are endocytosed through pathways involving CCVs or coated pits. Insulin was found to be internalized by CCVs by both electron microscopy (Fan et al., 1982) and biochemical analysis of purified liver CCVs (Pilch et al., 1983). The latter work is particularly noteworthy as the labelled

ligand was injected directly into the hepatic portal vein of a live liver prior to the isolation of CCVs. EGF was shown to be internalized by clathrin-coated vesicles in time course experiments incubating labelled EGF with live cells, and subsequently examining the internalized label by electron microscopy. Subsequent studies by Hanover et al. (1984) were conducted by immunoprecipitation. KB cells were incubated with EGF-HRP at 4 degrees C, warmed to 37 to allow internalization, and lysed at various times after internalization. Anti-clathrin antibodies coupled to Staphylococcus aureus were used to immunoprecipitate clathrin coated compartments from the lysates, and these were analyzed for HRP activity. Epidermal growth factor apparently passes through two clathrin-coated compartments on its way to the lysosome: one at 5 minutes after binding, and another at 20 to 25 minutes. The first compartment is undoubtedly an endocytic compartment. The second compartment may be involved in the transfer of EGF to the lysosome from the trans aspect of the Golgi cisternae (Hanover et al., 1984).

Platelet-derived growth factor appears to be internalized by both clathrin-coated and uncoated membranes, perhaps revealing two receptor classes (Rosenfeld et al., 1984). Somatostatin passes through CCVs shortly after internalization, although, oddly, it may not

be internalized by coated pits (Draznin et al., 1985). Modulators of cell growth that are not necessarily growth factors may also be internalized through CCV mediated pathways. These include alpha interferon (Zoon et al., 1983), and beta interferon (Kushnaryov et al., 1985). In addition, leutenizing hormone, follicle stimulating hormone, and chorionic gonadotropin are internalized by the gonadotropin receptor through a CCV-mediated pathway (Lin et al., 1982).

The acidic nature of the next compartment (the endosome, discussed below) encountered by an endocytosed ligand has facilitated the evolution of "double receptor" systems for the internalization of some small molecules. The model of this type of internalization is the transferrin system (Dautry-Varsat et al., 1983; Klausner et al., 1983; Harding and Stahl, 1983; and Morgan, 1981). Ferric ions in the medium bind apotransferrin to form diferric transferrin. The complex is internalized and transferrin is converted to apotransferrin by the acidic nature of the endosome. At acidic pH apotransferrin has a high affinity for the transferrin receptor, and while the released ferric ions are moved to the lysosome, this receptor-ligand complex is recycled to the cell surface. The apotransferrin-transferrin receptor complex is unstable at pH 7.4, and the apotransferrin is released into the

medium. This process has been called receptor-ligand recycling (Harding and Stahl, op cit.; Tietze et al., 1982).

The low-density-lipoprotein (LDL) receptor has perhaps served as the model for receptor-mediated endocytosis (Anderson et al., 1977). Initially studies of this receptor system were performed by light and eletron microscopic examination of cultured human fibroblasts incubated with labelled LDL (Anderson et al., op cit.). Subsequently, biochemically purified CCVs were shown to contain LDL and LDL receptor (Weintraub et al., 1985). Other significant endocytic cargo molecules of CCVs include alpha-2-macroglobulin (Willingham et al., 1980), asialoglycoprotein (Geuze et al., 1983), galactose particle receptor (Kolb et al., 1983), glucagon (Watanabe et al., 1984), thyroglobulin (Pearse et al., 1985), angiotensin II (Bianchi et al., 1986), the nicotinic acetylcholine receptor (Bursztajn and Fischhach, 1984), and some lysosomal membrane proteins (Tougard et al., 1985). Recent evidence has shown that receptor molecules localized to the adhesion plaques of Hela cells may interact with clathrin lattices. The lattices are apparently arrested in various phases of transformation to coats, and may be relevant to the structure of the plaque (Maupin and Pollard, 1983). Additional non-pathogenic endocytic cargo

are discussed in a later section on transcytosis.

Some viruses and pathogenic substances are internalized by CCVs and coated pits. For the most part, enveloped as opposed to naked viruses tend to be internalized by CCVs and coated pits. Naked viruses are either absorbed directly to the plasma membrane or internalized by cytoskeleton-dependent phagocytosis (Maul et al., 1978). Whether a particular virus enters a cell by a receptor-mediated endocytic pathway involving CCVs or coated pits depends in part on the pathway that the surface molecule acting as its receptor normally follows. Interference studies (DeLarco and Todaro, 1976) indicate that most enveloped viruses bind to a single receptor molecule that is recognized by the envelope glycoprotein of the virus in a manner that may be similar to or differ from a normal ligand. For example, the envelope of Influenza virus is covered by rod-shaped hemagglutinin (HA) molecules, a glycoprotein which binds host cell mucoprotein receptors (Matlin et al., 1981). These receptors are normally internalized by a CCV mediated pathway, and some evidence exists that Influenza virus follows a similar pathway (reviewed by White et al., 1983). Vesicular stomatitis virus (VSV) also appears to be internalized by CCVs or coated pits (Schlegel et al., 1982). Interestingly, although both viruses are

internalized by similar pathways, their newly synthesized envelope proteins are transported back to the plasma membrane by different pathways. This is best demonstrated by polarized epithelial cells, in which hemagglutinin is found exclusively on the apical face and VSV G-protein is found exclusively on the basolateral face (Rodriguez-Boulan, 1984). Recent experiments using CV-1 cells transfected with plasmids encoding HA-G-protein fusion proteins indicate that the cytoplasmic domains of the viral polypeptides target them to specific transport pathways (Roth et al., 1986). On the basolateral face of a polarized cell, the G-protein is constitutively recycled in CCVs and coated pits; on the apical face, however, HA is not. This implies that the endocytic pathway followed by a virus is determined by the cellular receptor for the virus, and this pathway may differ from that followed by the newly synthesized viral envelope proteins.

Several other viruses are internalized by CCVs and coated pits. These include: Sendai virus (Kim and Okada, 1982); Poliovirus type I (one of the few naked viruses to follow this pathway, Braunwald et al., 1985); mouse Elberfeld virus (Zeichhardt et al., 1985); Semliki Forest virus (Helenius and March, 1982); rabies virus (Superti et al., 1984); rotavirus SA 11 (Quan and Doane, 1983); and frog virus 3 (Braunwald et al., 1985).

Several studies have indicated that the endocytic pathway is essential for productive infection by enveloped viruses (togaviruses, orthomyxoviruses, retroviruses, and rhabdoviruses). In these cases, the low pH of the endosomal or lysosomal compartments induces conformational changes in the viral spike glycoproteins, resulting in membrane fusion of the envelope with these compartments and release nucleocapsids into the cytoplasm. (Helenius and Marsh, 1982). Drugs which inhibit the development of low pH by these compartments tend to inhibit viral infectivity (for example, Superti et al., 1984).

Bacterial toxins are also internalized by CCVs and coated pits. These include Pseudomonas exotoxin A (Morris et al., 1983), diptheria toxin (Morris and Saelinger, 1983: not by CCVs; Moya et al., 1985: by CCVs); and Staphylococcal toxic shock toxin (Kushnaryov et al., 1984). In contrast, cholera and tetanus toxins are clearly not internalized by CCVs or coated pits (Montesano et al., 1982). In addition, intracellular parasites such as Chlamydia trachomitis are internalized by the microfilament and microtubule-dependent phagocytosis mechanisms (Ward and Murray, 1984).

2. <u>After endocytosis</u>

Although CCVs and coated pits internalize molecules to be cycled through several different intracellular pathways, on the cell surface molecules internalized by CCVs or coated pits are only separated from those internalized by other pathways (Clathrin coated vesicles and endocytosis, above). In other words, several different receptor molecules may be internalized in the same coated pit. This means that any single CCV may have a heterogenous cargo (Maxfield et al., 1978; Carpentier et al., 1982). In addition to this, molecules internalized by absorptive or fluid phase endocytosis can also be internalized with receptor-ligand complexes, even though they may be bound for different intracellular destinations. After incubating cells with two or more differently labelled molecules, different pathways can be deciphered by following each label. Experiments such as these have indicated that membrane-bound molecules and molecules internalized by fluid-phase follow characteristic pathways in an organized fashion. In order to describe the organelles that participate in processing of receptors and ligands, a typical ligand will first be considered; then, variations will be described.

The asialoglycoprotein and other receptors: The endocytic pathway of the asialoglycoprtein receptor has

been described in detail (Schwartz et al., 1982). In the absence of ligand the receptor molecules are distributed diffusely over the cell surface. Binding of ligand is followed by rapid clustering of receptors and internalization through CCVs and coated pits. Biochemical studies have demonstrated that the intracellular half-life of asialo-orosomucoid (ASOR) is about 15 to 20 minutes whereas that of the receptor is 40 hours (Ashwell and Harford, 1982). Eight minutes after internalization, the receptor is returned to the surface (Schwartz et al., 1982). Degradation products of the ligand are detected in the medium of cultured HepG2 cells after at least one hour of incubation (Ciechanover et al., 1983). Together, these data indicate that the asialoglycoprotein receptor (AS-R) follows a much shorter pathway than the ligand (ASOR). As a result, a cellular compartment must exist in which the AS-R and ASOR are dissociated prior to the degradation of ASOR in the lysosome. One way to study the transport of ligands during endocytosis is to fractionate cell homogenates on Percoll gradients (Merion and Sly, 1983; Harford et al., 1983; Wileman et al., 1984). In the first 5 minutes radiolabelled ligand is concentrated in buoyant membrane fractions rich in plasma membrane markers. Internalization of AS-R-ASOR complexes requires a mean time of approximately 2 to 4 minutes (Ciechanover et al.,

1983). After approximately 5 minutes, most of the label is transferred to an intermediate density compartment, while after 20 to 30 minutes most of the ligand accumulates in the lysosomal fraction at the bottom of the gradient (Merion and Sly, 1983).

Correlates to the compartments identified by Percoll gradient centrifugation are identifiable by electron microscopy (Schwartz et al., 1982). Antibodies against both ASOR and AS-R were used with a double-labelling technique to follow both receptor and ligand during endocytosis and subsequent processing. These experiments indicated that soon after endocytosis both receptor and ligand were contained in large vesicles that did not bear clathrin coats. Subsequently, ligand was observed accumulating in the lysosomal compartments, while the receptor recycled to the cell surface. Helenius et al. (1983) used the term endosome to refer to the intermediate compartment, which corresponds to the intermediate density compartments detected by Percoll gradient centrifugation. Although no enzyme marker exists for the endosomal compartment, purification techniques have been proposed (Courtoy et al., 1984; Dickson et al., 1983). Purified endosomes do not contain clathrin (Dickson et al., op cit.). Direct evidence that the endosomal compartment is acidic has been provided by experiments using pH-dependent

emission shift of fluorescein labelled ligands (Tycko and Maxfield, 1982).

The endosomal compartment has been subdivided into three distinct cisternae (endosome I, II, and III; Helenius et al., 1983). Alternatively, this organelle can be classified by intracellular location (Hopkins, 1983, 1985). Endosome I is the earliest form, perhaps consisting of little more than a few uncoated vesicles. Endosome II and III have been compared to a compartment called the CURL (compartment of uncoupling of receptor and ligand) by Schwartz et al., 1982. In this compartment receptors are dissociated from ligands and recycled to the cell surface. Electron microscopic examination has indicated that endosomes II and III acquire arm-like extensions that appear to contain segregated receptor molecules (Geuze et al., 1983). The arm-like extensions are thought to detach and recycle the receptors to the cell surface, generating endosome III which contains the ligand bound for the lysosome (Harding et al., 1985).

Receptor recycling occurs through two pathways: a fast cycle, which proceeds through the endosome in 8 minutes, and a long cycle, which proceeds through some of the Golgi cisternae and GERL (Ciechanover et al., 1983), in approximately 2 hours. The slow pathway is only observed during prolonged incubation with ligand and can be detected

with asialotransferrin (which become re-sialylated; Snider and Rogers, 1985). These compartments are less acidic than earlier endocytic compartments (Yamishiro et al.,1984). There is no evidence suggesting that the slow pathway is induced by the receptor-ligand complex, and it may be a repair cycle followed by all membrane proteins. To this end, only 35% of the AS-Rs are confined to the plasma membrane, while the rest are found near the Golgi cisternae, smooth endoplasmic reticulum, and endosomal membranes (by electron microscopy, Geuze et al., 1983). Since these observations were made in the absence of ligand, this distribution is the result of constitutive recycling, which may include both the long and short pathways.

To summarize, the AS-R binds ligand, then clusters, and is internalized by CCVs and coated pits. The CCVs loose their coats and fuse to form endosome I. Endosome I is an acidifying compartment, which results in the dissociation of ligand and receptor. Endosome I transforms to endosome II (also called the CURL), which evolves an arm-like structure into which the receptor molecules are sorted. This structure mediates the fast return of the receptor to the cell surface (average time, 8 minutes) while the ligand passes to endosome III and subsequently to the lysosome. This pathway is followed by other receptor-

ligand systems, including LDL (Anderson et al., 1977) and mannose-6-phosphate (Geuze et al., 1984). In the case of mannose-6-phosphate, receptor-ligand complexes have been observed in the Golgi cisternae, although no receptor was observed in the lysosome (Geuze et al., op cit.). The ligand in this case was newly synthesized cathepsin D, and was not acquired through an endocytic pathway. As was mentioned earlier for the EGF receptor, the mannose-6-phosphate receptor expressed on the cell surface enters the endocytic pathway and passes through two clathrin-coated compartments: one 5 minutes after adding ligand, and another 20 to 30 minutes after adding ligand (Sahagian, 1984). The second represents transport from the trans aspect of the Golgi to the lysosome, a pathway in which the mannose-6-phosphate receptor mediates the transfer of newly synthesized lysosomal enzymes. The pathway is thought to involve endosomes and CURLs similar to the endocytic pathway (Sahagian, op cit.). In the case of the mannose-6-phosphate receptor, however, receptor recycling may be directed back to the Golgi cisternae.

Some receptors do not recycle. One example is the EGF receptor (Dunn et al., 1986). EGF is bound and internalized similarly to ASOR, arriving in an endosomal compartment 2 to 5 minutes after binding to the surface. Whereas of ASOR to the AS-R has little effect on the half-

life of the receptor (Breitfeld et al., 1985), exposure of liver to EGF results in a 4-fold reduction in the half-life of the EGF receptor (Dunn et al., op cit.). By several kinetic studies (for example, Hanover at al., 1984), it was determined that the EGF receptor accompanies EGF to the lysosome, where both are degraded. This leads to the well known phenomenon of receptor down regulation (Carpenter and Cohen, 1976). Interestingly, Hanover et al. (1984) found that EGF HRP passes through two clathrin-coated compartments on its way to the endosome, similarly to the mannose-6-phosphate receptor. The difference is that while the mannose-6-phosphate receptor is recycled to the Golgi, the EGF receptor remains to be degraded in the lysosome.

Other receptors that accompany their ligamds to the lysosome include the insulin receptor (Kasuga et al., 1981) and the Fc receptor (Mellman and Plutner, 1984; Ukkonen et al., 1986), although evidence exists that these receptors may recycle under some conditions. The internalization of the platelet-derived growth factor (PDGF) receptor is unusually slow (surface half-life of 20 minutes), perhaps due to the use of two pathways of internalization (clathrin-coated: 17%; smooth vesicle: 82%; Rosenfeld et al., 1984). Consistant with this, approximately 25% of the receptor is found in the lysosome after 20 minutes, indicating that the CCV-mediated pathway may behave

similarly to that of the EGF receptor (Rosenfeld et al., op cit.).

As discussed above for transferrin, some ligands are receptors themselves; these ligands participate in a process known as receptor-ligand processing (Morgan, 1981). In this case, the ligand itself is a receptor for another ligand, and the second ligand is transported to the lysosomes while the first ligand is recycled to the cell surface with the receptor. In the case of transferrin, this pathway may include a pass through the the Golgi (Yamashiro and Maxfield, 1984) during slow recycling, but no evidence exists that this is a receptor-ligand induced pathway. Asialotransferrin is resialylated during transport through this pathway (Regoeczi et al., 1982; Hanover et al., 1984; review: Dautry and Varsat, 1986). Interestingly, Pan et al. (1985) have observed that during the final stages of erythrocyte differentiation, recycling of transferrin stops, and the receptor accumulates in large multivesicular bodies. Receptor-ligand recycling is occasionally observed with ligands (such as LDL) that are normally transported to the lysosome while the receptor is recycled. These aberrancies, however, are only observed on the short recycling pathways in the presence of excess ligand (Townsend et al., 1984; Simmons and Schwartz, 1984; Greenspan and St. Clair, 1984; Tietze et al., 1982).

When labelled EGF (which accompanies its receptor to the lysosome) is coincubated with labelled transferrin (which recycles) both enter the same CCVs, and are localized in the same endosomal compartments 15 minutes later (Hanover et al., 1984). Subsequent to this, EGF is found in the Golgi cisternae or lysosomes, while apotransferrin recycles to the cell surface. Thus, the endosomal compartment serves as an intersection in which the recycling and non-recycling pathways segregate. The mechanisms by which some receptors are recycled by the arm-like projections of the CURL and others are sent to the lysosomes with their ligands are currently being investigated (for example, Harding et al., 1985). Interestingly, recent evidence from Mueller and Hubbard (1986) has indicated that ASOR is transferred through two different compartments of the endosome. These can be separated by sucrose density centrifugation, and one contains AS-R while the other does not.

Some toxins follow alternate pathways that may or may not reflect pathways followed by normal cellular membrane polypeptides. Staphylococcal toxic shock toxin, for example, is internalized by CCVs and coated pits, but subsequently is not brought to an endosomal compartment. Instead, the CCVs appear to coalesce with unusual transport vesicles that may be specialized elements of the human

epithelial cells being studied (Kushnaryov et al., 1984). Whether this is a normal endocytic pathway of endothelial cells remains to be determined.

Transcytosis: Clathrin-coated vesicles are involved in the transcytosis of membrane bound molecules across the yolk sac endoderm (Maxon and Wild, 1976; Mobbs and McMillan, 1981). In addition, an abundance of CCVs has been demonstrated in placental tissues (Ockleford and Whyte, 1977). These CCVs apparently traverse the placental trophoblasts and transfer maternal IgG, transferrin, and ferritin to the developing fetus (Wild, 1975). A similar process has been observed in the newborn rat intestine (Abrahamson and Rodewald, 1981), where Fc receptors on the lumenal membranes of intestinal epithelial cells mediate the transfer of IgG from the intestinal lumen to the circulation. The process apparently requires the binding of IgG to a special receptor which guides it across the cell. Molecules which co-internalize with IgG in the fluid phase are not transcytosed (HRP ends up in the lysosome). An interesting variation of transcytosis is observed with the poly-Ig receptor (Mostov and Blobel, 1982; Mostov and Simister, 1985). The receptor molecule is cleaved intracellularly to create a dimeric IgA molecule for secretion at the apical face of the hepatocyte. In this

way, IgA secreted by lymphocytes at the basolateral face acquires a secretory fragment and is released into the lumen.

Limet et al. (1985) injected polymeric IgA and galactosylated bovine serum albumin (polymeric IgA is transcytosed by hepatocytes; galactosylated bovine serum albumin is digested in the lysosomes) into rats. Ligand-HRP conjugates were used. Electron microscopic examination of sectioned livers revealed that subsequent to internalization polymeric IgA was transferred to distinct endosomal compartments. Thus, segregation of transcytotic and lysosomal cargo occurs before endosome II or the CURL. On the other hand, Geuze et al. (1984) compared the pathways in rat liver of asialoglycoprotein, mannose-6-phosphate receptor ligands, and polymeric IgA. All three were observed on the plasma membrane, in the Golgi apparatus and in the CURL (endosome II). Clearly, further work is needed to discern the compartments in which endocytic and transcytotic cargo are separated.

3. Clathrin-coated vesicles and secretion

Newly sythesized transmembrane and secretory polypeptides are synthesized on free ribosomes and inserted into the rough endoplasmic reticulum (RER) either co-

translationally or after synthesis is completed (Walter and Blobel, 1980; Walter et al, 1984; Wickner and Lodish, 1985). In the RER, polypeptides can acquire core mannose residues. Morphological studies have indicated that newly synthesized polypeptides are transferred from the RER to the cis aspect of the Golgi apparatus by "transition" vesicles (Palade, 1975). The polypeptides mature through the cisternae of the Golgi, which results in modification of their carbohydrate moieties. The polypeptides are transferred between adjacent cisternae of the Golgi by "Golgi vesicles" (Orci et al., 1986). Another population of vesicles transfers polypeptides from the trans aspect of the golgi to the plasma membrane. In some cases, further processing may occur in these vesicles (proinsulin to insulin, for example: Orci, 1986).

Palade and Fletcher (1977) originally suggested that CCVs may be involved in transferring RER contents to the Golgi cisternae. Subsequent immunochemical studies have not so far revealed the presence of clathrin coats on these vesicles or in the vesicles that mediate the transfer of polypeptides through the Golgi cisternae (Lin et al., 1982; Orci et al., 1986). The exit of newly synthesized membrane proteins from the trans cisternae of the Golgi complex, however, can be mediated by CCVs (Griffiths et al., 1985). When cells infected by VSV are incubated at 20

degrees centigrade, G protein accumulates in a Golgi-related cisternae that stains positively for acid phosphatase (the trans aspect and GERL). The enlarged structure which is generated at this temperature (or in the presence of monensin) is characterized by extensive areas of membrane buds which may or may not bear clathrin. In the case of VSV G protein, the buds do not bear clathrin (Griffiths et al., op cit.). This indicates that both clathrin-coated and non-coated membranes may mediate transport from the trans cisternae of the Golgi to the plasma membrane.

Clathrin-coated vesicles are also believed to mediate some phase of the transfer of cathepsin D from the trans aspect of the Golgi to the lysosome. In this capacity, CCVs may also be involved in the generation and maintenance of lysosomes. The rat ganglion nodosum was used to study processes that emerge into the perikarya from the axon (Holtzman et al., 1967). These areas accumulate GERL, Golgi, smooth endoplasmic reticulum, and lysosomes. Electron microscopic evidence (Holtzman et al., op cit.) indicates that the generation of lysosomes may be linked to the formation of multivesicular bodies from CCVs budding off the trans aspect of the Golgi cisternae. Friend and Farquhar (1967) demonstrated that in the epithelium of the rat vas deferens large CCVs (100+nm) serve as

heterophagosomes, transporting endocytic proteins to the lysosomes, and smaller (75nm) CCVs serve as primary lysosomes to transport hydrolytic enzymes from the Golgi to multivesicular bodies and the lysosomes. The work of Friend and Farquhar was visionary. Recent work by Geuze et al. (1985) has indicated that in transit from the trans aspect of the Golgi to the lysosome, mannose-6-phosphate receptor, cathepsin D, beta-hexosaminidase and alpha-glycosidase in HepG2 cells pass through CCVs and endosomal compartments. In these compartments, the mannose-6-phosphate receptors segregate in the CURL and are apparently recycled to the Golgi. Albumin, on the other hand, does not appear to pass through either CCVs or endosomal compartments.

Clathrin-coated vesicles may mediate the direct transfer of newly synthesized membrane polypeptides (or secretory polypeptides) to the plasma membrane, or intermediate vesicles may exist to which the CCVs fuse prior to their ultimate arrival at the plasma membrane. The secretory vesicles may bear clathrin lattices or diffuse clathrin coats reminiscent of the CCVs that fused to form these organelles (Orci et al., 1984). Processing of precursor secretory protein into their mature forms can occur in these compartments. Two examples are proinsulin to insulin conversion (Orci et al., 1985) and possibly

procollagen processing (Goldenberg and Fine, 1985). Direct transfer to the plasma membrane by CCVs has been suggested for secretion of acetylcholine receptor by muscle cells (Bursztajn and Fischbach, 1984). In addition, experimental evidence exists that newly synthesized acetylcholine esterase may be transported to the cell surface of muscle cells by CCVs. This observation was made using Karnovsky-Roots (1964) staining methodology, and is the basis of a method for separating exocytic and endocytic CCVs discussed below (Hemly et al., 1986). When molecules are part of a regulated secretory pathway, CCVs may be involved in the formation of secretory vesicles which actually release their content at another time (when the appropriate membrane depolarizing signal is present). This was recently demonstrated by Tooze and Tooze (1986), who showed that in AtT20 cells many of the CCVs budding from the trans aspect of the Golgi contain ACTH and its precursors. These polypeptides eventually end up in mature secretory vesicles which bear no clathrin.

4. After secretion

Membrane retrieval was originally hypothesized by Heuser and Reese (1973) as a mechanism by which CCVs mediate the recovery of membrane lost to the synaptic

plasma membrane by the fusion of synaptic vesicles. Heuser and Reese (1973) studied the neuromuscular junctions of frog sartorius muscle. The motor nerve terminals were stimulated to secrete electrically (10Hz for 15 minutes). After this, the synaptic vesicles of the terminal appeared to be replaced by numerous irregular cisternae. After resting for 15 minutes the synaptic vesicles reappeared concurrently with the disappearance of the irregular cisternae. Heuser and Reese assumed that these vesicles were regenerated from the irregular cisternae. When the live muscles were soaked in HRP and the procedure repeated, the irregular cisternae contained much HRP, and after resting some of the newly formed synaptic vesicles also contained HRP. Since the larger cisternae appeared to be generated by the fusion of CCVs, Heuser and Reese concluded that CCVs were recovering membrane to generate new synaptic vesicles. This process is supposed to occur continuously so that membrane accumulates in the large cisternae or remains in the synaptic vesicles. Later consideration of the same system by Meshul and Pappas (1984), however, indicated that CCVs may be responsible for recovery of only a fraction of the total membrane in the frog neuromuscular junction. In experiments again using frog sartorius muscle incubated with HRP, Meshul and Pappas found that stimulatory conditions similar to those used by Heuser and

Reese resulted in only 10% of the newly formed synaptic vesicles acquiring label. In addition, nearly 41% of the CCVs contained no label. These data indicate that only half the CCVs in the nerve ending may be generated directly from synaptic plasma membranes, and only 10% of the synaptic vesicle membranes are recycled by CCVs. Clearly alternate routes must exist.

Clathrin-coated vesicles may mediate membrane retrieval in other systems as well. Cholinergic stimulation of chromaffin cells induces rapid coating of the plasma membrane. The kinetics of stimulus-dependent formation of these CCVs and coated pits is closely similar to that for secretory granule membrane retrieval (Geisow et al., 1985; Patzak and Winkler; (1986). Kadota and Kadota (1982) have observed membrane retrieval by macropinocytosis (large CCVs, perhaps) in presynaptic terminals of cat sympathetic ganglia, while cortical granule exocytosis appear to be coupled with membrane retrieval in Brachydanio eggs (Donovan and Hart, 1986).

5. Structure and Biochemistry of Clathrin-Coated Vesicles

Pearse (1975) first demonstrated that CCVs isolated from porcine brain contain a major 180 kD polypeptide.

This polypeptide was named clathrin, from the Greek word meaning "basket." Kanaseki and Kadota (1969), although they did not identify the major structural protein, observed the geometric structure of the coat in partially purified Guinea pig brain CCVs. They proposed that the closed lattice was formed from a hexagonal lattice by the conversion of 12 hexagons to pentagons (necessitating the removal of clathrin trimers). This scheme, although experimentally unproven, has stood up to more rigorous theoretical analysis (Lisanti and Puszkin, 1985). Kanaseki and Kadota (1969) originally suggested that this transformation could "power" the formation of the vesicle.

Heuser (1980) used quick-freeze, deep-etch, rotary-replication methods to observe the clathrin coats underlying the plasma membrane at the putative start of receptor-mediated endocytosis. Using this methodology, the coats appeared as a network of 7 nm strands arranged in 30 nm polygons, consisting of mostly six and some five and seven-sided rings. The planar arrangement of the coat appeared to contain more hexagons, while the number of pentagons increased as the curvature of the lattice increased (Heuser, 1980). Other investigators have suggested that the flat lattice to curved lattice conversion does not occur, and that clathrin coats assemble from free clathrin in the cytosol similarly to the assembly

of cages *in vitro* (Kirchhausen and Harrison, 1981). This suggestion may be valid, since the lattices shown by Heuser (1980) resemble those arrested in development in the adhesion plaques of Hela cells (Maupin and Pollard, 1983).

Purification of clathrin-coated vesicles: The basic elements of CCV purification were originally devised by Pearse (1975), and, with refinements, are still useful. Clathrin-coated vesicles are most stable within a pH range of 6.0 to 6.8; therefore, tissues from which CCVs are to be isolated are first homogenized in 0.1M morpholinoethane sulfonic acid (MES) pH 6.5 (Pearse, op cit.). Magnesium chloride (1mM), EGTA (1mM), protease inhibitors, and azide are often added. Clathrin-coated vesicles are significantly smaller than nuclei, mitochondria, or large membrane fragments; therefore, differential centrifugation can be used to separate the CCVs from these particles. Since CCVs pellet at 100,000 xg, several centrifugations below this force may be used to clear the homogenate. The most convenient is a single spin at 20,000 xg for 30 minutes. The remaining membranes and CCVs can be spun from the supernatant at 100,000 xg for 1 hour. This pellet is often called a crude membrane preparation (Pearse, op cit.) and several variation on further processing have been introduced. Originally, Pearse suggested processing the

Figure 2. Generalized scheme for purification of bovine brain clathrin coated vesicles.

P(1, 2, 3, or 4): pellets: S(1, 2, 3, 4): supernatants

diagram by Walter Silva

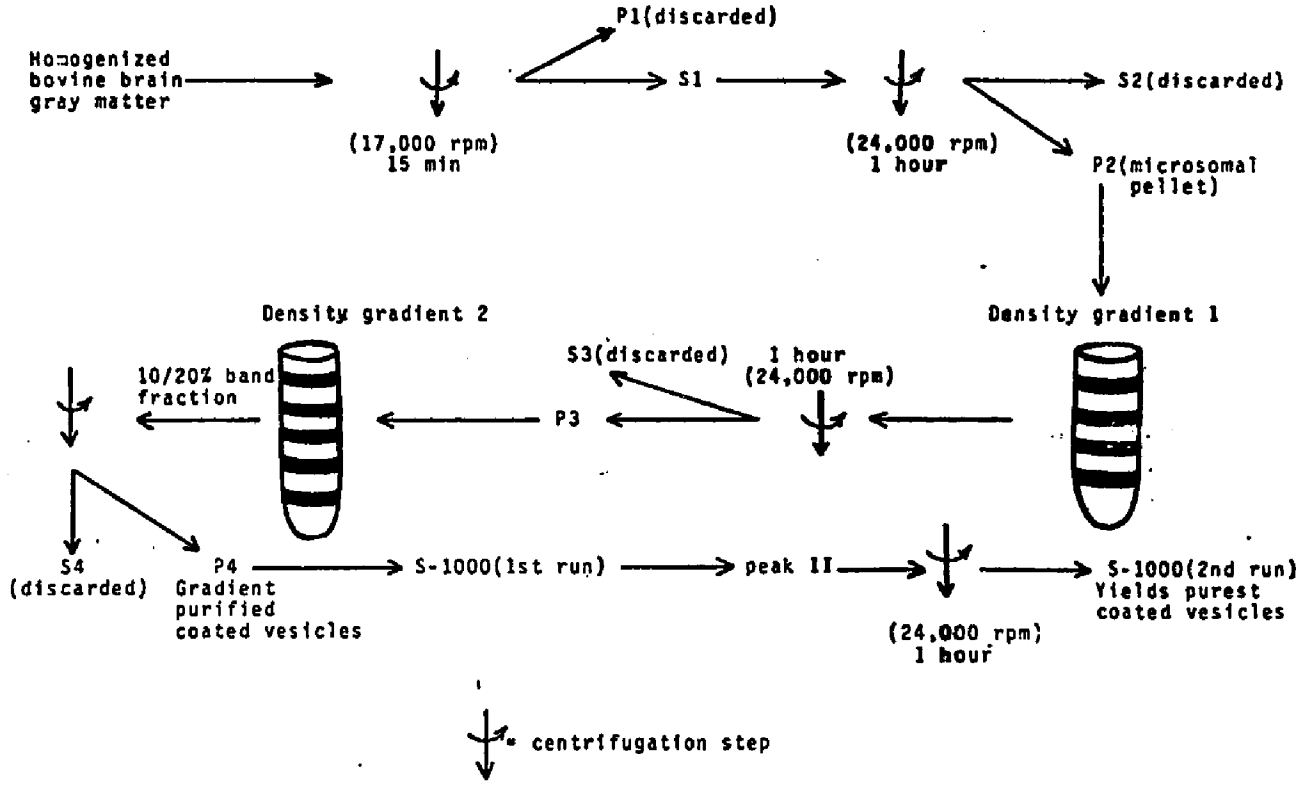
17,000 rpm: (HS34 rotor, Sorvall, 20,000 xg)

24,000 rpm: (Type 30 rotor, Beckman, 100,000 xg)

Density gradient 1: variations are described in the text

Density gradient 2: 5 - 30% sucrose for 1 hour

FIGURE 4

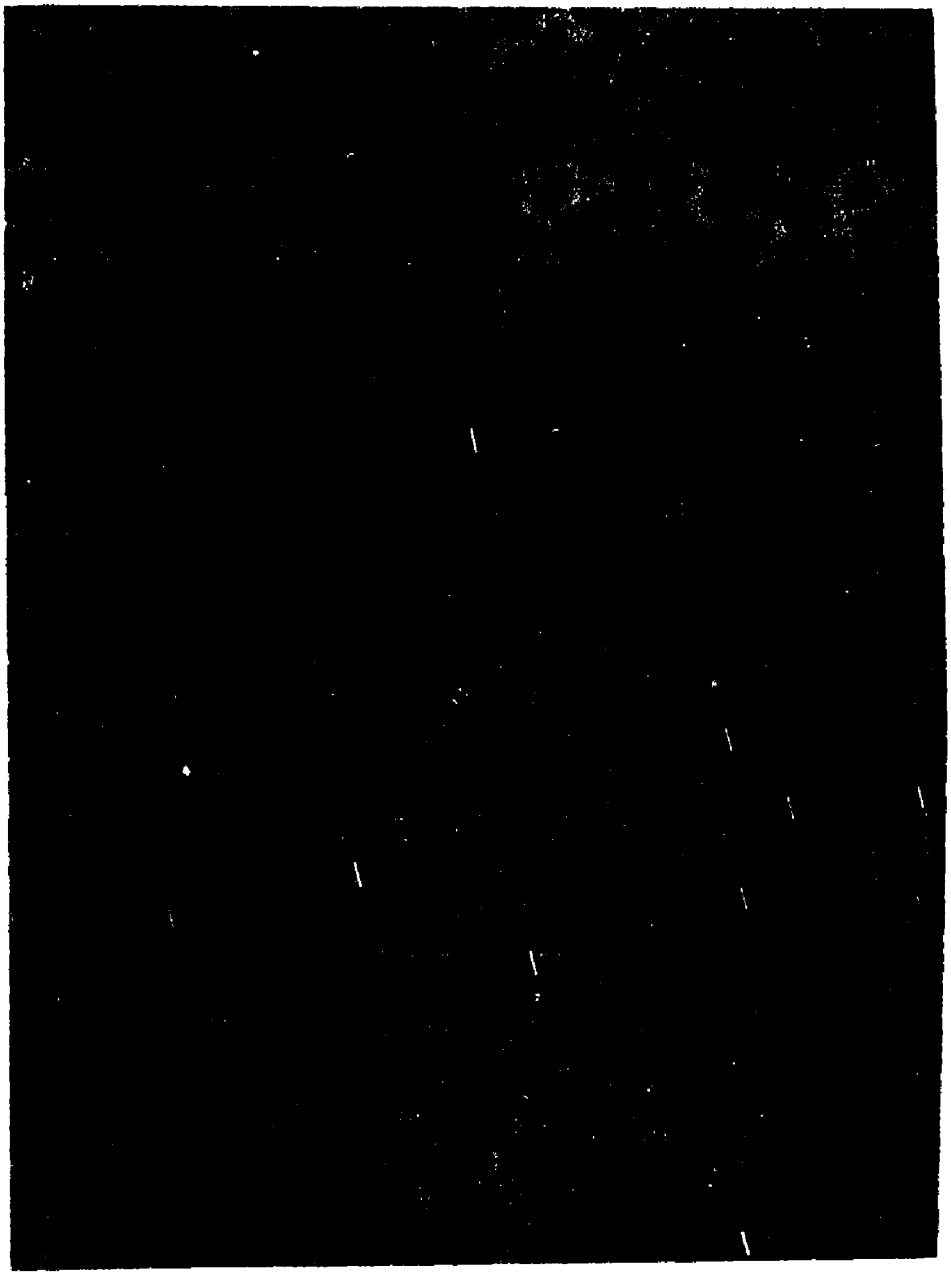

P1(discarded)
Homogenized bovine brain gray matter
(17,000 rpm) 15 min
S1
(24,000 rpm) 1 hour
S2(discarded)
P2(microsomal pellet)
Density gradient 2
Density gradient 1
S3(discarded)
1 hour (24,000 rpm)
10/20% band fraction
P3
S4 (discarded)
P4 Gradient purified coated vesicles
S-1000(1st run)
peak II
(24,000 rpm) 1 hour
S-1000(2nd run) Yields purest coated vesicles
centrifugation step

crude membrane pellet through three sucrose gradients: 5 to 60% at 50,000 xg for two hours (keep top half); 20 to 60% at 50,000 xg for 16 hours (top clear blue-white band); 5 to 30% for 1 hour at 100,000 xg (top turbid area). This method has been modified by Keen et al. (1979) to include only two sucrose gradients. This increases yield, but the lesser purity necessitates further purification steps.

Evidence of damage to brain CCVs by high sucrose concentrations has been noted by Nandi et al. (1983). This characteristic does not appear to be shared by CCVs from liver (Fine and Ockleford, 1984). As a result of this observation, Nandi et al. (1983) suggested the use of 8% sucrose, 100% D_2O for the sedimentation of brain CCVs instead of sucrose gradients. The use of isosmotic Percoll gradients has also been used instead of sucrose gradient centrifugation (Pauloin et al., 1982). Pearse (1982) has suggested the alternative use of either 9 to 90% D_2O/H_2O or Ficoll/D_2O gradients to replace sucrose gradient centrifugation. Finally, 12% Ficoll/12%sucrose centrifugation can be used, which appears to combine the preservation of Ficoll/D_2O with the high purity of sucrose gradient centrifugation. This can be followed by centrifugation over 5 to 30% sucrose for 1 hour.

A number of procedures can be used to further purify CCVs after gradient centrifugation. S-1000 column

Figure 3. Purified bovine brain clathrin coated vesicles. Clathrin coated vesicles were pelleted at 100,000 x g, sectioned, and analyzed by transmission electron microscopy. Total magnification 110,000 X.



chromatography has been used to purify CCVs from yeast (Mueller and Branton, 1982), bovine brain (Schook and Puszkin, 1985), and bovine heart (Goldman et al., in preparation). After this, controlled pore glass bead exclusion chromatography can be used to further purify CCVs (Kelly et al., 1983). Pearse (1975) has suggested the use of Triton X-100 to dissolve uncoated membranes. Recent evidence, however, indicates that Triton treatment removes several membrane bound proteins from CCVs (Wiedenmann et al., 1985). Since CCVs have a negative net electric charge, agarose electrophoresis has been used to further purify them from contaminating membranes (Rubenstein et al., 1981). Results obtained with this methodology should be tempered with the knowledge that sulfur compounds in agarose could inhibit endogenous enzyme activities in the CCV. Agarose electrophoresis has been used to purify CCVs from brain, liver, and CHO cells (Rubenstein et al., op cit.), and to separate subpopulations of CCVs from liver (Kedersha et al., 1986). Methods for further fractionation of CCVs into subpopulations will be discussed in a later section.

Clathrin: Clathrin coated vesicles have been purified from brain (Pearse, 1975), human placenta (Whyte, 1978; Pearse, 1982), rat liver (Campbell et al., 1983;

Figure 4. Clathrin cages, as visualized by negative staining. Clathrin triskelions were reassembled at pH 6.5 in the presence of 1 mM Mg^{2+}, then briefly digested with chymotrypsin, pelleted, and visualized. Total magnification 510,000 X.

Pilch et al., 1983), bovine adrenal cortex (Weintraub et al., 1985), oocytes (Woods et al., 1978), yeast (Mueller and Branton, 1982; Payne and Schekman, 1985), and cultured lymphoma (Pearse, 1976) and CHO (Rothman and Fine, 1980) cells. Clathrin appears as a 180 kD polypeptide in CCVs isolated from all of these sources. Immunochemical studies have indicated that the structure of clathrin is highly conserved between species, although diversity has been noted between clathrin from higher and lower mammals (Croze et al., 1982). One dimensional peptide mapping of clathrin derived from human, porcine, and bovine tissues revealed very similar profiles (Rothman et al., 1980). In addition, anti-sera have been developed which react specifically with avian and reptile cells (Robinson, 1982). In plant cells clathrin has an apparent molecular mass of 190 kD (Mersey et al., 1982).

Clathrin can be solubilized from CCVs by treatment with either 2M urea (Blitz et al., 1977) or 0.5M Tris (Keen et al., 1979), or by increasing the pH (Robinson, 1982) or lowering the ionic stregnth (Nandi et al., 1980) of the suspension buffer. Uncoating under these conditions causes transformation of the cage structure into triskelions composed of 3 clathrin molecules and three clathrin light chain molecules. When the triskelions are analyzed by low angle platinum rotary shadowing (Ungewickell and Branton,

1981) they appear as three elbows attached by their tips to a single axis. Each elbow represents one clathrin heavy chain molecule. The complex is commonly referred to as a triskelion. The clathrin light chain (3 per triskelion) are bound to the hub of the triskelion. Each elbow of the triskelion is 45 nm long, and bent almost precisely in the middle (Kirchhausen and Harrison, 1981). The purified triskelions spontaneously reassemble into cages (clathrin coats without the membrane vesicle; Keen et al., 1979; Blitz et al., 1977). Reassembly occurs optimally at concentrations of clathrin above 50 ug/ml, at pH 6.0 to 6.8, in the presence of Mg^{2+} or Ca^{2+}. Monoclonal antibodies to various antigenic sites on clathrin can be coincubated with reassembling triskelions to produce abberant lattice structures (Blank and Brodsky, 1986) indicating that assembly may be orchestrated in a site dependent manner. Interestingly, reassembly conditions such as salt concentration and temperature can affect the nature of the cage complex that is formed. This was recently shown by Sorger et al. (1986). Addition of 12% ammonium sulfate to an assembly mixture kept at 4 °C induced the formation of cubes consisting of eight clathrin triskelions. An average cage consists of about 40 triskelions with an equal number of vertices (Crowther et al., 1976).

Recent deep-etch visualization of clathrin absorbed to mica has revealed more detailed features of the triskelion (Heuser and Kirchhausen, 1985), such as scroll shaped hooks that can open and close to vary the lengths of the arms. The actual role of these "scroll" moieties in cage assembly is unclear, since partial digestion of the cages with trypsin yields a truncated 110 kD clathrin fragment that retains the ability to reassemble into cages (Schmid et al., 1982). The triskelions generated from this fragment appear to have trucated arms, missing the distal ends which contain the "scroll" structures. The correlates of *in vitro* and *in vivo* assembly, however, are unclear. Structures which are irrelevant to assembly *in vitro* may be critical *in vivo*. Elastase digested cages remain intact after digestion, but cannot reassemble (Lisanti et al., 1981). In this case, however, the clathrin molecules are left intact, but the light chains are digested. Thus, the clathrin light chains may have a role in orchestrating assembly *in vitro*.

Since CCVs appear to exist transiently *in vivo* (Willingham and Pastan, 1983), it would seem apparent *a priori* that an enzyme capable of disassembling clathrin coats and/or cages should exist. Schlossman et al. (1984) originally developed an assay for cage disassembly, and this was used as the basis of a scheme to purify an

"uncoating" enzyme. A 70 kD polypeptide was eventually isolated with an ATP-dependent ability to disassemble clathrin cages (Braell et al., 1984). Triskelions are released in a stoichiometric complex with the uncoating enzyme, and the released complexes are incapable of reassembling (Braell et al., op cit.). Later studies indicated that uncoating activity is mediated by the clathrin light chains, and although the enzyme binds clathrin tightly at two distinct sites, cages stripped of light chains are not disassembled (Schmid et al., 1985; Schmid and Rothman, 1985).

The clathrin "uncoating" protein is closely related to (if not identical to) a heat shock protein, hsc 70 (Ungewickell, 1985; Rothman and Schmid, 1986). Recent evidence (reviewed by Pelham, 1986) has indicated that one function of this protein is to break up hydrophobic aggregates of protein. Since the expression of these proteins is modulated by heat, one of the major targets of the heat shock proteins <u>in vivo</u> may be heat denatured protein aggregates. To hsc 70, then, the clathrin cage may appear to be simply a denatured aggregation of protein. Since hsc 70 can comprise as much as 1% of the protein in a growing cell (a 30-fold excess over clathrin) it is unlikely that uncoating is its sole function (Pelham, 1986).

Clathrin light chains (or clathrin-associated proteins): The clathrin light chains (or clathrin-associated proteins: CAPs; Lisanti et al., 1981) were originally observed by Pearse (1978) co-purifying with extracted clathrin. There are two forms of CAPs in higher animals: CAP_1 migrates slower during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with an apparent molecular mass of 36 kD in neuronal tissues and 32 kD in non-neuronal tissues; CAP_2 has an apparent molecular mass of 33 kD in neuronal tissues and 30 kD in non-neuronal tissues. In most tissues of higher animals the CAP_1 and CAP_2 are found in a 1:2 molar ratio (Ungewickell and Branton, 1981). A possible exception is erythrocyte plasma membranes, which appear to contain only CAP_1 (Davis et al., 1986). Each clathrin triskelion contains three heavy chain molecules and three CAP molecules. Chemical crosslinking studies indicate that each CAP molecule is associated with one heavy chain molecule (Pearse, 1976). Direct electron microscopic visualization of the CAPs using anti-CAP_1 monoclonal antibodies indicated that they are located near the hub of the triskelion (Kirchhausen et al., 1983). Monoclonal antibodies specifically reactive with either form (CAP_1: Kirchhausen et al., 1983; Brodsky, 1986; CAP_2: Kohtz et al., 1985, herewithin) have attested to the

structural diversity between CAP_1 and CAP_2. Direct competitive binding studies, however, with clathrin cages stripped of CAPs and affinity purified CAP_1 and CAP_2 have indicated that both CAPs bind the same site on clathrin (Winkler and Stanley, 1983). In addition, both CAPs mediate equally well the action of the "uncoating" enzyme (Schmid et al., 1985).

As was indicated in the previous section, the CAPs appear to confer geometric regularity to the triskelion structure, thereby facilitating its assembly into cages. Limited elastase (Kirchhausen and Harrison, 1981) or chymotrypsin (Lisanti et al., 1981) digestion of clathrin cages leaves the clathrin heavy chain intact but removes the CAPs. Clathrin-associated proteins can also be removed from cages by sodium isothiocyanate treatment followed by size exclusion chromatography (Winkler and Stanley, 1983). When the CAP-free cages are dissociated the resulting triskelions cannot be reassembled into organized cages, although aggregates are formed (Kirchhausen and Harrison, 1981). Schmid et al. (1982) have noted that while 60% of triskelion with CAPs have all three elbows pointing in the same direction, only 30% maintain this conformation in their absence (statistically random). Also noteworthy in this respect is the inability of the "uncoating" enzyme to disassemble clathrin cages lacking

CAPs. Therefore, the CAPs may influence the geometric conformation of the clathrin triskelion in a manner that is essential to efficient assembly and disassembly *in vitro*.

Modulation of clathrin geometry and mediating the action of the "uncoating" enzyme are functions which are performed equally well by either CAP_1 or CAP_2. Functions associated with their diversity are not well understood. A kinase activity has recently been associated exclusively with CAP_2 (Usami et al., 1985; Schook and Puszkin, 1985). This activity requires the presence of either polylysine or other polybasic substances, and has been recently attributed to a CCV-bound casein kinase II (Bar-Zvi and Branton, 1986). The functional significance of this kinase activity is unknown. Kirchhausen et al. (1983) have indicated that in purified clathrin triskelions the two forms of CAP are distributed in a statistically random manner; recent studies have indicated that CCV subpopulations exist in brain that are enriched in either CAP_1 or CAP_2 (Kohtz et al., 1985, herewithin). These results indicate that the diversity between the two proteins may be associated with sorting or transport of CCVs in different intracellular locations. It is perhaps noteworthy that CCVs isolated from yeast bear only one form of CAP (Payne and Schekman, 1985). This implies that two forms of CAP may have evolved to mediate the specialized

functions of highly differentiated cells in complex organisms.

Assembly Complex Polypeptides: The 110 kD and 50 to 55 kD polypeptides of the CCV assembly complex were first observed remaining associated with vesicle membranes after they were stripped of clathrin by treatment with 2M urea (Blitz et al., 1977) or 0.5M Tris (Keen et al., 1979). Unanue et al. (1981) originally demonstrated that the 110 kD and 50 to 55 kD polypeptides form a complex that mediates the binding of clathrin to the vesicle membrane. The association of clathrin with membrane-bound assembly complex has been shown in studies by Hanspal et al. (1984) using a filter paper assay to separate bound from unbound labelled clathrin. 112 kD and 124 kD tryptic fragments of clathrin were found to inhibit clathrin binding as well as whole clathrin, indicating that the hub portion of the triskelion mediates binding to the assembly complex. In addition, Scatchard plots indicated that the high affinity interaction of clathrin and CCV membranes occurs through a positively cooperative process (Hanspal et al., op cit.). This is a welcome observation, since it provides an explanation for the localized and rapid association of clathrin with regions of the membrane being internalized.

<u>In vitro</u> reassembly of clathrin cages in the presence

of assembly polypeptides has revealed their relevance to the assembly process. Irace et al. (1982) first observed that two sizes of cage could be formed, depending on the presence or absence of the assembly polypeptides. 110 kD assembly polypeptide was purified by lysine-Sepharose chromatography, and its addition to reassembling clathrin induced the formation of 150S cages. In its absence, 300S cages formed. Also, Ca^{2+} or Mg^{2+} stimulated formation of 300S cages in the presence of 110 kD polypeptide. Zaremba and Keen (1983), making similar obserations about the sizes of CCVs induced by the 110 kD polypeptide, also observed that in the presence of assembly polypeptide significant assembly occurs at low concetrations of clathrin, at various temperatures (above freezing), and at pH values of 7.2 to 7.5. In its absence, little assembly is observed above pH 6.7. Therefore, assembly polypeptides may mediate CCV assembly under physiological conditions.

Purification of bovine brain assembly complex was accomplished by Pearse and Robinson (1984) using Triton X-100 extracted CCV and a combination of gel filtration, hydroxyapatite, and DE-52 cellulose chromatography. These complexes consist of 100 kD and 50 kD polypeptides in a 1:1 molar complex. Pearse observed three distinguishable species (by hydroxyapatite chromatography) of 100 kD polypeptide that asssociate with 50 kD polypeptide, and

three species of 100 kD polypeptide that were not associated with this protein. Clathrin cages assembled in the presence of the 100/50 kD complexes yielded a homogeneous population of cages similar to the 150S cages observed by Irace et al. (1982). Clathrin assembled in the presence of the 100 kD polypeptides that lack 50 kD polypeptide produced a wide variety of cages, icosahedra, barrels, and complexes larger than 100 nm. Both types of assembly complexes induced clathrin assembly under putatively physiological conditions. Robinson and Pearse (1984) also noted that saturation of clathrin cages with assembly complex required approximately 3 molecules of 100 kD polypeptide (with or without 50 kD polypeptide) per clathrin triskelion. Thus, assembly complex and clathrin are present in equivalent molar amounts in CCVs.. Zaremba and Keen (1985) have indicated that brief elastase treatment of the assembly complex degrades the 100 kD polypeptide but leaves the 50 kD and a previously unidentified 16.5 kD assembly complex polypeptide intact. This treatment abolishes the ability of the complex to mediate the assembly of cages, indicating that the 50 kD and 16.5 kD polypeptides alone cannot facilitate this process.

Pearse and Bretscher (1981) have proposed that the assembly complex proteins mediate signaling between the

receptor-ligand complex on the cell surface or cargo molecules in the Golgi and the clathrin coat. The term "adaptor" molecule was proposed to refer to molecules which signal the assembly of clathrin coats on specific membranes, a function attributed to the various forms of the assembly complex polypeptides. Consistent with this, Pearse (1985) showed that mannose-6-phosphate receptors are integrated into clathrin cages reassembled in the presence of assembly polypeptides. The diversity observed by Pearse and Robinson (1984; described above) may be associated with the generation of CCVs on different intracellular membranes or with different receptors. Recent results from Robinson and Pearse (1986) have revealed immunochemical diversity between different forms of assembly complex proteins. Peptide mapping experiments also revealed that three forms of brain 100 kD polypeptide have different amino acid sequences. Two anti-sera to the 100 kD polypetides were produced by Robinson and Pearse (1986). One of the two anti-sera reacted preferentially with perinuclear membranes while the other reacted with both perinuclear and plasma membranes. This result, although preliminary, reveals that 100 kD diversity may be associated with transport and assembly at specific intracellular membranes.

Diversity has also been observed among the 50 to 55 kD polypeptides. In brain, this protein constitutes one 50

kD band during SDS-PAGE, while in liver it constitutes two, 53 kD and 55 kD (Campbell et al., 1984). These polypeptides have been mistaken for tubulin (which is a significant component of bovine brain CCVs) and tau protein; however, recent evidence has clearly shown that the assembly polypeptides are unrelated to these cytoskeletal proteins.

In purified CCVs the 50 to 55 kD assembly polypeptides are phosphorylated by an endogenous cAMP-independent, Ca^{2+}/calmodulin-independent kinase. In brain CCVs this kinase phosphorylates the single 50 kD (Pauloin and Jolles, 1984) assembly polypeptide; in liver, it phosphorylates the 53 kD and 55 kD polypeptides (Campbell et al., 1984). *In vitro* reassembly studies using elastase treated, phosphorylated and unphosphorylated 50 kD complex have indicated that this kinase activity has no obvious effect on cage assembly (Zaremba and Keen, 1985). Recent evidence has indicated, however, that *in vivo* the 100 kD polypeptide may actually be a more relevant substrate for phosphorylation. A co-assembly assay was devised by Keen and Black (1986) to facilitate purification of assembly complex polypeptides from cultured cells. Briefly, cultured rat sympathetic ganglion cells were labelled with ^{32}P-inorganic phosphate, homogenized, and combined with unlabelled reassembling clathrin cages. The cages were

purified, and the labelled CCV polypeptides integrated into these structures were analyzed by two dimensional gel chromatography and autoradiography. These experiments revealed two groups of phosphoproteins: 100 to 110 kD polypeptides (assembly polypeptides) and a 155 kD polypeptide. No phosphorylation of the 50 kD polypeptide was observed. These results imply that the biochemistry of CCVs *in vivo* and *in vitro* may differ considerably. Phosphorylation of the 100 to 110 kD polypeptide, although not observed *in vitro*, may regulate assembly *in vivo*.

Other polypeptides and enzymes associated with CCVs: Several cellular proteins, other than cargo molecules, may be associated with CCVs. Actin was originally noted as a component of CCVs purified from Wil2 cells (Salisbury et al., 1980). An 18.5 kD polypeptide, which comigrated with calmodulin, was also noted in these preparations. Stelazine (a calmodulin inhibitor, trifluoroperazine dihydrochloride) was also shown in these studies to inhibit recruitment of clathrin to receptor complexes in Wil2 cells, although it had no effect on subsequent events. Dihydrocytochalasin B (an actin-binding drug) had no effect on clathrin recruitment in these cells, but it partly inhibited CCV formation. The non-CCV mediated pathways of internalization were completely

inhibited by both drugs. Calmodulin binding elements have also been noted by Geisow and Burgoyne (1984) in bovine adrenal medulla CCVs.

Tau-like proteins were once thought to be components of CCVs, although these results have fallen into disfavor (Pfeffer et al., 1983). Recent evidence indicates that alpha-actinin and calmodulin may interact directly with the clathrin heavy chain (Merisko, 1985). Tubulin is clearly a component of both brain and liver CCVs, as has been shown by analysis of CCVs highly purified by controlled pore glass bead exclusion column chromatograpjhy (Kelly et al., 1983). As expected, alpha and beta tubulin are present in a 1:1 molar ratio (Kelly et al., op cit.). Membrane polypeptides have been identified in brain CCVs (38 kD and 29 kD) that may be shared by synaptic vesicles (Pfeffer and Kelly, 1985). Two additional membrane polypeptides have been identified in brain CCVs by Triton extraction (24 kD and 10 kD; Weidenmann et al., 1985).

Two kinase activities have been associated with CCVs, and these are discussed in the sections on clathrin light chains and assembly complex polypeptides. Other enzyme activities have also been associated with CCVs. Perhaps the most significant is an ATP-dependent proton pump which is thought to acidify compartments involved in receptor recycling. This enzyme activity in CCVs was described by

Forgac et al. (1983). Proton movement was monitored by measuring ^{14}C-methylamine distribution. Addition of Mg^{2+} and ATP to purified CCVs resulted in the generation of a 4 to 5 fold gradient (equivalent to a .6 to .7 unit pH gradient). Proton ionophores and N-ethylmaleimide inhibited methylamine uptake, but the enzyme was unaffected by vanadate and strophanthidium (Na^+/K^+ ATPase inhibitors), or oligomycin and aurovertin (mitochondrial ATPase inhibitors). Stone et al. (1984) independently showed that the enzyme operates in parallel with a chloride transporter, and that CCV acidification can be inhibited by the antibiotic duramycin. Duramycin apparently acts at low concentrations on the chloride transporter, and at high concentrations on the proton pump. Xie et al. (1984) observed that the partially purified enzyme was activated by phosphatidylserine but not other phospholipids that were tested. Van Dyke et al. (1985) have indicated that vesicles prepared from bovine synaptic plasma membranes contain a proton pump biochemically similar to that of CCVs. Finally, Xie and Stone (1986) have purified a 530 kD complex which, when reconstituted with phosphatidylserine lipid lyposomes, catalyzes the azide-resistant, N-ethylmaleimide sensitive generation of a pH gradient. This is believed to represent the CCV enzyme.

Other enzyme activites associated with CCVs include:

a Mg-ATP/ADP dependent phosphatase (Pauloin and Jolles, 1986) which dephosphorylates the 50 kD element of the assembly complex in brain CCVs; a cyclic nucleotide phosphodiesterase activity that is stimulated by cGMP (Silva et al., 1985); and a phosphatidylinositol kinase (Campbell et al., 1985).

Characteristics of clathrin-coated vesicle membranes: The membrane lipid bilayer of CCVs and coated pits differs considerably from that of plasma membrane or smooth vesicles. Filipin is a sterol-specific antibiotic that can be used to study the distribution of cholesterol in cellular membranes. Montesano et al. (1979) used filipin and freeze-fracture techniques to study the distribution of cholesterol in plasma membrane. Filipin-cholesterol complexes appear as 25 to 30 nm protuberances in the plasma membrane. Although abundant in the plasma membrane, they were conspicuously absent from CCVs and coated pits. Later studies (Montesano et al., 1981) established the absence of filipin-sterol complexes in CCVs and coated pits, despite their presence in plasma membrane, smooth vesicles, and phagocytic membranes. Eventually, more detailed studies (Gravotta and Marcioni, 1985) indicated that the cholesterol to phospholipid molar ratio in brain CCVs is .47 +/- .07 as compared to 1.06 +/- .08 in

synaptic plasma membrane. In addition, Gravotta and Marcioni (1985) noted that the ganglioside species present in CCVs and synaptic plasma membrane are essentially the same. Finally, Alfsen et al. (1984) have shown that CCV membranes contain a higher percentage of phosphatidylethanolamine than plasma membrane.

The clathrin coat can directly affect the chemistry of membranes with which it is associated. Steer et al. (1984) using infrared spectroscopy, have shown that an associated clathrin coat specifically locks the lipid moieties of the bilayer matrix into stereochemical conformations that are different from those in uncoated vesicles, synaptic vesicles, and synaptic plasma membrane. In addition, Alfsen et al. (1984), using the fluorescent probes pyrene and parinaric acid to study lateral mobility and rotational motion in the lipid bilayer of CCV membranes, have noted that a clathrin coat introduces disorder into the surrounding lipids. This observation supports the original notion of Kanaseki and Kadota (1969) that the assembly of the clathrin coat may actually "power" the formation of the vesicle.

Subpopulations of clathrin-coated vesicles: Friend and Farquhar (1967) originally suggested that subpopulations of CCVs may mediate specific events at

different intracellular organelles. Since then considerable heterogeneity has been observed in the parameters of isolated CCVs and in the appearance of CCVs in situ. Steven et al. (1983) have noted that purified bovine brain CCVs vary in mass from 20 to 100 megadaltons, with peaks of distribution at 26 megadaltons and 35 megadaltons. In comparison, liver CCVs range in size from 20 to 220 megadaltons, with a single weighted average of 66 megadaltons. Smith and Smith (1984), using serial sections of frog cutaneous pectoris neuromuscular junction, observed two subpopulations of CCVs: one the size of synaptic vesicles (60 to 70 nm), the other (8% of total) of larger diameter (more than 100 nm). In addition, only 20% of the CCVs actually appeared to be associated with the synaptic plasma membrane. A similar percentage was determined by Pfeffer and Kelly (1985), who isolated a subpopulation of brain CCVs carrying synaptic vesicle cargo molecules by immunoprecipitation with monoclonal antibodies directed at the cytoplasmic domains of two synaptic vesicle membrane antigens. In a typical preparation of bovine CCVs, this subpopulation represented 20% of the total. Pfeffer and Kelly (1985) noted the enrichment of 38 kD and 29 kD protein in the subpopulation of CCVs carrying synaptic vesicle cargo molecules.

Weintraub et al. (1985) purified two subpopulations

of CCVs differing in size from bovine adrenal cortex. Bomsel et al. (1986) separated two homogeneous subpopulations of CCVs from bovine adrenal cortex, also on the basis of size (100 nm and 70 nm). Visualization of LDL receptor by ligand blotting and enzyme linked immunosorbent assay revealed increased binding capacity in the smaller CCVs.

Hemly et al. (1986) made the first significant association of a purified subpopulation of CCVs with a specific function. Clathrin-coated vesicles were purifed from liver perfused with diisopropylfluorophosphate to inactivate cholinesterase in the endocytic subpopulation. The population containing newly synthesized cholinesterase (an exocytic subpopulation) was then isolated by treating the CCVs with Karnovsky-Roots reagents (1964) and fractionating those containing the dense reaction product by gradient centrifugation. No significant differences in polypeptide composition were observed between the endocytic and exocytic subpopulations; however, the endocytic population had a significantly lower ratio of cholesterol to phospholipid.

Kedersha et al. (1986) have separated subpopulations of purified liver CCVs using non-sieving concentrations of agarose (separating particles on the basis of surface charge). This method was facilitated by the removal of the

clathrin coats prior to electrophoresis, since most of the charge heterogeneity is generated by the membrane and membrane cargo molecules. Slow-migrating and fast migrating subpopulations were isolated and analyzed by immunoblotting for receptor content. In additon, CCVs isolated from livers perfused with ^{125}I-asialoorosomucoid were analyzed, and the labelled subpopulation of vesicles (the slow migrating vesicles) were labelled as endocytic.

Immunoprecipitations of purified bovine brain CCVs using mAbs directed against actin, tubulin, CAP_1, CAP_2, or specific cargo molecules have revealed subpopulations of CCVs generated by heterogeneous distribution of the coat proteins (Kohtz et al., 1985; herewithin).

METHODS

VI. Immunological Methods

Preparation of hybridomas: Female Balb/C mice (6 to 8 weeks of age) were initially immunized intraperitoneally with 100 ug of heat denatured coated vesicle proteins 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 on days four and three before fusion on day one.

Sp 2/0-Ag14 myeloma cells 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-Ag14 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 10^4 myeloma cells per well (0.2 ml) and selected by adding 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 screened with an enzyme-linked immunosorbant assay (ELISA). Briefly, polyvinyl chloride plates (Linbro) were coated with antigen at concentrations varying fron 5 ug/ml (purified CAPs) to 50 ug/ml (whole coated vesicles) in 5 mM Tris pH 8.0. Binding was performed at 37°C for one hour followed by a 4°C 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°C, washed three times with TBS containing either .5% Tween 20 (purified antigens) or .05% Tween 20 (Whole coated vesicles). Wells were incubated with goat anti-mouse IgG/IgM conjugates to alkaline phosphatase (Boehringer) for one hour at 37°C, washed three times, then incubated for one hour at 37°C with para-nitrophenyl. Absorbance (405 nm) was quantitated with a Titertek Multiskan microelisa reader.

Positive hybridomas were subcloned by limiting dilution. Ascites fluid was generated in pristane (Aldrich) primed Balb/C mice. Antibodies were purified from ascites fluid by hydroxyapatite high pressure liquid chromatography (BioRad). Antibodies were coupled to cyanogen bromide activated Sephaose 4B as described by the

manufacturer (Pharmacia). Monoclonal antibodies were subtyped immunochemically:

IgG1: C-4E5 (k), C-10B2 (k), C-6C1 (l), C-7H12 (l)
S-8G8 (k), S-6G7 (k)

IgM: 2D9 (k), S-11D9 (k), A-7C11 (k), T-2H9 (k)

IgG2b: S-10C7 (k)

Monoclonal antibody CVC-1 (Kirchhausen et al., 1983) was acquired from the American Type Culture Collection (TIB 135).

Sandwich ELISAs: High-pressure liquid chromatography purified mAbs were cross-linked to alkaline phosphatase in the following manner. Antibody and alkaline phosphatase (bovine intestinal mucosa [Sigma]) were combined in phosphate-buffered saline, pH 7.4, at 0.5 mg/ml each. The mixture was placed in a dialysis bag and dialyzed against a 500-fold excess volume of phosphate-buffered saline containing 0.04% glutaraldehyde. After eight hours at 4°C, the bag was moved to 1,000-fold excess volume phosphate-buffered saline without glutaraldehyde and dialyzed overnight at 4°C. Samples were run through a Bio-Gel P-200 column equilibrated with phosphate-buffreed saline containing 0.1% BSA. Column fractions were assayed directly by ELISA for CAP binding and enzyme activity.

Polyvinyl chloride, 96-well plates were incubated

overnight at 4°C with 50 ug/ml purified mAb in 5 mM Tris, pH 7.0. Plates were incubated the next day for one hour at room temperature with TBS containing 0.5% Tween 20 (TBS-Tween) and incubated overnight at 4°C with 25 ug/ml CAPs in TBS-Tween. The next day the plates were washed twice with TBS-Tween and incubated two hours at 37°C with 5 ug/ml alkaline phosphatase-conjugated mAbs in 1% BSA in TBS. The plates were washed twice with TBS-Tween and once with distilled H_2O. Reactions were developed with paranitrophenyl phosphate (Sigma) in 100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.4. Color development was quantitated with a titertek multiskan microelisa reader set at 405 nm.

Immunoprecipitation of CAPs: CAPs and phosphorylated CAPs were incubated with Sepharose 4B-coupled mAbs in HEPES, pH 7.0, containing 1% BSA, 100 mM sodium chloride and .01% sodium azide. A typical experiment consisted of .05 ml of 5% Sepharose 4B incubated with either 100 ug of unlabeled CAPs or 10^6 cpm of radioiodinated CAPs. After incubating overnight at 4°C with agitation, the beads were pelleted briefly in a Brinkman 3200 centrifuge, washed three times with 0.5 ml of 20 mM HEPES, pH 7.0, 1% BSA, then twice with 0.5 ml of 20 mM HEPES, pH 7.0, containing 100 mM sodium chloride. Samples were dissociated in

Laemmli sample buffer (0.1% SDS, 50 mM Tris pH 6.5, 100 mM dithiothreitol, 10% glycerol) and counted.

Gel Electrophoresis and Immunoblotting: Sodium dodecyl sulfate-PAGE was performed as described by Laemmli (1972). Two-dimensional gel electrophoresis was performed as described by O'Farrell et al. (1975). Gels were stained with Coomassie brilliant blue and/or dried and autoradiographed using a Cronex lightening plus intensifying screen (Dupont) and Cronex X-ray film (Dupont).

Proteins were transferred to nitrocellulose (BioRad) electrophoretically using a BioRad transfer apparatus and the procedure described by the manufacturer. Nitrocellulose filters were fixed, then saturated with 0.1% BSA and 0.05% Tween 20 in TBS, pH 7.5. Filters were incubated at 4°C overnight with primary antibodies suspended 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, then incubated one hour at 4°C with peroxidase-conjugated goat anti-mouse IgG/IgM (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, .5M sodium chloride pH 7.5, reactive bands were visualized with 4-chloro-1-napthol

(BioRad) and 0.015% hydrogen peroxide.

Immunoprecipitation of CCVs: Unlabelled or radioiodinated CCVs were incubated with Sepharose 4B-coupled monoclonal antibodies in HEPES, pH 7.0, containing 1% BSA, 100 mM sodium chloride and .01% sodium azide. A typical experiment consisted of .05 ml of 5% Sepharose 4B incubated with either 100 ug of unlabeled CCVs or 10^6 cpm of radioiodinated vesicles. After incubating overnight at 4°C with agitation, the beads were pelleted briefly in a Brinkman 3200 centrifuge, washed three times with 0.5 ml of 20 mM HEPES, pH 7.0, 1% BSA, then twice with 0.5 ml of 20 mM HEPES, pH 7.0, containing 100 mM sodium chloride. Samples were dissociated in 0.1% SDS, 50 mM Tris pH 6.5, 100 mM dithiothreitol, 10% glycerol. Percent total vesicles precipitated was calculated indirectly from the amount of 180 kD protein remaining in the first supernatant as determined by SDS-PAGE. A total clathrin-coated vesicle precipitate as calculated by this method resulted in recovery of 30-40% of the total counts. This is probably due to losses during washing.

Immunofluorescence Analysis: Cells were cultured either directly on glass coverslips or on coverslips coated with rat tail collagen as described (Greene and Tischler,

1976). Cells were fixed with freshly prepared 3.75% paraformaldehyde in phosphate buffered saline (PBS) for 15 minutes at room temperature, then treated with .1% Triton X-100 in PBS for 5 minutes. Cells were incubated with ascites fluid diluted 1:500 in PBS containing 1% BSA or in undiluted culture supernatant for 45 minutes at 37 °C with either rhodamine- or fluorescein-conjugated goat anti-mouse IgG/IgM (Boehringer) diluted 1:100 in PBS containing 1% BSA. Cells were washed and mounted in Fluoromount (Southern Biotechnology Associates). Slides were viewed through a Zeiss fluorescent microscope using a Zeiss oil emersion 63x lens. Sections of human heart (autopsy material) were processed as described (Bloom and Puszkin, 1983), using mAb S-11D9.

VII. Culturing and Differentiation of Cells.

Cell Culture: MDBK cells were obtained from Dr. Peter Palese (Department of Microbiology, Mount Sinai School of Medicine). Neuroblastoma (SH-SY5Y) cells were obtained from Dr. S. Berl (Department of Neurology, Mount Sinai School of Medicine). SH-SY5Y cells were carried in RPMI contaning 20% fetal bovine serum, 1 mM l-glutamine, 1 mM sodium pyruvate, penicillin, streptomycin, and gentamycin. MDBK cells were cultured on glass coverslips

in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% CLEX (Dextran), 1mM L-glutamine, .1 mM sodium pyruvate, penicillin, streptomycin and gentamycin. **Human cardiac myoblast** (HCM) cells were cultured on glass coverslips in DMEM supplemented with 20% fetal bovine serum, .5% chick embryo extract (Gibco), 1mM L-glutamine, 1mM sodium pyruvate, .1 mM MEM non-essential amino acids and MEM vitamins. SH-SY5Y cells were co-cultured at a 5:1 ratio with HCM cells in myoblast medium. All hybridoma cells were carried in RPMI containing 15% fetal bovine serum, 1mM L-glutamine, 1 mM sodium pyruvate, .1 mM MEM non-essential amino acids, MEM vitamins, 15 ug/ml hypoxanthine, 7.6 ug/ml thymidine. HL60 cells were grown in RPMI containing 20% fetal bovine serum, 1 mM L-gutamine, 1 mM sodium pyruvate and .1 mM non-essential amino acids. To differentiate HL60 cells, serum was reduced to 10% and DMSO was added to 1.5%. Medium was changed daily during differentiation.

C_2 mouse myoblasts (Blau et al, 1983; C_2M were obtained from Dr. Edward Johnson, Mt. Sinai School of Medicine) were cultured in the same medium as the human cardiac myoblasts. To differentiate C_2M cells, the medium was changed to DMEM containing 4% clarified horse serum, 1 mM L-glutamine, 1 mM sodium pyruvate, 15 ug/ml hypoxanthine, and .5 mM non-essential amino acids.

PC12 cells were adapted to culture in RPMI containing 20% fetal serum, 1 mM L-glutamine, 1 mM sodium pyruvate, and .1 mM non-essential amino acids. To differentiate PC12 cells, 50 ng/ml nerve growth factor was added every 2 days. Medium was changed every 4 days during differentiation. For immunofluorescence, PC12 cells were cultured on glass coverslips coated with rat tail collagen (Greene and Tischler, 1976).

VIII. Subcellular Fractionation.

Preparation of CCVs, CAPs, Synaptic Vesicles, Golgi fractions and Synaptic Plasma Membranes: Bovine brain coated vesicles were prepared by a modification of a method described recently by Schook and Puszkin (1985). Brains were obtained five hours or less after slaughtering. The meninges were removed and the gray matter was excised by aspiration. Gray matter was suspended in 100 mM morpholinoethanesulfonic acid buffer (MES) pH 6.5 containing 1 mM ethylene glycol-bis-(beta-amino ethyl ether) N, N'-tetraacetic acid, 0.5 mM $MgCl_2$, 0.02% sodium azide, 0.3 mM phenylmethylsulfonyl fluoride, 1 mM benzaidine, 0.005 mM leupeptin, and 2 ug/ml soybean trypsin inhibitor. After homogenization in a Waring blendor, the suspension was spun 15 minutes (4°C) at 17,000 rpm in a SS-

34 rotor (Sorvall). The supernatant was recovered and spun for one hour (4°C) at 24,000 rpm in a type 30 rotor (Sorvall). 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 x g 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 Ti45 rotor (Beckman) at 33,000 rpm for one hour. The pellet was suspended in MES buffer and run twice over a S-1000 (Pharmacia) column. Protein peaks were monitored by absorbance at 280 nm. This procedure yields approximately 10 mg of purified coated vesicles per 500 grams starting material.

Enriched synaptic vesicles were prepared from bovine brain gray matter as described by DeLorenzo and Freedman (1978), or Zisapel and Zurgil (1979). Both procedures yielded synaptic vesicles containing a 44 kD polypeptide reactive with S-11D9. Synaptic plasma membranes were prepared from rat and bovine cerebra as described by Jones and Matus (1974). Golgi fractions were prepared as described by Blanch et al. (1984). Enzyme profiles of synaptic vesicles, synaptic plasma membranes, Golgi fractions, and coated vesicles are reported elsewhere (Silva et al. 1986). Cultured cell homogenates were

produced by washing cells twice with TBS, then treating the adherent cells directly on the plates with 50 mM Tris pH 6.5, 1% SDS, 100 mM dithiothreitol, 20% glycerol, 0.25% phenol red, 0.3 mM phenymethlysulfonyl fluoride. The extracts were immersed in boiling water for ten minutes prior to gel electrophoresis.

Extraction and Purification of Clathrin and NP185: Clathrin was extracted from purified bovine CCVs by either 0.5M Tris or 2M Urea. Membranes were removed by centrifugation at 100,000 xg for 1 hour, and the clathrin in solution was concetrated either by ammonium sulfate precipitation or by Amicon "Centricon" dialysis. Clathrin was further purified by Sepharose 4B chromatography. Two cycles of assembly/disassembly were used to further purify clathrin.

NP185 was extracted from CCVs or SVs by the addition of a 5M NaCl solution to 0.9M final concentration. CCVs and SVs were incubated in high salt for 10 minutes, then spun at 100,000 xg to remove membranes. NP185 was immunoprecipitated similarly to CAPs (see above).

IX. Biochemical Methods.

Chymotryptic Digests: Chymotrypsin was dissolved

first in 50 mM Tris, 10 mM $MgCl_2$, pH 7.5, at 1 mg/ml, then diluted to 1.7 ug/ml in a 1 mg/ml clathrin-coated vesicles (CCV) preparation in 100 mM morpholinoethanesulfonic acid buffer, pH 6.5. Digestions were performed for 30 seconds to one hour at room temperature before being stopped with 1 mM phenylmethylsulfonyl fluoride. Samples were then spun at 100,000 xg in an airfuge and pellets and supernatants were dissolved in Laemmli sample buffer.

Radioiodination of CAPs: CAPs were radioiodinated using lactoperoxidase/glucose-coupled beads as described (BioRad). Prior to radioiodination, CCVs and CAPs were exhaustively dialyzed against pH 6.5 phosphate buffer. Subsequent to radioiodination, CAPs were resuspended in 20 mM HEPES (Sigma), pH 7.0, 1% BSA, 100 mM sodium chloride, .01% sodium azide, and purified twice by G-50 column chromatography.

Phosphorylation of CAPs: Phosphorylation of CCVs was conducted in 100 mM Tris, 10 mM $MgCl_2$, 100 mM morpholinoethanesulfonic acid, pH 7.5 Reaction mixtures (0.1 ml) contained 0.1 mg of CCVs, 0.02 mM ATP and 0.001 mCi gamma [^{32}P]-labeled ATP. Some samples were supplemented by 0.01 mg/ml poly-L-lysine (14 kD [Sigma]). Incubations were performed for 10 minutes at room

temperature. Samples were heated at 100°C for 5 minutes and spun at 100,000 x g for one hour prior to immunoprecipitation.

Radioiodination of CCVs: Coated vesicles were radioiodinated using lactoperoxidase/glucose oxidase-coupled beads (BioRad) as described by the manufacturer. Prior to radioiodination, CCVs were exhaustively dialyzed against pH 6.5 phosphate buffer. Subsequent to radioiodination, CCVs were resuspended in 20 mM HEPES (Sigma), pH 7.0, 1% BSA, 100 mM sodium chloride, .01% sodium azide, and purified twice by Sepharose 4B column chromatography. Specific activity of CCVs radioiodinated in this manner was 10^8-10^9 cpm/ug.

In vitro Translation and Pulse Chase Experiments: Rabbit reticulocyte lysates were prepared as described by Maniatis et al. (1982), except for the addition of 25 ug/ml yeast transfer RNA. Reaction cocktail was prepared as described, and the translation reactions were conducted as described (Maniatis et al., 1982), with the co-translational or post-translational addition of canine microsomal membranes as described by the manufacturer (Amersham). Total cellular RNA was isolated by the guanidinium isothiocyanate-cesium chloride method; mRNA was isolated by two passes over an oligo-dT cellulose column

(all performed as described by Maniatis et al., 1982, and references within).

Translation reaction products were immunoprecipitated after 1:3 dilution into LB buffer [.25% Triton X-100, 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 5 mM EGTA [ethylenebis(oxyethylenenitrilo)]tetraacetic acid pH 7.2.] Monoclonal antibody S-11D9 was added (20 ug/ml) and incubated overnight at 4 degrees C. Rabbit anti-mouse IgM (Boehringer) covalently coupled to Sepharose was then added, and incubated with agitation at 4 degrees C for 8 hours. The beads were spun out and washed three times in dilution buffer. High salt buffers were omitted as they tended to inhibit binding by this mAb. Samples were then dissociated by boiling in Laemmli sample buffer [100 mM Tris, 2.0% SDS (sodium dodecyl sulfate), 20% glycerol, 0.2% phenol red, 100 mM dithiothreital), resolved by SDS-PAGE, and autofluorographed with EnHance (New England Nuclear).

Pulse chase experments were performed with day 7 nerve growth factor-differentiated PC12 cells. Cells were incubated in methionine-free medium for 15 minutes, then pulsed with .25 mCi/ml ^{35}S-methionine for 15 minutes. The label was chased by the addition of cold methionine to a concentration of 1 mM. At various times during the chase, cells were lysed with LB buffer, centrifuged for 15 minutes at 20,000 xg, and immunoprecipitated with mAb S-11D9

according to the above protocol.

Cell Surface Radioiodination: HL60 cells were washed three times with phosphate buffered saline (PBS: 100mM sodium monophosphate/diphosphate, .9M sodium chloride, 1 mM magnesium chloride pH 7.4). Cells were radioiodinated by Enzymobeads (Pharmicia: lactoperoxidase/glucose oxidase coupled to Sepharose 4B) in PBS without magnesium chloride. After the reaction was completed, the cells were lysed with LB buffer and centrifuged at 20,000 xg for 15 minutes. Supernatants were immunoprecipitated with mAb S-11D9 as described above.

Phosphorylation of Tubulin: Purified rat brain tubulin was a kind gift from Dr. Ronald Liem, Department of Pharmocology, New York University. The tubulin was purified by temperature-dependent cycles of polymerization-depolymerization. Endogenous Ca^{2+}/calmodulin-dependent kinase phophorylation of tubulin was performed by suspending tubulin (at concentrations greater than 500 ug/ml) in 10 mM PIPES [piperazine-N-N'-bis(2-ethanesulfonic acid], 10 mM magnesium chloride, 2 mM EGTA, pH 7.4]. Gamma-^{32}P-labelled-ATP (.002 mCi) was added to a 20 ug sample of tubulin at a .02 mM final concentration of ATP. Incubation were performedt at room temperature for 20

minutes and stopped by the addition of .25M EDTA (1:10).

A standard phosphorylation of tubulin by casein kinase II was performed in a .1 ml reaction volume. The reaction buffer consists of 100 mM Tris, 10 mM magnesium chloride, and 1 mM potassium chloride. Standard reaction mixtures consisted of .01 mg of tubulin, .005 mg NP185 (or 1M sodium chloride extract of CCV) and .001 mg polylysine. Gamma-^{32}P-labelled ATP (.002 mCi) was added with a .02mM final concentration of ATP. Incubation were performed at room temperature for 20 minutes and stopped by the addition of .25M EDTA (1:10).

Phosphorylation of tubulin by the Ca^{2+}/calmodulin-dependent kinase or casein kinase II was also performed in batch for the competitive binding assays. In these instances 0.5 mM cold ATP was used.

NP185-Tubulin Binding Assays: Assays were performed using radioiodinated tubulin as labelled ligand (4 x 10^6 cpm/ug specific activity). Ca^{2+}/calmodulin-dependent kinase or casein kinase II phosphorylated tubulin were used as competing ligands. Binding reactions were performed in 20 mM HEPES buffer, pH 7.4. Labelled ligand was incubated alone or with various dilutions of competing ligand. Samples were incubated with NP185 pre-bound to monoclonal antibodies S-8G8 and S-6G7 (which were coupled to Sepharose

4B). Incubations were performed at 4 degrees C for 20 to 30 minutes (kinetic conditions) or 4 hours (equilibrium conditions) with constant agitation. Sepharose beads were then pelleted (10 second spin at 10,000 xg), washed twice with ice cold HEPES buffer, and counted in a gamma counter. Background binding was assessed using S-8G8 and S-6G7-coupled Sepharose 4B in the absence of NP185 for each dilution of competing ligand. Every determination was performed in triplicate, and the experiments were repeated with two different preparations of CCV-NP185 and SV-NP185.

In order to determine the specific binding of tubulin as a function of NP185 concentration, a stock of NP185-coated, S-8G8 and S-6G7-coupled Sepharose 4B was analyzed for NP185 content by SDS-PAGE, Coomassie staining, and comparison with a clathrin standard. This stock was then diluted into suspensions of S-8G8 and S-6G7-coupled Sepharose 4B beads. Binding assays were performed with these dilutions as described above.

Data Analysis: Competition experments were analyzed for fit with either a one site or two site binding model:

$$\text{One site model:} \quad \%B = 100/[1 + (K_d/x)]$$

$$\text{Two site model:} \quad \%B = \%H/[1 + (K_dh/x)] + 100 - \%H/[1 + (K_dl/x)]$$

%B = percent bound labelled ligand; %H = precent high affinity sites; $K_m h$ = affinity of the high afinity site; $K_m l$ = affinity of the low affinity site; 100 - %H = % low affinity sites; X is the variable for percentage bound ligand. A best fit curve (constrained by these functions) was determined by an iterative nonlinear regression analysis program available in the National Institutes of Health Prophet System.

RESULTS

X. Immunochemical Analysis of Structure and Function in the Clathrin Light Chains

Introduction: The cage structure enclosing clathrin coated vesicles (CCVs) self-assembles from triskelions composed of the three clathrin heavy chain and three clathrin light chain molecules (also called clathrin associated proteins or CAPs [Kirchhausen and Harrison, 1981, and Lisanti et al., 1982]). The production of monoclonal antibodies (mAbs) reactive only with CAP_1 (Brodsky, 1985, and Kirchhausen et al., 1983) provided evidence of the structural diversity of these two polypeptides. In this report, mAbs reactive with CAP_2 and with CAP_1 and CAP_2 are presented and their characterization reveals that distinct epitopes are involved in mediating the functional parameters of CAPs.

Four epitopes of CAP_2: Four unique mAbs to CAP_2 were eventually generated from the fusion of immune Balb/C spenocytes to Sp2/0 myeloma cells. Two of them, mAbs C-7H12 and C-6C1, were produced from a Balb/C mouse immunized with CAPs isolated from CCVs by heating at 100°C for five

Table 1. Sandwich ELISA of Anti-CAP_2 mAbs.

Coated mAb	Soluble mAb-alkaline phosphatase Conjugate C-7H12	C-10B2	C-6C1	C-4E5
C-7H12	-	++	+	++
C-10B2	+++	-	+++	+
C-6C1	+	+++	-	++
C-4E5	+++	+	++	-
CAPs	+++	+++	+++	+++

Purified mAbs were coated on PVC plates, incubated with CAPs and subsequently incubated with alkaline phosphatase conjugated mAbs. Reactivity was quantitated colorimetrically using paranitrophenyl phosphate. Results represent the mean of three data points in typical experiments. The concentration of plate-bound CAPs (last row) was adjusted stoichiometrically to 40% of the amount of plate-bound antibody. A control value using an unrelated alkaline phosphatase conjugated IgG1 murine mAb was determined for each row, and subtracted to give the reported reults:

+++ 66%-100% of absorbance using PVC plate-bound CAPs.

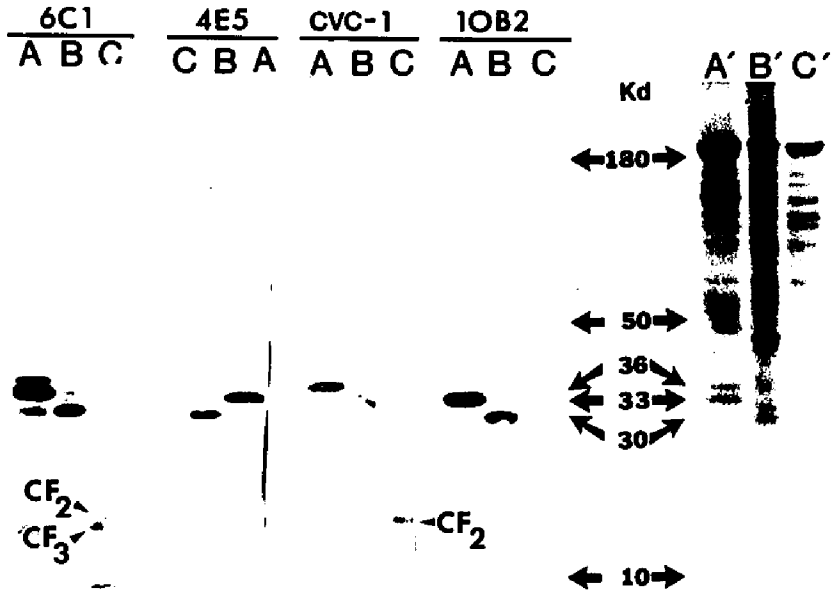
++ 65%-32% of absorbance using PVC plate-bound CAPs.

+ 10%-32% of absorbance using PVC plate-bound CAPs.

- <10% of absorbance using PVC plate-bound CAPs.

minutes while mAbs C-10B2 and C-4E5 were produced from a mouse immunized with whole clathrin. All four mAbs reacted with CAPs from both neuronal and non-neuronal sources (Figures 5 and 6). To localize reactive sites of the mAbs, sandwich ELISAs were performed with purified mAbs coupled to alkaline phosphatase. Polyvinyl chloride plates were coated with a purified mAb, incubated with a preparation of both CAPs, and subsequently incubated with a purified mAb coupled to alkaline phosphatase. The results of these experiments are summarized by the matrix in Table 1. All of the mAbs completely inhibited themselves in the assay but only variably inhibited each other. The mechanics of the sandwich ELISA prevent varying affinities of mAbs from affecting interpretation of the results. In other words, if the plate-bound antibody has a higher affinity for the same epitope as the competing antibody, the competing antibody will not bind and no reaction will be observed. If the opposite is true, the competing antibody will displace the bound CAP into solution and again no reaction will be observed in the solid phase. Therefore, the results of the sandwich ELISA matrix indicate that binding sites of mAbs C-7H12, C-6C1, C-10B2 and C-4E5 are distinct. The epitopes bound by C-7H12 and C-6C1 may be situated in the same domain of the CAP molecule and this would explain their partial inhibition in the sandwich

Figure 5. Reactivity of mAbs C-6C1, C-4E5, CVC-1 and C-10B2 with bovine brain and adrenal CCVs. (A) brain CCVs; (B) adrenal CCVs; and (C) brain CVs digested 40 minutes with chymotrypsin (as described in Figure 2). A', B', C': Coomassie blue stain; A, B, C: immunoblots. The different mobilities of neuronal and non-neuronal CAPs are evident in A' and B'. The mAbs C-6C1 and CVC-1 also reacted with some of the lower molecular weight fragments in lane C. Key for fragment abbreviations is given in Figure 6.


6C1
A B C
4E5
C B A
CVC-1
A B C
1OB2
A B C
A′ B′ C′
Kd
180
50
36
33
30
10
CF2
CF3
CF2

ELISA. Similiar results were obtained for mAbs C-10B2 and C-4E5, which completely inhibited themselves but only partially inhibited each other in the sandwich ELISA.

Close inspection of the sandwich ELISA matrix revealed that plate-bound mAbs C-7H12 and C-6C1 appear to slighlty reduce binding by mAbs C-4E5 and C-10B2. The reciprocal experiments using plate-bound C-4E5 and C-10B2, however, did not reveal significantly reduced binding by C-6C1 or C-7H12. The inhibition may be attributed to binding of C-4E5 and C-10B2, which bind only CAP_2. This effect is not observed in the reciprocal experiment inasmuch as C-7H12 and C-6C1 efficiently bind CAP_2 sandwiched by either C-10B2 or C-4E5.

Assigning mAb Binding Sites to Chymotryptic Fragments of CAPs: To characterize the reactive sites of each mAb on the CAP molecule, CCVs were chymotryptically digested and the vesicle-bound proteolytic fragments were isolated by centrifugation and subsequently analyzed by SDS-PAGE and immunoblot. By varying the digestion times, CAP fragments are generated that are trimmed increasingly close to the heavy chain binding site. Immunoblotting experiments using these fragments and the anti-CAP mAbs facilitated construction of a cleavage map of the CAPs using the clathrin binding site as a reference point.

Figure 6. Reactivity of mAb C-7H12 with chymotryptic fragments of CAPs bound to clathrin heavy chains. CCVs, reassembled clathrin cages, reassembled cages containing only CAP_2 and purified CAPs (total protein concentrations: 1 mg/ml in 100 mM morpholinoethanesulfonic acid buffer, pH 6.5) were incubated with 1.7 ug/ml chymotrypsin for various time periods, treated with phenylmethyl sulfonyl fluoride, pelleted at 100,000 x g in an airfuge (except the purified CAPs) and analyzed by SDS-PAGE and immunoblotted with mAb C-7H12. A: Coomassie stain of digested CCVs; B: immunoblot of gel loaded as in A, using mAb C-7H12;

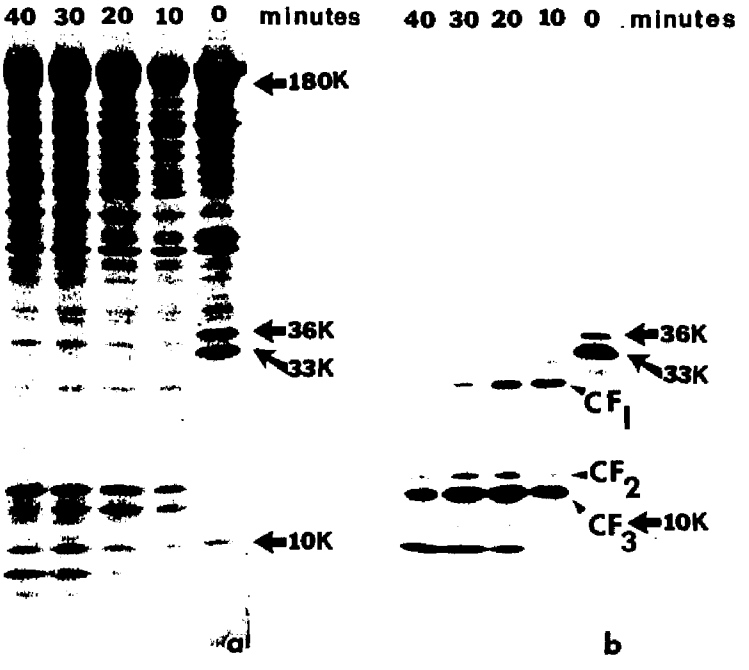

40 30 20 10 0 minutes
180K
36K
33K
10K
a
40 30 20 10 0 minutes
36K
33K
CF1
CF2
CF3
10K
b

Figure 6. Reactivity of mAb C-7H12 with chymotryptic fragments of CAPs bound to clathrin heavy chains. C: immunoblot using mAb C-7H12 of chymotryptically digested cages containing both CAP_1 and CAP_2; E: immunoblot using mAb C-7H12 of chymotryptically digested free CAPs. In Figure 2D and 2E CAP_1 was obscured by CAP_2 reactivity. Figures 2A and 2B were run using 7-15% gradient gels, which allowed greater separation of the CAPs than the 8-16% gels used in Figures 2C through 2E. Key: CF: common fragments between digested cages and CCVs; CF_1 and CF_3: fragments of CAP_2; CF_2: fragment of CAP_1.

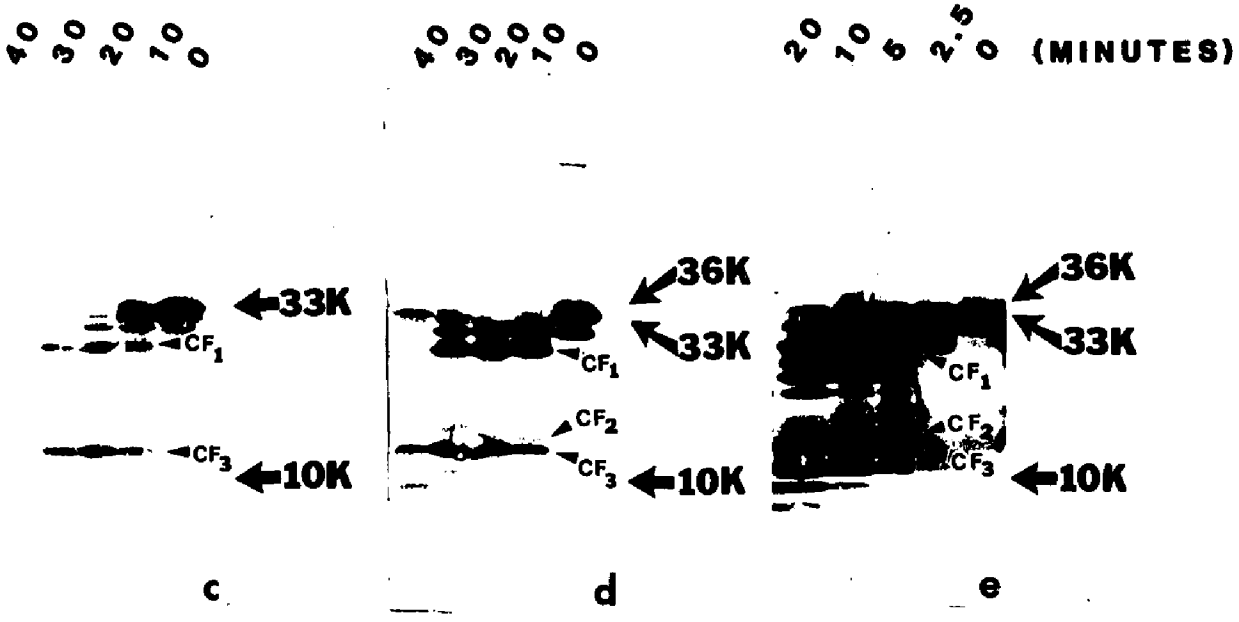

40 30 20 10 0
40 30 20 10 0
20 10 5 2.5 0 (MINUTES)
33K
CF1
CF3
10K
36K
33K
CF1
CF2
CF3
10K
36K
33K
CF1
CF2
CF3
10K
c
d
e

Figure 6A shows chymotryptic fragments that were generated when CCVs were used as substrate. Figure 6B is an immunoblot of an identically loaded gel using mAb C-7H12. For convenience, we have divided the CAP fragments into two catgories: fragments common to CCVs and reassembled cages (CFs) and unique fragments (UFs). The CFs consist of 30kD (CF_1), 18kD (CF_2) and 15kD (CF_3) fragments. Previous experiments (Lisanti et al., 1981 and 1982) have shown that CF_2 and CF_3 are produced by chymotryptic digestion of either CCVs or reassembled clathrin cages. We show in Figure 2 that CF_1 cam also be generated from either structure.

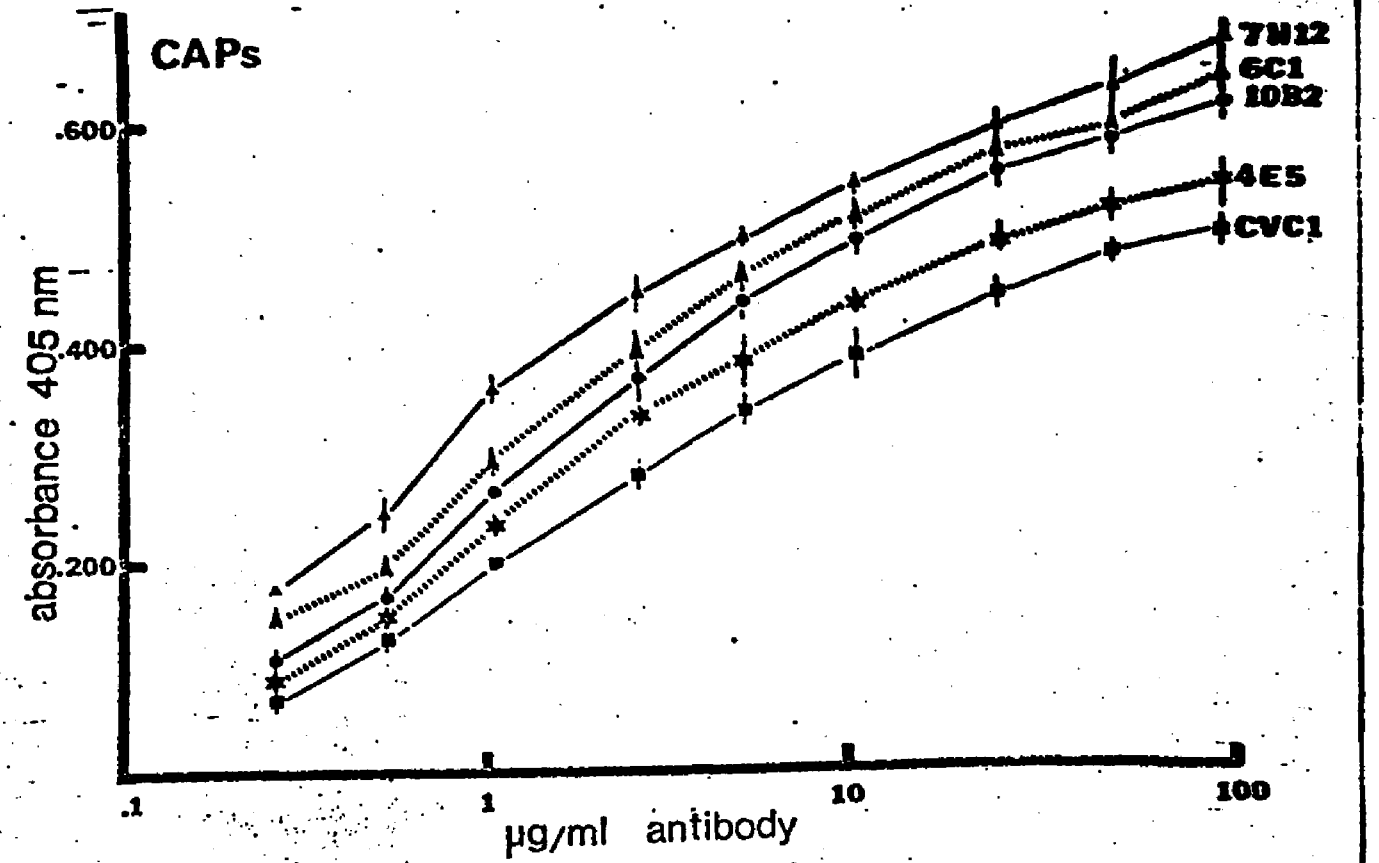
To determine which fragments are generated from CAP_1 and which are generated from CAP_2, clathrin cages were stripped of CAPs with sodium isothiocyanate as described (Winkler et al., 1983), and reassociated with mAb C-4E5 affinity-purified CAP_2. The reassembled cages were digested with chymotrypsin as before and blotted with mAb C-7H12. As shown in Figures 6C and 6D, CF_1 and CF_3 are produced by cages containing only CAP_2; thus, these fragments must be generated by proteolysis of CAP_2, while CF_2 must be generated from CAP_1. These results support the notion that CAP_1 and CAP_2 bind clathrin with structurally similiar domains shared by both proteins. Interestingly, CVC-1 (Kirchhausen et al., 1983) also binds CF_2 (Figure 5),

confirming its identity as a fragment of CAP_1. CVC-1 does not react with CAP_2, CF_3 or CF_1, but does react with clathrin-bound CAP_1. These data indicate that CF_2 contains at least two distinct epitopes: one is shared by CAP_2 and appears to be involved in clathrin binding; the other is unique to CAP_1.

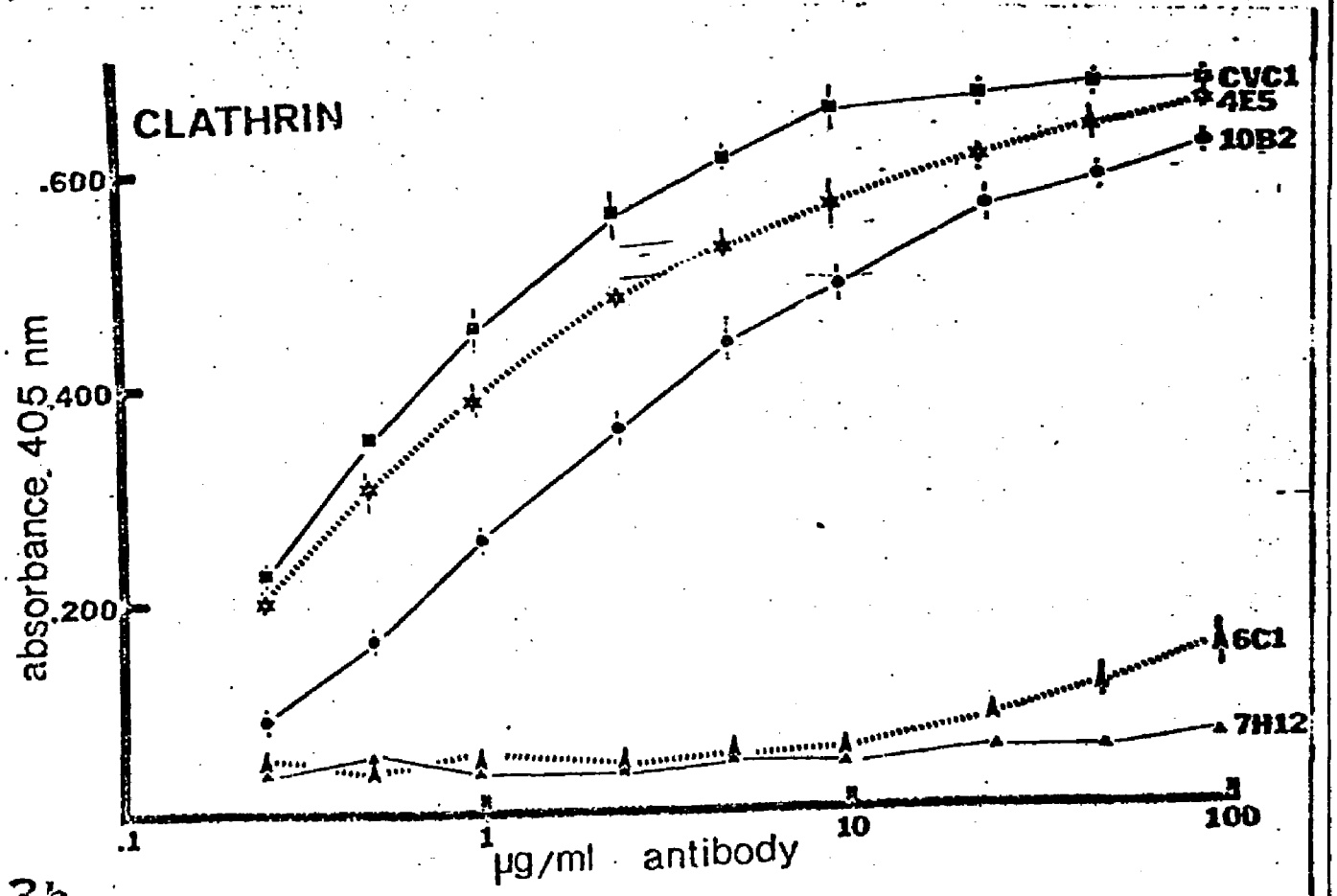
Several CAP fragments are generated with molecular masses less than 10 kD using either CCVs or reassembled cages as substrates. The common fragments in this range are difficult to identify, although CCVs appear to produce a smaller set of fragments than reassembled cages. This implies that CAPs are less accessible in CCVs than they are in reassembled cages, which is not surprising considering the more complex structure of the vesicle. The small size of UFs generated from either reassembled cages or CCVs has led us to suggest that mAb C-7H12 binds an epitope involved in clathrin heavy chain binding. Unless this epitope constitutes a linear string of amino acids, structural conservation of this region of the molecules may not be obvious from primary sequence analysis.

Anti-CAP reactivity was not detected in supernatants of chymotryptic digestion experiments using C-10B2, C-4E5 or anti-CAP_1 mAb CVC-1 (data not shown). Either the released fragments are digested beyond recognition by these mAbs or the reactive epitopes span residues on both

Figure 7. Reactivity of anti-CAP mAbs with free and clathrin heavy chain-bound CAPs. ELISAs were performed using PVC plates coated with either free CAPs or clathrin-bound CAPs. Purified mAbs, at various concentrations, were used and bound mAb was quantitated using an alkaline phosphatase conjugated second antibody (Boehringer).


CAPs
7H12
6C1
10B2
4E5
CVC1
.600
.400
.200
absorbance 405 nm
.1
1
10
100
µg/ml antibody

3a

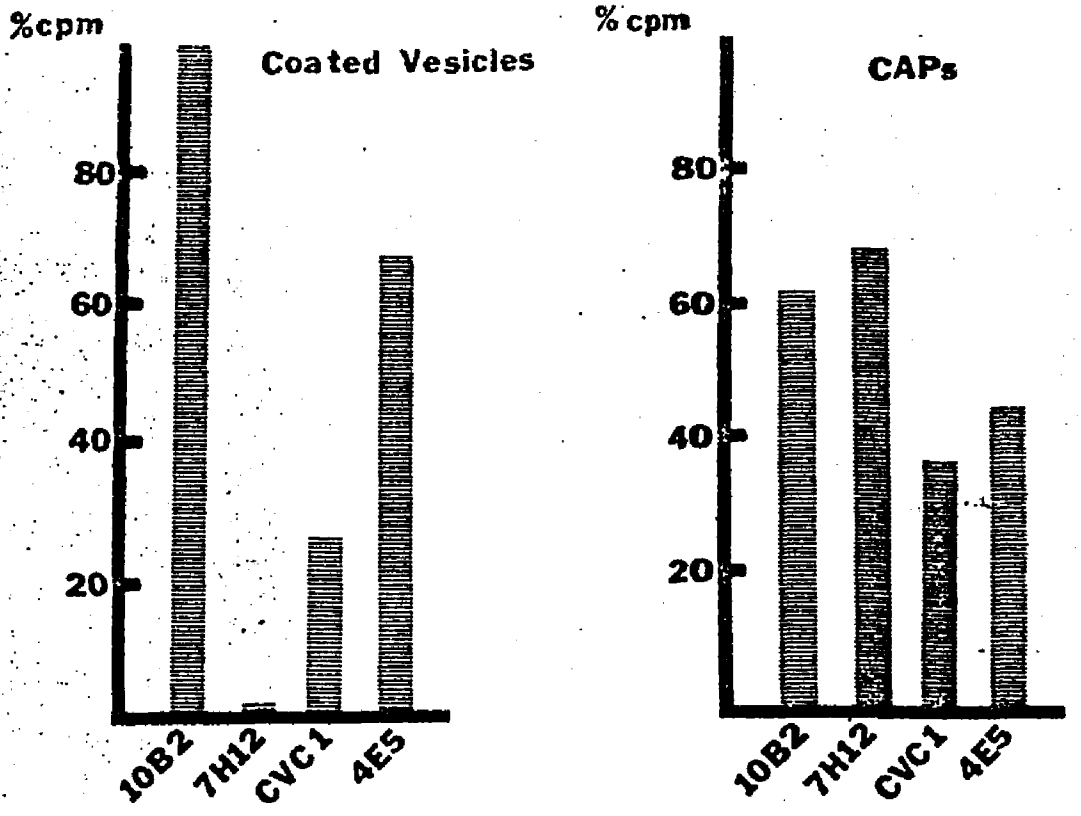

CLATHRIN
CVC1
4E5
10B2
6C1
7H12
.600
.400
.200
absorbance 405 nm
.1
1
10
100
µg/ml antibody

3b

released and bound fragments. Clathrin-associated proteins isolated by boiling and digested with chymotrypsin produce a plethora of new fragments in addition to CF_1, CF_2, CF_3 (Figure 6E). Hence, binding by clathrin affords considerable protection against proteolysis by chymotrypsin.

Monoclonal Antibody C-7H12 Reacts with the Clathrin Binding Region of Both CAPs: The immunoblot in Figure 6 shows that mAb C-7H12 reacts with both CAPs. Until now, all reported mAbs have reacted specifically with one of the CAPs (Kirchhausen op cit.). The existence of cross-reactive epitopes may be predicted, however, from the biochemical characteristics of the proteins. In particular, both CAPs appear to bind a common site on the clathrin heavy chain (Winkler et al., 1983). This implies that the clathrin binding region of both CAPs share structural homology, and antibodies to this region of the molecules should cross-react with both forms. With these considerations in mind, an ELISA was used to determine the relative reactivity of each of the mAbs with free CAPs and clathrin-bound CAPs. Figure 7 shows mAbs CVC-1, C-4E5, C-10B2, C-6C1 and C-7H12 reacting with free CAPs. On the other hand, only mAbs CVC-1, C-4E5 and C-10B2 react with the clathrin-bound CAPs present in purified preparations of

Figure 8. Fraction of total radioiodinated CCVs or radioiodinated CAPs precipitated by anti-CAP mAbs coupled to Sepharose 4B. The mAbs used were at 10^3-fold excess over the amount needed to precipitate an equivalent mass of unlabeled CAPs. Iodination of free CAPs variably affected the efficiency of immunoprecipitation by each mAb.


%cpm
Coated Vesicles
80
60
40
20
10B2
7H12
CVC1
4E5
% cpm
CAPs
80
60
40
20
10B2
7H12
CVC1
4E5

4

triskelions. Monoclonal antibody C-6C1 reacts poorly with clathrin-bound CAPs, while mAb C-7H12 does not react with clathrin-bound CAPs. These results were obtained only when the clathrin-bound CAPs were pre-spun as cages or CCVs prior to coating on PVC plates. Clathrin-associated proteins apparently dissociate from clathrin at a slow rate during storage at 4°C.

To document further the lack of reactivity of mAb C-7H12 with clathrin-bound CAPs, immunoprecipitations were performed using radioiodinated CCVs and radioiodinated free CAPs. Purified mAbs C-7H12, C-10B2, CVC-1 and C-4E5 covalently coupled to Sepharose 4B were used for these experiments. These mAbs precipitate radioiodinated free CAPs with varying efficiencies (Figure 8). Monoclonal antibody C-7H12, however, does not precipitate detectable counts of radioiodinated CCVs. This indicates that in the conformation present on the CCVs, CAPs are not recognized by mAb C-7H12 but are recognized by mAbs CVC-1, C-10B2 and C-4E5. These results differ from those reported recently (Brodsky, 1985) in which mAbs to CAP_1 were only shown to be hindered from binding the clathrin-CAP complex. In total, our data suggest that mAb C-7H12 reacts directly with the clathrin binding sites shared by <u>both</u> CAPs.

Mapping the CAP Phosphorylation Site to an Accessible

Region of CAP_2: A kinase activity recently has been associated with CAP_2 (Schook and Puszkin, 1985, and Usami et al., 1985). This activity was recently confirmed in another report and attributed to an endogenous CCV casein kinase II (Bar-Zvi and Branton, 1986). Although this activity is intensified by polybasic compunds such as polylysine or histone 1 (Schook and Puszkin, op cit.), phosphorylation can be detected in the absence of these compounds by immunoprecipitation and long-term autoradiography (Kohtz and Puszkin, unpublished results). A significant feature of this phosphorylation is its substrate preference for CAP_2 (Schook and Puszkin, op cit.). Nevertheless, phosphorylation of CAP_1 was detected in the presence of polylysine after radioimmune precipitation with anti-CAP_1 mAb CVC-1 and long term autoradiography (data not shown). Whether phosphorylation sites are partially conserved between the two forms of CAP is unclear at present.

An ELISA was devised to determine how efficiently phosphorylated CAPs react with each of the mAbs in comparison to unphosphorylated CAPs. Polyvinyl chloride plates were coated with unphosphorylated CAPs. The titer of purified mAb was adjusted in preliminary experiments to produce an optical density of 2 at 405 nm after two hours of incubation (using a 1:1000 dilution of alkaline-

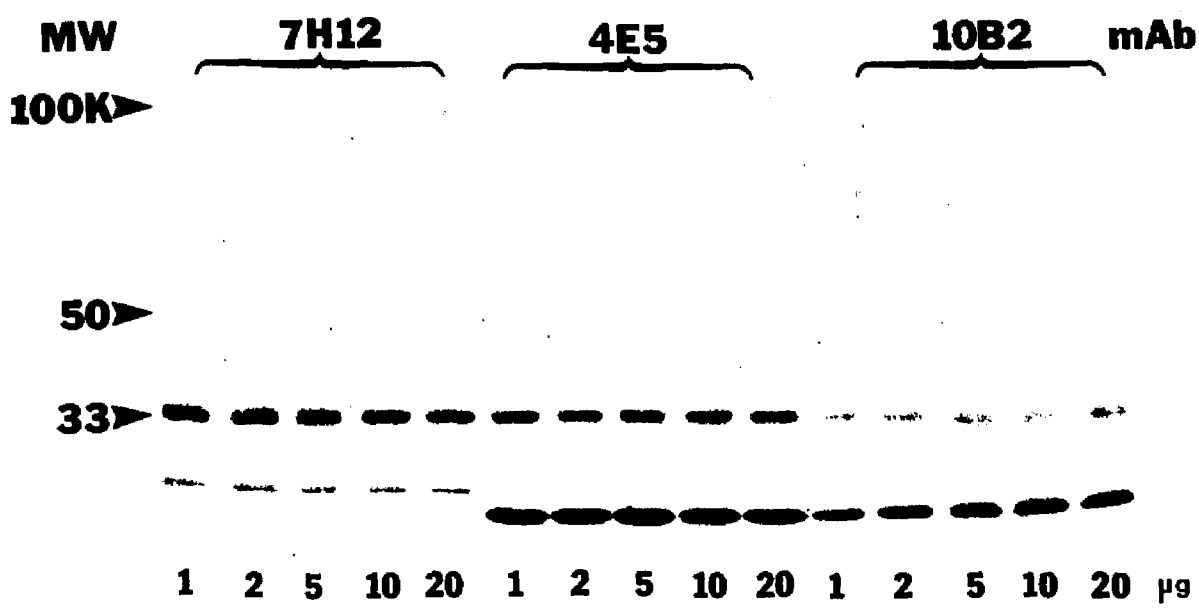
Table 2. Competitive ELISA using phosphorylated and unphosphorylated CAPs

PO_4-CAP or CAP micrograms	Antibody C-4E5	C-10B2	C-7H12	C-6C1
	Absorbance 405 nm (PO_4-CAP/CAP)			
50	.1/.1	.3/.2	.2/.1	.2/.1
25	.2/.2	.6/.5	.5/.4	.5/.5
10	.5/.4	1.2/1.0	.7/.7	.8/.7
5	1.1/1.0	1.5/1.3	1.1/1.1	1.2/1.1
1	1.6/1.5	1.8/1.6	1.3/1.2	1.4/1.3
.5	1.7/1.6	1.8/1.8	1.6/1.6	1.7/1.6
.1	1.9/1.9	1.9/1.9+	1.8/1.8	1.9/1.8
0	1.8/1.9+	1.9/1.9+	1.9/1.9	1.9+/1.9

PVC plates were coated with unphosphorylated CAPs, then incubated with mAbs in the presence of the indicated amount of phosphorylated or unphosphorylated CAPs (phosphorylated/unphosphorylated). Binding was quantitated using an alkaline phosphatase-conjugated anti-mouse second antibody. Results represent the means of three values from a typical experiment. A control value using an unrelated IgG1 murine mAb was determined for each row, and subtracted to give the reported results.

phosphatase-conjugated second antibody; Boehringer). Purified mAbs at this dilution were pre-incubated with varying amounts of either phosphorylated or unphosphorylated CAPs. These preparations were subsequently incubated with the PVC plate-bound CAPs, and bound antibody was quantitated with the alkaline phosphatase conjugated anti-mouse second antibody. As shown in Table 2, phosphorylated and unphosphorylated CAPs bind almost equally well to all of the mAbs. Phosphorylated CAPs do not appear to compete as well for mAb C-10B2 as unphosphorylated CAPs, although the difference is small. The similiar affinities of phosphorylated and unphosphorylated CAPs for all the mAbs is reflected also in the efficiency of immunoprecipitation of phosphorylated CAPs. Clathrin-associated $protein_2$ was phosphorylated by the CCV-associated kinase activity in the presence of polylysine. The samples were heated, denatured proteins removed by centrifugation, and the CAPs precipitated with mAbs coupled to Sepharose 4B. Since the beads were coupled to equal amounts of purified antibody we assumed that their specific binding activities were equivalent. The autoradiograph in Figure 11 shows that mAbs C-10B2, C-4E5 and C-7H12 all precipitate phosphorylated CAPs in a quantitative manner. Interestingly, a doublet was precipitated by C-10B2. Using

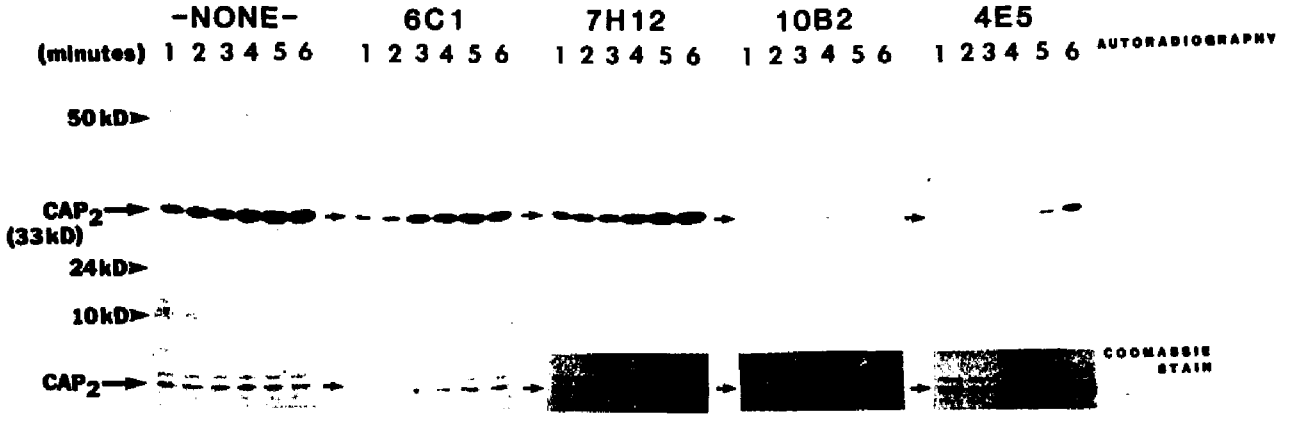
Figure 9. Relative efficiency of immunoprecipitation of phosphorylated and unphosphorylated CAPs by mAbs C-10B2, C-4E5 and C-7H12. A limiting titer of coupled antibody was used to precipitate a total of 20 ug of CAP_2. The various amounts of phosphorylated CAP_2 in the total amount are indicated in ug. Were phosphorylated CAP_2 precipitated less effeciently than unphosphorylated CAP_2, the total precipitated protein would be reduced as the proportion of phosphorylated CAP_2 increased. The lower molecular mass bands are antibody light chains, which apparently do not couple efficiently and are released in Laemmli sample buffer containing dithiothreitol. Arrow indicates CAP_x precipitated by mAb C-10B2.


MW
7H12
4E5
10B2
mAb
100K
50
33
1 2 5 10 20 1 2 5 10 20 1 2 5 10 20 µg

a limiting concentration of CAPs and varying the proportion of phosphorylated CAP_2 also does not appear to quantitatively affect the efficiency of CAP_2 immunoprecipitation by any of the mAbs (Figure 9).

In order to determine whether any of the mAbs reacted with epitopes relevant to the phosphorylation process, kinase inhibition experiments were performed (Figure 10). For this, mAbs at 3.5-fold molar excess to CAP_2 were incubated with CCVs in phosphorylation buffer overnight at 4°C. Polylysine and gamma radiolabeled ATP were added and the reactions were stopped at various time points with ethylenediaminetetracetic acid (EDTA). Samples were heated to 100°C for ten minutes and pelleted. The supernatants were analyzed by SDS-PAGE and autoradiography. None of the mAbs inhibited phosphorylation of casein by the endogenous CCV casein kinase II (data not shown). Both mAbs C-4E5 and C-10B2 inhibited phosphorylation of CAP_2 as long as five minutes after addition of ATP. The inhibition profiles of these mAbs appear to differ slightly: CCVs preincubated with C-10B2 acquire a small amount of CAP_2-bound phosphate before being completely inhibited, while CCVs preincubated with C-4E5 slowly but progressively acquire CAP_2-bound phosphate. The pattern of inhibition produced by mAb C-4E5 could be interpreted as less complete than that produced by mAb C-10B2. This would result from differences in affinity

Figure 10. Autoradiography showing the inhibition of CAP_2 phosphorylation by mAb C-10B2 and C-4E5. mAbs at 3.5-fold molar excess to CAPs were pre-incubated with overnight at 4°C. Time periods for the phosphorylation reaction (descirbed in Methods) ranged from one minute to six minutes. Reactions were stopped by adding 250 mM EDTA (1:10, pH 8.0), heating to 100°C for 10 minutes, and pelleting. Supernatants were analyzed by SDS-PAGE.

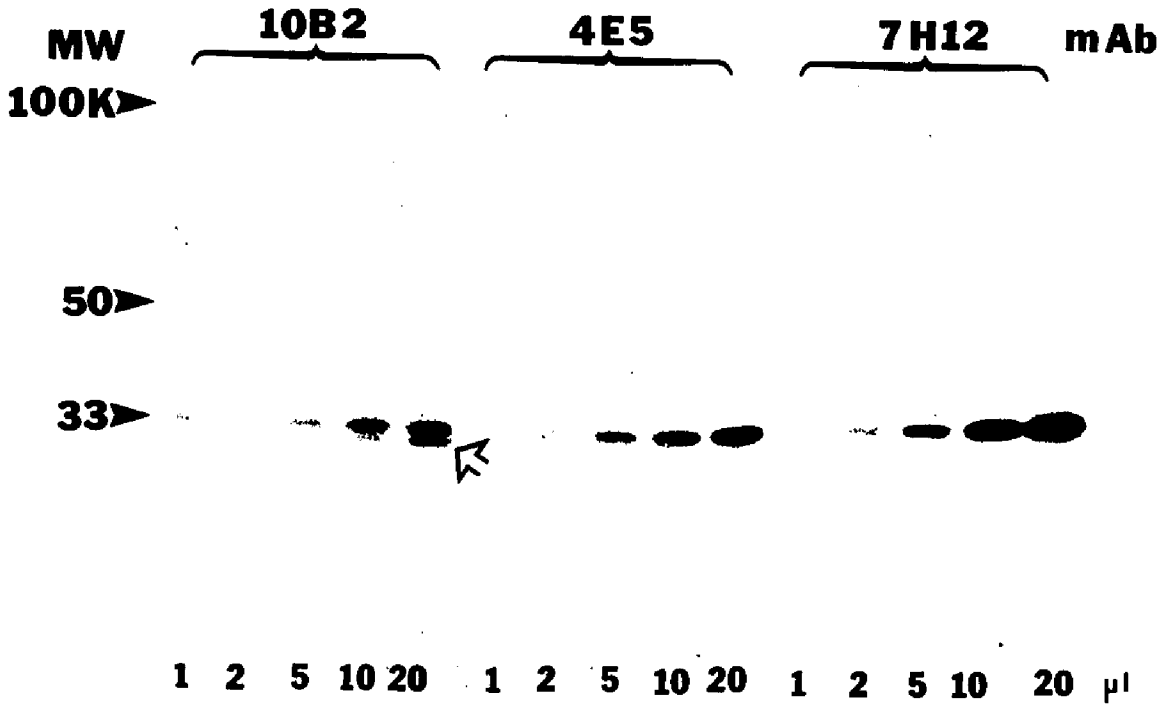

-NONE-
6C1
7H12
10B2
4E5
(minutes) 1 2 3 4 5 6
AUTORADIOGRAPHY
50kD
CAP2
(33kD)
24kD
10kD
CAP2
COOMASSIE STAIN

between the two mAbs, or differences in the proximity of the antigenic sites to the phosphorylation site. Alternatively, since it has been suggested that CAP_2 contains more than one phoshorylation (Usami et al., 1985), the antibodies may be inhibiting the phosphorylation of different residues. Further experiments are in progress to determine which of these possibilities is valid (Kohtz et al., in preparation).

A 32kD Phosphoprotein Cross-reacts with Anti-CAP_2 mAb C-10B2: Curiously, mAb C-10B2 co-precipitates another heat stable phosphoprotein from the CCV preparation of 32kD molecular mass. As indicated by the open arrow on the autoradiography in Figure 11, the co-precipitated phosphoprotein migrates immediately below the phosphorylated CAP_2. This phosphoprotein apparently competes very effectively with CAP_2 for mAb C-10B2, since it is not detectable in preparations of total CAPs (not shown). In C-10B2 precipitates, the 32 kD phosphoprotein is substantially enriched, indicating an affinity for mAb C-10B2 at least as high as that of CAP_2; this is apparent in both the autoradiograph in Figure 11 and the Coomassie stain in Figure 9 .

The 32kD protein does not react with CAP_1 nor with mAbs reactive with DARPP 32 (kindly provided by Dr. P.

Figure 11. Immunoprecipitation of phosphorylated CAP_2 and CAP_x by mAbs coupled to Sepharose 4B. CCVs were phosphorylated in the presence of polylysine, heated at 100°C for five minutes and pelleted. Aliquots of supernatant from a single phosphorylation reaction were immunoprecipitated by various volumes (in ug) of a 10% solution of Sepharose 4B-coupled mAbs. Approximately 1 ug of mAb is bound per microliter of 10% Sepharose 4B suspension. Precipitates were dissociated with Laemmli sample buffer and analyzed by SDS-Page and autoradiography. CAP_x is indicated by open arrow.


MW
10B2
4E5
7H12
mAb
100K
50
33
1 2 5 10 20 1 2 5 10 20 1 2 5 10 20 µl

Greengard, Rockefeller University). Also, it only reacts with one of the four mAbs recognizing CAP_2 (although traces may be present in mAb C-4E5 precipitates, not shown), implying that it is not a proteolytic fragment of this protein. We have tentatively labeled this phosphoprotein CAP_x (or LC_x), and are presently determining whether it is a CCV component or a soluble contaminant of our preparations. Its stability to heat denaturation (the vesicles are heated and centrifuged prior to precipitation), phosphorylation properties (it is phosphorylated in the presence of polylysine), and cross-reactivity with an anti-CAP_2 mAb (C-10B2) indicate that it may be structurally related to CAP_2.

Discussion: The characterization of mAbs to CAPs by our laboratory and others (Brodsky, 1985, and Kirchhausen et al., 1983) has revealed considerable structural diversity between the two forms (CAP_1 and CAP_2). The functional significance of this diversity is probably relevant to the evolution of higher organisms, since yeast appear to possess only one form of CAP. At presnt, the explicit function associated with this diversity have evaded elucidation. Instead, two properties possessed by both forms of CAPs have been described: a) an ATP-dependent uncoating enzyme works by initially binding to either of

single site on the clathrin heavy chain binds equally well to either form of CAP (Winkler and Stanley, 1983). Hence, mAbs which distinguish the two forms of CAPs would not be as likely to recognize epitopes involved in these conserved functions as mAbs reacting with both CAPs.

In this report, two mAbs (C-6C1 and C-7H12) are presented which react with epitopes shared by both CAPs. The term "conserved domain" may be used to describe the structural region of the CAPs reactive with these mAbs. The significance of the epitope recognized by C-6C1 has not been detailed, although chymotryptic mapping experiments revealed that it is near the clathrin binding region of the CAPs. The mAb C-7H12 appears to react with an epitope directly on the clathrin binding domain as evidenced by chymotryptic mapping, ELISAs using clathrin-bound CAPs and free CAPs, and immunoprecipitation experiments using radioiodinated CCVs and CAPs. Sandwich ELISAs indicate that, despite their proximity, the epitopes recognized by mAbs C-6C1 and C-7H12 are distinct.

Sandwich ELISAs indicate that mAbs C-10B2, C-4E5, C-7H12, and C-6C1 react with distinct epitopes on CAP_2. As may be inferred from the degree of sterically induced inhibition observed in these assays, mAbs C-10B2 and C-4E5 appear to react with different epitopes in the same region of CAP_2. As judged by its sensitivity to chymotryptic

digestion, this region of CAP_2 is on an accessible end of the molecule. The term "non-conserved domain" may be used to refer to this accessible edge of the CAP_2 and its putative counterpart on CAP_1.

Two kinase activities are associated with CCVs. The first is Ca^{2+}/calmodulin- and cAMP-independent, and utilizes the 50 kD assembly complex polypeptide as a substrate; the second is accentuated by the presence of polybasic compounds and can use CAP_2 as a substrate. Direct inhibition experiments indicate that mAb C-10B2 and C-4E5 react with an epitope relevant to the phosphorylation process. In particular, C-4E5 inhibits phosphorylation better in the short term, while C-10B2 inhibits better in the long term. Inhibition of CAP_2 phosphorylation by these mAbs could be produced by several mechanisms: steric interference of the bound antibody and the enzyme; allosteric structural alterations produced in CAPs by bound antibody; direct competition for the enzyme binding site by the antibody; and, if it is distinct from the enzyme binding site, direct blocking of the phosphorylation site by bound antibody. Further work is necessary to determine the mechanisms responsible for inhibition of CAP_2 phosphorylation by each mAb.

Immunoprecipitation experiments using mAb C-10B2 revealed a cross-reactive phosphoprotein with a molecular

mass 32 kD. This phosphoprotein is referred to as CAP_x or LC_x since it co-precipitates with CAP_2. This fragment is not co-precipitated by by C-4E5, CVC-1, C-7H12, or C-6C1, indicating that it is not a CAP fragment. Since CAP_x is a phosphoprotein and C-10B2 does inhibit CAP_2 phosphorylation, it appears that C-10B2 cross-reacts with the phosphorylated epitopes on both polypeptides. Similarities between the polypeptides suggest that it is a member of the CAP family. In particular, CAP_x is heat stable; it can be isolated from CCV preparations; and it is phosphorylated under the same conditions as CAP_2. When a CAP_x-specific antibody is produced it may be possible to ascertain if the polypeptide is an integral vesicle protein.

Immunoprecipitation experiments using C-4E5 and CVC-1 revealed two other properties of CAP phosphorylation: CAP_2 is phosphorylated in absence of effectors, but to a considerably lesser degree; CAP_1 also is phosphorylated in the presence of polylysine, but not to the same extent as CAP_2. The latter observation implies that the kinase is not absolutely specific for CAP_2 and in the presence of other mediators or enzymes, CAP_1 may be equally well phosphorylated. Alternatively, Usami et al 1985, have indicated that CAP_2 bears three sites of phosphorylation; CAP_1 may have conserved only a fraction of these sites.

Further work should elucidate the physiological significance of each site of phosphorylation and their distribution on each CAP.

XI. Subpopulations of Clathrin-coated vesicles:

1. Subpopulations of Clathrin-coated vesicles Generated by Clathrin light chains

Introduction: The biochemical elements generating CCV and clathrin-coated granule diversity are not well characterized. An example of tissue-type heterogeneity is observed in the size of CAPs extracted from bovine brain (33 kD and 36 kD) and from other bovine tissues (29 kD and 32 kD). This difference in size may reflect a greater functional diversity in brain. Pfeffer and Kelly (1985) observed microheterogeneity in the composition of bovine brain CCVs by using monoclonal antibodies directed against two different synaptic vesicle transmembrane cargo proteins. SDS-PAGE of purified bovine brain CCVs immunoprecipitated by these antibodies revealed the subpopulation of CCVs carrying synaptic vesicle cargo proteins to be enriched in two previously uncharacterized vesicle proteins (32 kD and 29 kD).

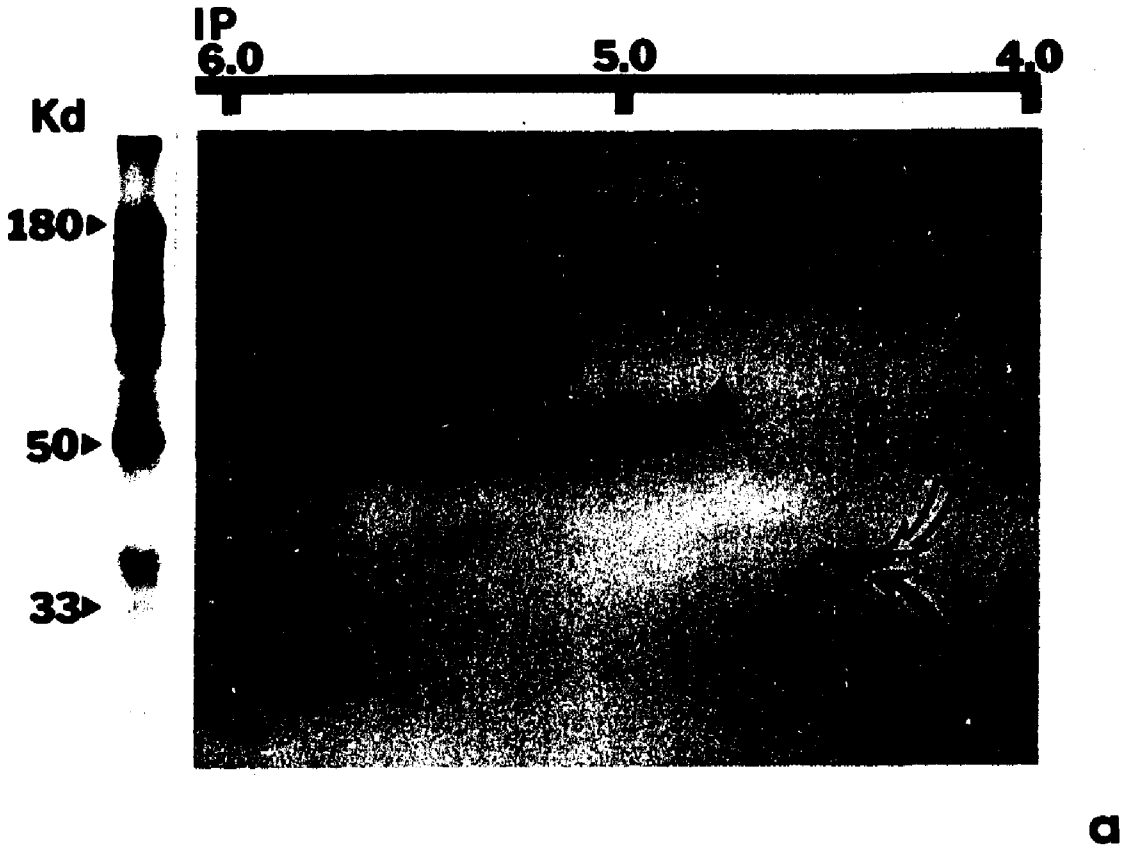
In this report smaller vesicles enclosed in a clathrin cage are referred to as CCVs, while larger vesicles bearing either an open clathrin lattice or a diffuse clathrin coat are referred to as CCGs. The roles of CCVs and CCGs in endocytic and secretory systems are examined here using novel monoclonal antibodies (mAbs)

directed against subpopulation-specific determinants. Subpopulations of CCGs may be represented in preparations of purified CCVs since condensation of the lattice structure can occur during subcellular fractionation in buffers which enhance the stability of CCVs. Bearing this in mind, immunoprecipitation and biochemical analysis were used to examine subpopulations in purified preparations of bovine brain CCVs, and immunofluorescent microscopy was used to examine to a limited extent the intracellular distribution of these subpopulations. Clathrin-coated vesicles are resolved into subpopulations by mAbs reactive with cytoskeletal proteins, membrane cargo molecules, and constitutive vesicle proteins. The technical aspects of CCV immunoprecipitation were modified from previous protocols (Pfeffer and Kelly, op cit.) to facilitate the selection of subpopulations based on the copy-number of certain antigens as well as their presence or absence in the vesicle. In part 1 this approach allows the immunochemical discrimination of subpopulations of CCVs enriched in either the upper or lower molecular mass CAP, despite the presence of both forms in every vesicle. In part 2 subpopulations of CCVs containing the most actin (actin$^+$) are separated from those with the least actin (actin$^-$) by immunoprecipitation with mAb reactive with beta actin. These subpopulations may be derived from

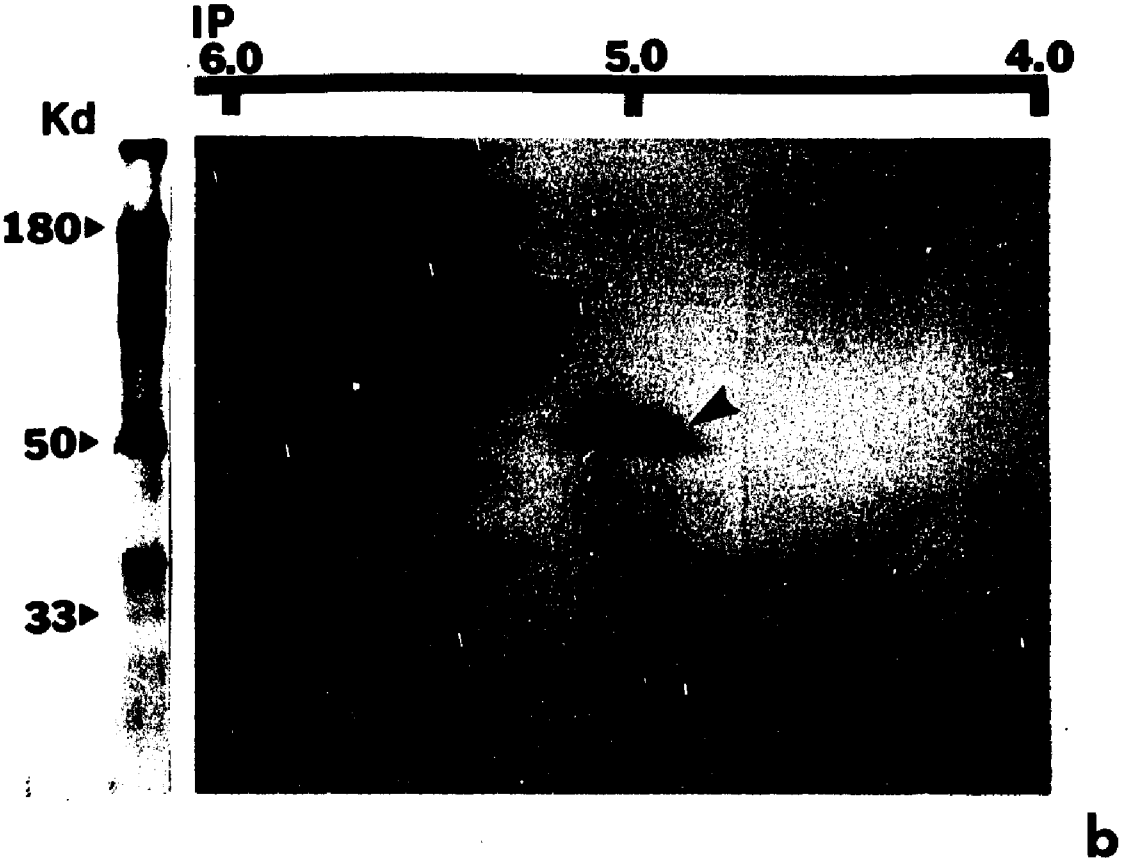
Figure 12. Autoradiography of one- and two-dimensional gel analysis of radioiodinated CCVs precipitated by mAbs C-4E5 and CVC-1. Open arrows indicate positions of the upper CAP; solid arrows indicate the positions of the lower CAP. Arrowhead indicates position of tubulin.

A: CCV profile precipitated by mAb CVC-1.

B: CVC profile precipitated by mAb C-4E5.



a



b

intracellular regions containing either high (i.e., plasma membrane) or low (i.e., perinuclear cytoplasm) concentrations of actin, respectively. Finally, mAbs reactive with CCV membrane antigens are used to demonstrate by immunoprecipitation that cargo molecules may be sorted between actin$^+$ and actin$^-$ subpopulations.

Generation of CCV and Coated Granule Diversity by CAPs: The distribution of CAPs in coated vesicle subpopulations was investigated using mAbs specifically reactive with either the 33 kD or 36 kD forms present in bovine brain CCVs. Immunoblot analysis of the anti-CAP mAbs used is presented in Figure 5. Results from other laboratories indicate that the two forms of CAPs are distributed in a random manner among purified clathrin triskelions (Kirchhausen et al., 1983). However, this does not imply that the CAP-containing triskelions are distributed in a random fashion among CCVs and CCGs. To address this point, purified radioiodinated bovine brain CCVs were immunoprecipitated by mAbs specifically reactive with either the 33 kD (C-4E5) or 36 kD (CVC-1) forms of CAP. The titers of anti-CAP were limited in these experiments by adding unlabelled CCVs until the total precipitated counts was half the value precipitated in antibody excess. Experiments done in antibody excess,

however, did not precipitate all CCVs (Table 3), presumably due to the relatively low affinity of mAbs CVC-1 and C-4E5. Two dimensional gel electrophoresis of the CCVs precipitated at limiting titers revealed subpopulations of brain CCVs enriched in either the 33 kD or 36 kD form of CAP (Figure 12). It is unlikely that this result is generated by free CAPs co-precipitating with the vesicles as these preparations were purified twice by Sepharose 4B column chromatography following radioiodination. In addition, mAb C-7H12, which recognizes only free CAPs, precipitated no counts above background from these samples.

Experiments with an actin^{+} subpopulation (discussed below) revealed that CAPs in different subpopulations of CCVs may be radioiodinated with differing efficiencies. It is therefore necessary to assess the extent of CAP_1 or CAP_2 enrichment in subpopulations precipitated by mAbs CVC-1 or C-4E5 by an alternate means. Clathrin coated vesicles were precipitated with mAb CVC-1 or C-4E5 and incubated with polylysine and gamma ^{32}P-labeled ATP to phosphorylate CAP_2. After dissociating labeled CCVs with acid, equivalent microgram amounts of dissociated protein were heated to 100°C, centrifuged, and equivalent volumes of supernatant were analyzed by SDS-PAGE and autoradiography. As shown by Coomassie stain in Figure 13, CCVs precipitated by mAb CVC-1 are enriched in CAP_1, while

Figure 13. Analysis of CAPs extracted from CCVs immunoprecipitated by mAbs CVC-1 (anti-CAP_1) or C-4E5 (anti-CAP_2). Subsequent to precipitation by the mAbs, CCVs were suspended in 100 mM Tris, 10 mM $MgCl_2$, pH 7.5, with 10 ug/ml polylysine. .001 mCi gamma ^{32}P-labeled ATP was added (.02 mM final concentration ATP), and the mixtures were heated to 100°C for 5 minutes, centrifuged, and supernatants analyzed by SDS-PAGE.

A: total CCVs.

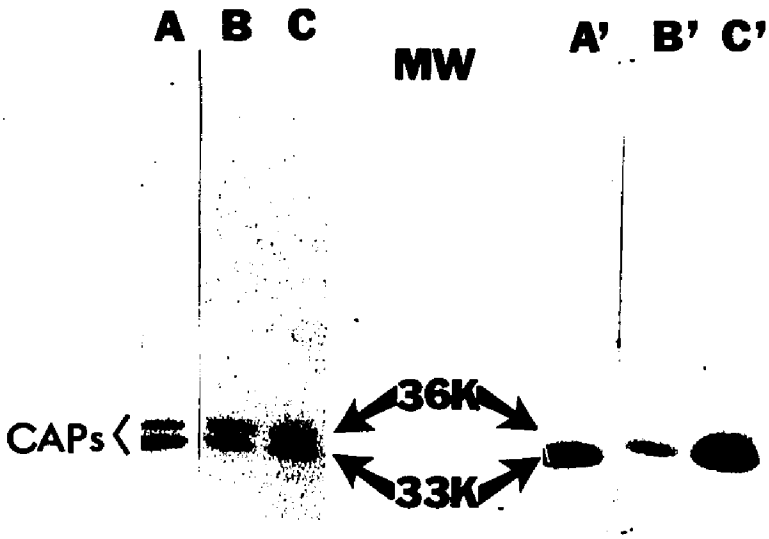
B: CVC-1 precipitated CCVs.

C: C-4E5 precipitated CCVs

Phosphorylation is observed only in CAP_2 (33 kD).

A, B, and C: Coomassie blue stain.

A', B' and C': Autoradiography of same gels.


A
B
C
MW
A'
B'
C'
CAPs
36K
33K

CCVs precipitated by mAb C-4E5 are enriched in CAP_2. This is emphasized by autoradiography of the same gels: CCVs precipitated by C-4E5 also contain a considerably greater amount of labeled CAP_2. Whether this difference is only due to the greater quantity of CAP_2 in these CCVs or to a greater quantity of enzyme is currently being determined.

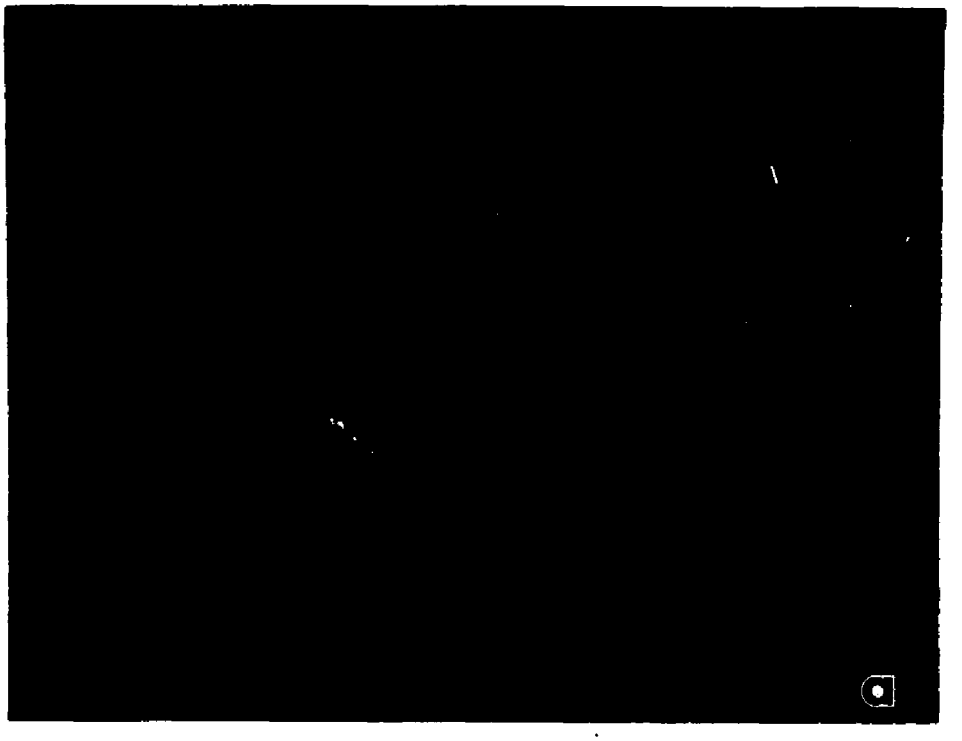
Since the upper and lower CAPs are not distributed in a 1:2 ratio among all CCVs, we investigated histologically the distribution of these molecules in various cultured cell lines. The scope of this investigation was limited by the capacity of these mAbs to react exclusively with CAPs from higher mammals. In most of the cell lines examined, the distribution of upper and lower CAPs was indistinguishable. This is typified by patterns observed in MDBK cells, shown in Figures 14A and 14B. A strain of human cardiac myoblasts proved to be the exception, displaying different distributions of upper and lower CAPs during the synthetic phase of their growth cycle. As shown in Figures 15A and 15B, intense CAP immunofluorescence was observed on large granules amidst the abundant protein synthesis organelles. These granules are reactive with antibodies to either CAP and they are visible by phase light microscopy (not shown). In contrast to the larger granules, the smaller peripheral vesicles are recognized only by mAb against the lower CAP (Figure 15B). When the

Figure 14. Immunofluorescence microscopy using mAbs specific to CAP_1 or CAP_2.

A: MDBK cells, mAb CVC-1.

B: MDBK cells, mAb 10B2.

Cells were fixed with 3.7% paraformaldehyde and treated with 0.1% Triton X-100 before being incubated with primary antibody. Rhodamine-conjugated rabbit anti-mouse IgG second antibody was used. Total magnification: 800X.


a

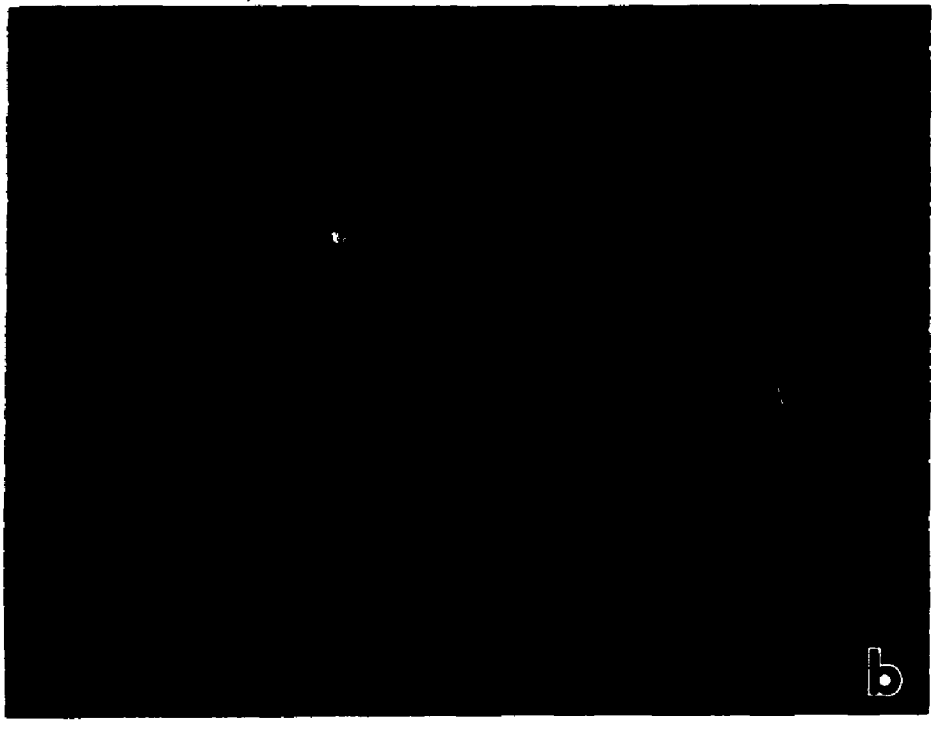

b

Figure 15. Immunofluorescence microscopy using mAbs specific to CAP_1 or CAP_2.

A: HCM cell, synthetic phase, mAb CVC-1.

B: HCM cell, synthetic phase, mAb C-4E5.

Cells were fixed with 3.7% paraformaldehyde and treated with 0.1% Triton X-100 before being incubated with primary antibody. Rhodamine-conjugated rabbit anti-mouse IgG second antibody was used. Total magnification: 800X.

A

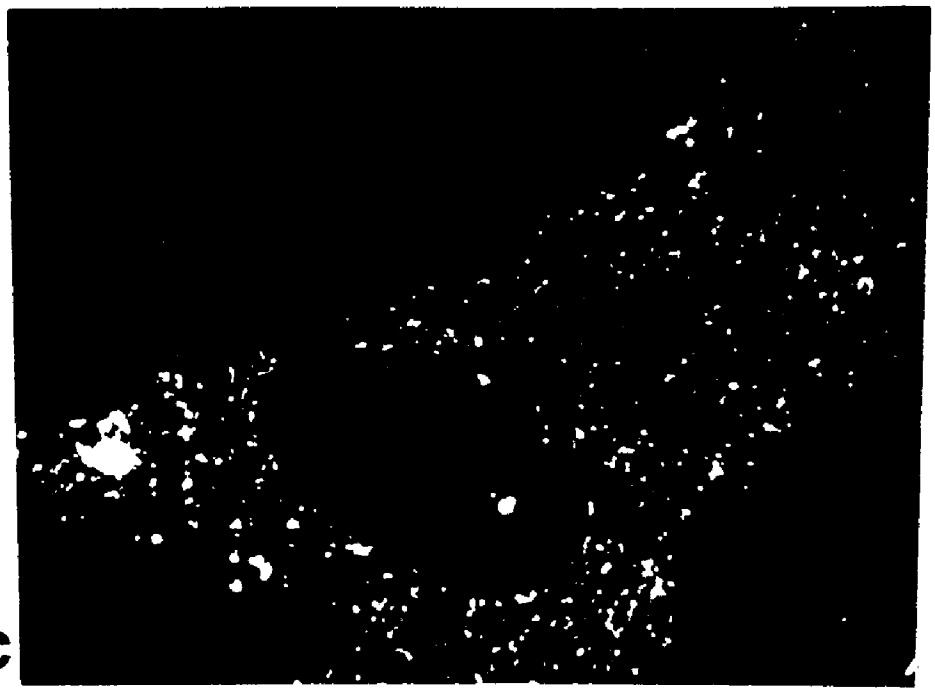
B

Figure 15. Immunofluorescence microscopy using mAbs specific to CAP_1 or CAP_2.

C: HCM cell, pseudo-contractile phase, mAb CVC-1.

D: HCM cell, pseudo-contractile phase, mAb C-4E5.

Cells were fixed with 3.7% paraformaldehyde and treated with 0.1% Triton X-100 before being incubated with primary antibody. Rhodamine-conjugated rabbit anti-mouse IgG second antibody was used. Total magnification: 800X.



C

D

cells progress to a pseudo-contractile phase (produced by co-culture with SH-SY5Y neuroblastoma cells) the distribution of the two forms of CAPs appears more homogeneous (Figures 15C and 15D), although lower CAP immunofluorescence appears more dense. This may result from the larger vesicles transforming into smaller ones and redistributing themselves throughout the cell.

XII. Subpopulations of Clathrin-coated vesicles:
2. Subpopulations of Clathrin-coated vesicles Generated by Actin

A distinct subpopulation of clathrin-coated vesicles contains actin: Monoclonal antibodies reactive with three types of antigens were used in this study to immunoprecipitate subpopulations of CCVs: 1. mAbs reactive with cytoskeletal proteins; 2. mAbs reactive with vesicle cargo proteins; and 3. mAbs reactive with constitutive vesicle proteins. Cytoskeletal proteins are generally restricted to certain areas of the cell; additionally, polysomes translating mRNA of these proteins appear to be coincidentally distributed (Lawrence and Singer, 1986). Clathrin coated vesicles are known to contain at least two cytoskeletal proteins: actin and tubulin (Pfeffer and Kelly, 1981). Since actin and microfilaments are distributed throughout the cell periphery (Small and Celis, 1978), it is reasonable to assume that CCVs associated with the cell surface may be enriched in this protein. On the other hand, tubulin and microtubules are distributed more uniformly through the cell; therefore, tubulin should be more randomly distributed among CCVs.

To examine the actual distribution of actin and

Table 3. Summary of Monoclonal Antibodies

mAb	Class	Antigen	Pellet: CCVs	Supernatant: A-7C11	T-2H9
A-7C11	N.D.	actin	25%	<5%	70%
T-2H9	N.D.	tubulin	>95%	<5%	<5%
S-11D9	IgM;k	44kD	25%	<5%	70%
C-4E5*	IgG1;k	CAP_2	65%	15%	25%
C-10B2	IgG1;k	CAP_2	90%	5%	5%
(CVC-1)+	IgG1;k	CAP_1	25%	15%	70%
S-10C7	IgG2b;k	N.D.	20%	25%	75%
C-7H12	IgG1;l	free CAPs	<5%	25%	>95%
C-6C1	IgG1;l	free CAPs	N.D.	N.D.	N.D.
S-8G8	IgG1;k	NP 185	>95%	<5%	<5%
S-6G7	IgG1;k	NP 185	>95%	<5%	<5%
2D9	IgM;k	clathrin heavy chain	N.D.	N.D	N.D

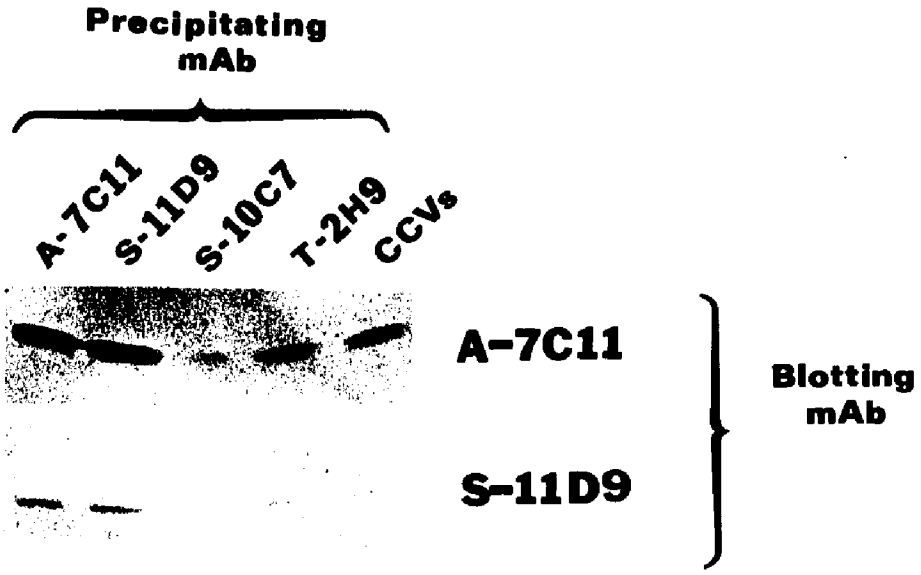
Results indicate percentage CCVs precipitated (pellet) from a typical preparartion utilizing an excess of antibody. Remaining CCVs were precipitated by either mAb A-7C11 or T-2H9 (supernatant). CCV preparation may represent a selected population, not the total CCV cell profile. Values were rounded to the nearest 5%.

* mAbs C-4E5 and CVC-1 precipitate less than 100% of the CCV due to their relatively low affinities (which may be a result of CNBr coupling to Sepharose 4B), not to the absence of either form of CAP in any CCV subpopulation.

+mAb CVC-1 was made by Kirchhausen et al. (1983).

tubulin among purified CCVs, anti-actin mAb A-7C11 and anti-tubulin mAb T-2H9 (Kohtz and Puszkin, unpublished results) were purified, coupled to Sepharose 4B, and used to immunoprecipitate radioiodinated bovine brain CCVs. These mAbs were characterized by immunoblotting with purified brain actin (kind gift of Dr. S. Berl, Dept. of Neurology, Mount Sinai School of Medicine), tubulin (kind gift of Dr. R. Liem, Dept. of Pharmacology, New York University School of Medicine) and CCVs, and were found to produce characteristic patterns of reactivity for the cytoskeletal components in immunofluorescence analysis (not shown). The quantitative results of the radioimmune precipitation experiments are shown in Table 3. Anti-tubulin mAb T-2H9 consistently precipitated >95% of brain CCVs, while anti-actin mAb A-7C11 generally precipitated only 25%. These values indicate that nearly all brain CCVs contain substantial amounts of tubulin, while only a fraction contain enough actin to mediate their immunoprecipitation. This result is supported by the immunoblot in Figure 16. Equal amounts of CCVs precipitated by mAbs A-7C11 and T-2H9 were resolved by SDS-PAGE and blotted with anti-actin mAb. The subpopulation of CCVs immunoprecipitated by mAb A-7C11 contains more actin than an equal quantity of the total population of CCVs precipitated by mAb T-2H9. As is indicated in Table 3, the

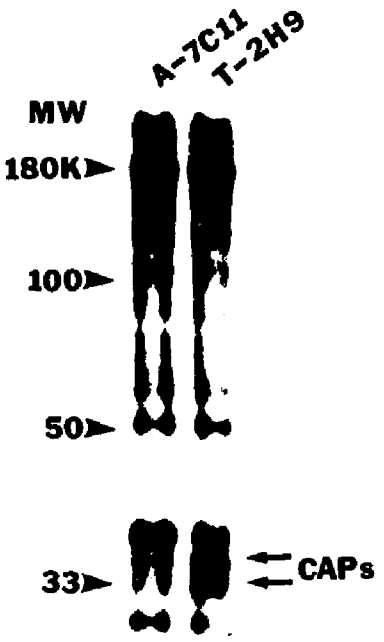
Figure 16. Immunoblot analysis of immunoprecipitated CCVs. CCVs were immunoprecipitated by the indicated mAbs, dissociated and tested for total protein content, then loaded in equivalent amount for analysis by SDS-PAGE and immunoblotting. Separate duplicate blots were used to analyze actin (mAb A-7C11) and 44 kD antigen (mAb S-11D9) content. Enlargements of segments of the immunoblots are shown to facilitate comparison of the intensities of reactive bands at 43 kD (actin) and 44 kD (S-11D9 antigen).


Precipitating mAb
A-7C11
S-11D9
S-10C7
T-2H9
CCVs
A-7C11
S-11D9
Blotting mAb

subpopulation of CCVs precipitated by mAb A-7C11 is contained within the putative total brain population precipitated by mAb T-2H9. This explains the presence of actin in CCVs precipitated by T-2H9.

Radioiodinated brain CCVs were immunoprecipitated by Sepharose 4B-coupled mAbs A-7C11 and T-2H9, and equivalent counts analyzed by SDS-PAGE (Figure 17). Clathrin-coated vesicles precipitated by mAb A-7C11 apparently contain considerably less radiolabeled CAPs than CCVs precipitated by mAb T-2H9. This difference may reflect a quantitative difference in the amount of CAPs, or, alternatively, a structural difference in the CCVs precipitated by A-7C11 which affects the efficiency of CAP radioiodination. Another difference between the SDS-PAGE profiles in Figure 2 is the relative intensity of bands at 38 kD and 29 kD molecular mass. These bands are enhanced in the CCVs precipitated by mAb A-7C11. Polypeptides of similar molecular mass were previously found to be enriched in a putatively endocytic subpopulayion of CCVs carrying synaptic vesicle cargo antigens (Pfeffer and Kelly, 1985). Increased amounts of these two putative brain endocytic CCV markers in actin^{+} CCVs would corroborate the notion that this subpopulation is derived from intracellular regions proximal to the plasma membrane. We have found, however, that the actual levels of these two

Figure 17. Autoradiography of radioiodinated CCVs immunoprecipitated by mAbs A-7C11 (anti-actin) and T-2H9 (anti-tubulin) and analyzed by SDS-PAGE.


A-7C11
T-2H9
MW
180K
100
50
33
CAPs

bands varies considerably with the immunoprecipitation protocol used, and while the difference in the CAPs bands is consistent, the difference in 38 kD and 29 kD bands is not always apparent.

Clathrin-coated vesicle cargo antigens are sorted between actin$^+$ and actin$^-$ subpopulations: Monoclonal antibody S-11D9 reacts with a 44 kD CCV cargo molecule that is also present in brain synaptic vesicles (Figure 21, discussed in detail below). Immunoblots of whole cell extracts revealed that this antigen is present in a variety of cells and tissues (Figures 23A, 25 and 26). When fractionated synaptic plasma membranes were analyzed by SDS-PAGE and immunoblotting with S-11D9, a prominent 56 kD band was observed in addition to the 44 kD band. The higher molecular weight band in the plasma membrane may represent a precursor of the vesicle 44 kD polypeptide (Figure 21). The generation of the two forms of this antigen is considered in the next chapter.

Brain CCVs can acquire synaptic vesicle cargo molecules, such as the S-11D9 antigen, by at least two pathways. The first occurs subsequent to synaptogenesis, when CCVs are thought to recover vesicle membrane lost to the synaptic plasma membrane during transmitter secretion (Heuser and Reese, 1973). The second occurs during

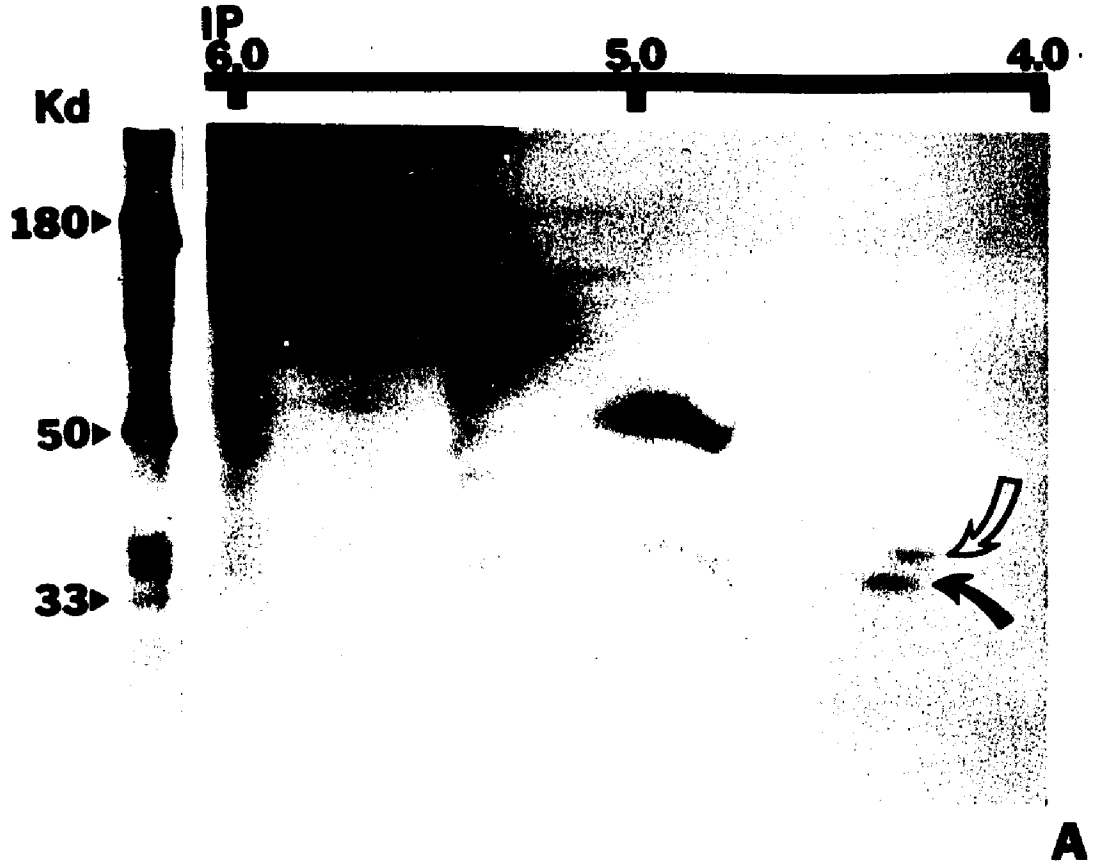
transport, when CCVs may be involved in transferring synaptic vesicle cargo molecules from the Golgi to the nerve ending. To determine whether actin$^+$ or actin$^-$ CCVs carry the antigen reactive with S-11D9, purified brain CCVs were immunoprecipitated with mAB S-11D9, resolved by SDS-PAGE, and immunoblotted with anti-actin mAb A-7C11. When compared with equivalent microgram amounts of CCVs precipitated by anti-tubulin mAb T-2H9 or the total brain CCV population (Figure 16), CCVs precipitated by S-11D9 appear to contain as much actin as CCVs precipitated by anti-actin mAb A-7C11. These data imply that the subpopulation of CCVs carrying the antigen reactive with mAb S-11D9 derive from an intracellular location enriched in actin, possibly beneath the synaptic plasma membrane (Mahler, 1977). Therefore, brain CCVs may have acquired this antigen from synaptic vesicles during membrane retrieval at the synaptic junction.

Since the actin$^+$ subpopulations of CCVs precipitated by mAb A-7C11 have diminished amounts of CAPs, radioimmunoprecipitation experiments were performed to determine whether the amount of CAPs is similiarly reduced in CCVs precipitated by mAb S-11D9. Purified brain CCVs were radioiodinated, immunoprecipitated by mAb S-11D9 or mAb C-10B2, and analyzed by two-dimensional gel electrophoresis. As is shown by the autoradiographs in

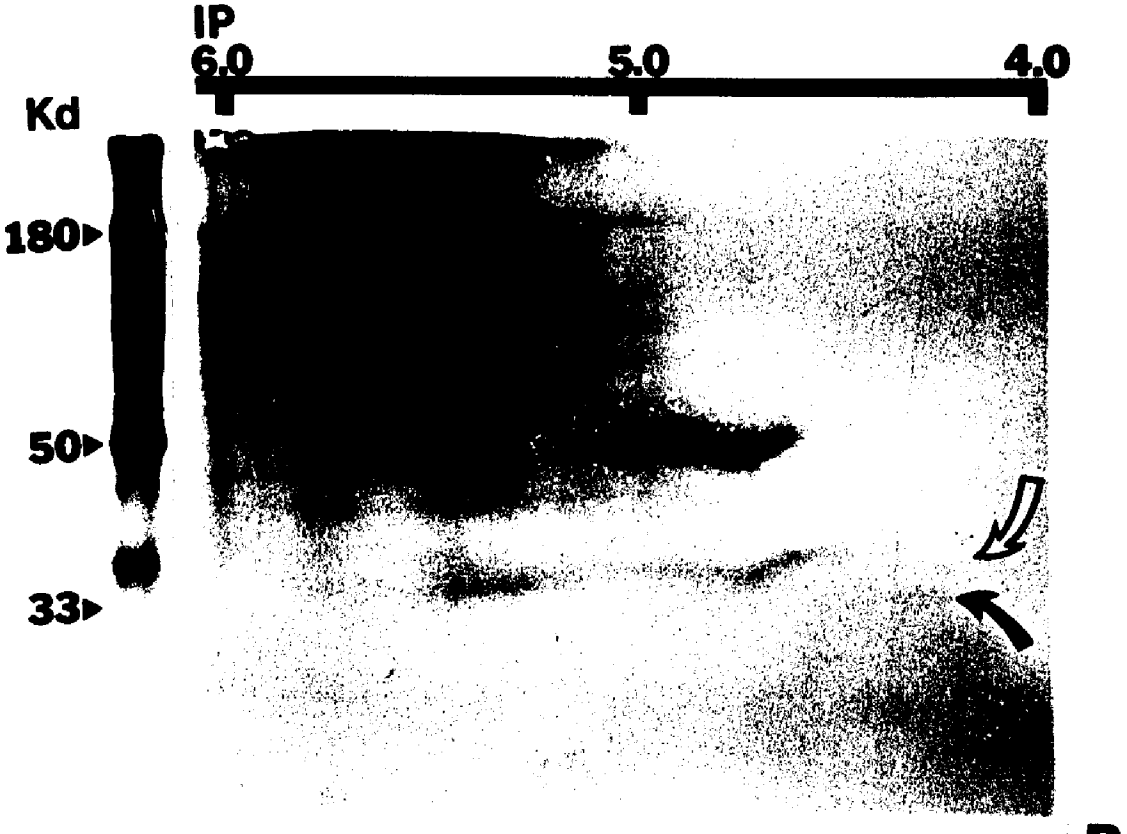
Figure 18. Autoradiography of one- and two-dimensional gel analysis of radioiodinated CCVs precipitated by mAbs S-11D9 and C-10B2. Open arrows indicate positions of upper CAP; solid arrows indicate the positions of lower CAP. Arrowhead indicates position of tubulin.

A: CCV profile precipitated by mAb S-11D9.

B: CCV profile precipitated by mAb C-10B2.



A

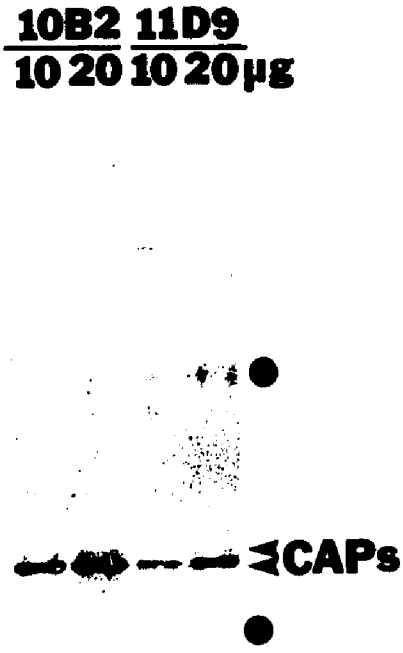


B

Figure 18, the subpopulation of CCVs precipitated by mAb S-11D9 appears to have substantially less CAPs than the total population precipitated by C-10B2 (as indicated in Table 3, C-10B2 precipitates at least 90% of the total CCVs). The reduced intensity of the CAPs bands in the actin$^+$ subpopulation of CCVs may merely reflect the effects of differental iodination. To test this hypothesis, equivalent microgram quantities of CCVs precipitated by these mAbs S-11D9 and C-10B2 were resolved by SDS-PAGE and immunoblotted with mAb C-6C1 (a mAb reactive with both CAPs, Figure 19). Using this protocol, the CCVs precipitated by mAb S-11D9 also appeared to contain less CAPs than the total population; however, the change in profile was not as profound as that observed when using radioiodinated CCVs. This indicates that while the actin$^+$ subpopulation of CCVs may contain measurably less CAPs than the total population, the appearance of this difference is accentuated by radioiodination techniques. This is probably the result of structural differences between the actin$^+$ and actin$^-$ subpopulations. It is important to note that actin$^+$ CCVs do contain some CAPs, and that the entire subpopulation can be precipitated by anti-CAP$_2$ mAb C-10B2 (Table 3).

The actin$^+$ subpopulations of CCVs precipitated by mAbs S-11D9 and A-7C11 comprise approximately 25% of total

Figure 19. Immunoblot analysis of CCVs precipitated by mAbs S-11D9 and C-10B2. Total precipitated protein loaded into each lane is indicated. Solid dots indicate bands contributed by dissociated immunoglobulins. Immunoglobulin heavy chains preferentially bind to cyanogen bromide activated Sepharose 4B allowing release of some light chains after reduction in sample buffer containing dithiothreitol. Due to pentameric structure of IgM, S-11D9-coupled beads also release some heavy chain from portions of the molecule that remain uncoupled. Arrows indicate positions of CAPs. This blot was performed using mAb 6C1 (reactive with both CAPs).


10B2 11D9
10 20 10 20 µg
CAPs

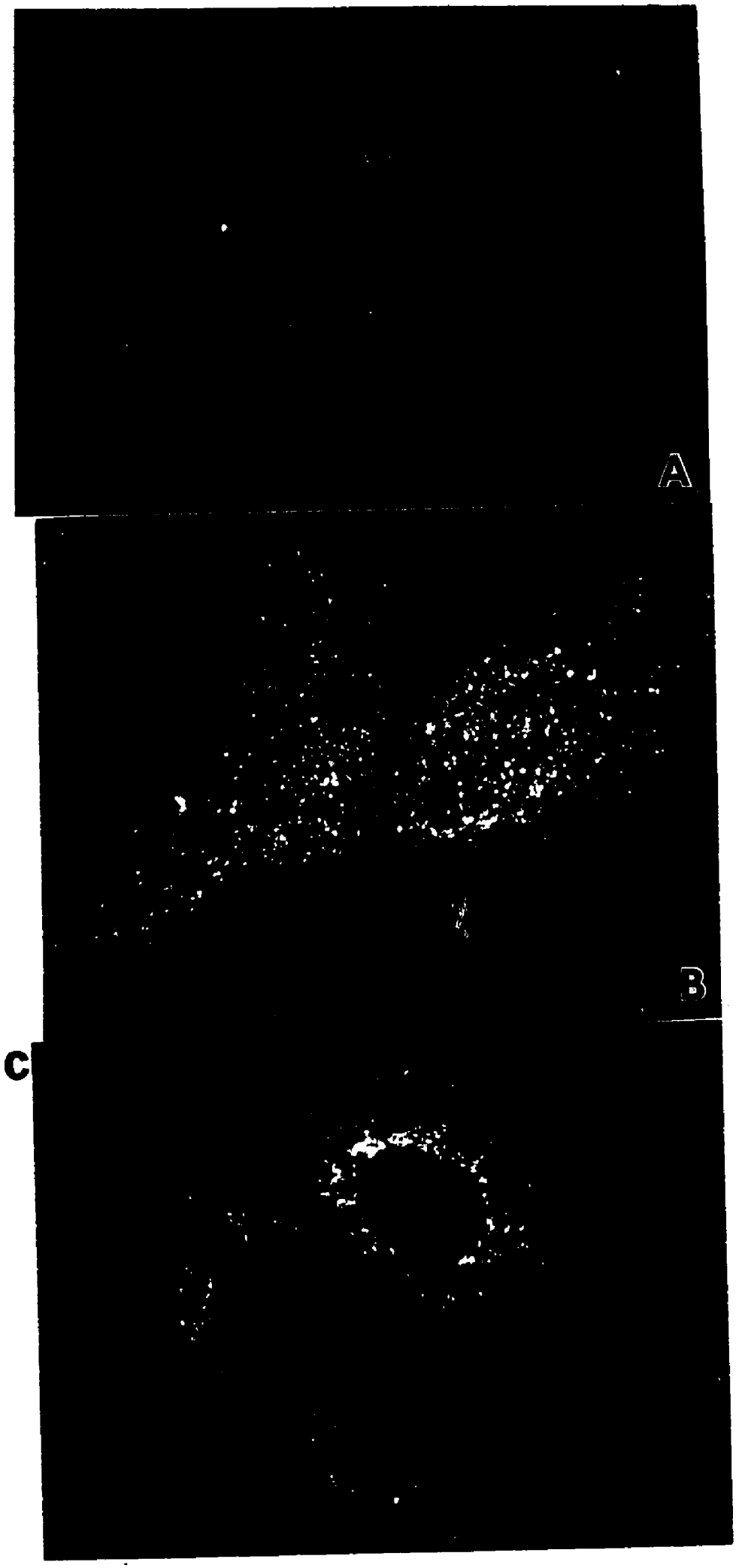
brain CCVs (Table 3). To determine whether CCV cargo molecules are preferentially sorted to the actin$^-$ subpopulation, mAbs generated against CCV membrane antigens were screened for enrichment of the actin$^-$ subpopulation by differential immunoprecipitation. As shown in Table 3, mAb S-10C7 precipitates a subpopulation of approximately 20% of brain CCVs that is completely distinct from the subpopulations precipitated by either mAb A-7C11 or S-11D9. Two dimensional gel electrophoresis indicates that S-10C7 radioimmune precipitates are composed of CCV proteins and not contaminating membranes (not shown). When CCVs immunoprecipitated by S-10C7 are resolved by SDS-PAGE and immunoblotted with anti-actin mAb A-7C11 (Figure 16) it is apparent that they consist of actin$^-$ vesicles. Clathrin-coated vesicles precipitated by S-10C7 contain less actin than the total populations and considerably less than CCVs precipitated by S-11D9 or A-7C11. Clathrin-coated vesicles carrying the S-10C7 antigen are apparently derived from an intracellular location which contains relatively little actin, such as the perinuclear cytoplasm.

Monoclonal antibody S-10C7 was originally observed to react with CCV membranes in an ELISA, but immunoblots of CCV membranes or whole cell extracts failed to identify the antigen. The native conformation of the reactive antigen may be requisite for binding by mAb S-10C7, and this is

probably lost during SDS-PAGE prior to blotting. Attempts to immunoprecipitate the antigen from radioiodinated CCV membranes dissolved by Triton X-100 have also been unsuccessful. The possibility that mAb S-10C7 reacts with a glycolipid cannot be excluded.

From synthesis to eventual degradation, the antigens recognized by mAbs S-10C7 and S-11D9 are shuttled throgh the cell by a variety of vesicles of which CCVs represent only a discrete subset. To better evaluate the complete transport pathways traveled by these antigens, MDBK cells were fixed with paraformaldehyde, treated with Triton X-100 and analyzed by immunofluorescence with S-10C7 and S-11D9. As shown in Figure 20, mAbs S-11D9 and S-10C7 stain distinct sets of vesicles: S-11D9 stains a smaller surface population, while S-10C7 stains a cytoplasmic and perinuclear population of larger vesicles. Monoclonal antibody C-4E5 (anti-CAP_2) should react with most CCVs, and the fluorescent pattern generated by this mAb refects their distribution in MDBK cells. This pattern differs from the fluorescent patterns produced by either mAb S-10C7 or S-11D9. The intracellular location on CCVs immunoprecipitated by either mAb S-11D9 or S-10C7 may be represented by common regions of staining in the patterns produced by each of these mAbs and that produced by mAb C-4E5.

Figure 20. Immunofluorescence microscopy of cultured MDBK cells using mAbs S-11D9 (A), S-10C7 (B), and C-4E5 (C). mAbs S-11D9 and S-10C7 react with a variety of vesicles, while C-4E5 presumably reacts exclusively with CCVs. Cells were fixed with 3.7% paraformaldehyde and treated with 0.1% Triton X-100 before being incubated with primary antibody. Rhodamine-conjugated rabbit anti-mouse IgG second antibody was used. Total magnification: 800X.


A
B
C

Discussion: Clathrin-coated vesicles probably participate in several diverse events associated with membrane sorting and transport. At least four criteria may be used to generate subpopulations of CCVs: function, morphology, polypeptide content and intracellular location. The first criterium was used successful by Hemly et al. (1986) to separate endocytic from secretory CCVs based on the source of their cholinesterase cargo. The second was used by Kedersha et al. (1986) to resolve several distinct subpopulations of CCVs differing primarily in surface surface. The third was originally used by Pfeffer and Kelly (1985) to immunoprecipitate a subpopulation of brain CCVs carrying synaptic vesicle cargo molecules. In this report, CCV subpopulations are also resolved on the basis of polypeptide content by immunoprecipitation with mAbs. Three classes of antigen are used: cargo molecules, constitutive CCV polypeptides, and cytoskeletal elements. By immunoprecipitating CCVs with mAbs reactive with cytoskeletal elements, subpopulations are generated that originate from distinct intracellular locations.

Immunoprecipitation of brain CCVs with anti-actin mAb A-7C11 resolved two subpopulations: actin$^+$ and actin$^-$ CCVs. Two other mAbs, S-11D9 and S-10C7, identify cargo molecules that are preferentially sorted into the actin$^+$

and actin$^-$ subpopulations, respectively. Since nerve endings and cell processes generally contain relatively high concentrations of actin (DeLorenzo and Freedman, 1978), it is clearly possible that actin$^+$ CCVs are derived from these cellular domains. On the other hand, the perinuclear cytoplasm contains relatively little actin, and it is reasonable to assume that actin$^-$ CCVs originate from this domain. Actin$^+$ CCVs should therefore consist primarily of endocytic vesicles, while actin$^-$ CCVs should consist of secretory vesicles. Kedersha et al. (1986) recently reported that endocytic CCVs contain an unidentified acidic 43 kD polypeptide not present in exocytic CCVs. The results reported here imply that this polypeptide is actin. Whether actin acts functionally in the actin$^+$ subpopulation or merely adheres to these vesicles preferentially during the extraction prodcedure remains to determined.

Immunoprecipitation experiments using mAbs against the upper and lower CAP have revealed subpopulations of CCVs that are enriched in either the upper or lower CAP. The significance of these results is corroborated by immunofluorescence experiments using CAP_1 and CAP_2 specific mAbs and human cardiac myoblasts. During the synthetic phase of the growth cycle of these cells two distinct types of vesicles are clearly present: large granular perinuclear

vesicles and smaller peripheral vesicles. Both CAP_1 and CAP_2 are present in the larger vesicles while only CAP_2 is present in the smaller ones. Together, these data imply that CAPs may be actively sorted between CCVs and CCGs in some cell types. In addition, actin$^+$ CCVs precipitated by either mAb S-11D9 or A-7C11 appear to have less CAPs than other CCVs. The functional significance of this distribution and the structural alterations which result in diminished radioiodination of CAPs in actin$^+$ CCVs are presently being investigated. These observations may reflect a mechanism by which the stability of these vesicles is enhanced and their sensitivity to the uncoating protein (Schmid et al., 1984) is reduced.

XIII. Post-translational Modifications and the Intracellular Distribution of a Clathrin-coated vesicle Cargo Molecule

Introduction: In eukaryotic cells, membrane-bound and secretory proteins are synthesized and inserted into the rough endoplasmic reticulum (RER), modified by the Golgi apparatus, and either transported to the appropriate membrane organelle or secreted (Palade, 1975). After synthesis, membrane receptors can be recycled through either endosomes or the Golgi apparatus (Helenius et al., 1983; Ciechanover et al., 1983) or they can accompany their ligands to the lysosome (Hanover et al., 1984). Double labelling experiments using ligands specific for receptors with different intracellular routes of transport have indicated that the transport processes are highly organized, and that membrane polypeptides are directed through their intracellular routes in a regulated manner (Hanover et al., op cit.).

The structural elements targeting membrane polypeptides to specific transport pathways are either encoded in the primary sequence of the protein or added post-translationally. Site-directed mutational analysis has begun to decipher some of the primary sequence elements

that are responsible for targeting proteins to specific intracellular locations (for example, Prywes et al., 1986). In addition, the role of carbohydrate modification in directing intracellular protein transport is currently being investigated (Guan et al., 1985; Matlin and Simons, 1983).

In this report, two monoclonal antibodies are presented that react with a membrane antigen which undergoes a series of post-translational modifications. Each modification appears to target the protein to a specific intracellular location. The modifications vary with cell and tissue type, apparently to mediate the transport of this antigen to specialized membrane organelles. The data imply that a hierarchy exists in targeting signals, since proteolysis or carbohydrate cleavage target the antigen to new intracellular locations despite the lack of any additional signals. The data also indicate that the generation of specialized membrane organelles during differentiation may be accompanied by the generation of specialized modification processes that target proteins to these organelles.

Subcellular membrane distribution of SA_1, SA_2, and SA_3: Monoclonal antibodies S-11D9 and XB26 were generated from two different fusions of spleen cells from Balb/C mice

immunized with CCV membranes and Sp2/0 Ag14 myeloma cells. Initial screening of the original clones and subsequent subclones was performed with an ELISA using CCV membranes as antigen. Positive clones were further analyzed by immunoblotting of SDS-PAGE resolved CCV protins. Both mAbs S-11D9 and XB26 reacted with a CCV membrane polypeptide of approximately 38 to 44 kD molecular mass (SA_2; Figure 21). This antigen could readily be extracted from the CCV by .5% Triton X-100 but not 1M NaCl (data not shown). Previous work (herewithin, Figure 18) showed that a subpopulation of radioiodinated CCVs can be immunoprecipitated by mAb S-11D9, indicating that the antigen is not present only in contaminating smooth membranes. Since the subpopulation of CCVs containing SA_2 were actin$^+$, SA_2 is acquired by CCVs through the endocytic pathway.

The next experiments were designed to map the intracellular distribution of SA_2. Preparations of bovine brain synaptic plasma membrane (SPM), synaptic vesicles (SV), and two different preparations of Golgi membranes were resolved by SDS-PAGE and analyzed immunoblotting with mAb S-11D9 (preparations and enzyme profiles are given in the methods section). Two additional polypeptides were revealed by this analysis: SA_1, with an apparent molecular mass of 56 kD; and SA_3, with an apparent molecular mass of

Figure 21. Analysis of the distribution of SA proteins on fractionated intracellular membranes. Membrane or vesicle fractions were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with mAb S-11D9. Key: SPM - synaptic plasma membrane (100 ug); CPM - cardiac plasma membrane (100 ug); SV - synaptic veicles (100 ug); CCV - clathrin coated vesicles (50 ug), NCM - serum free culture medium of neuroblastoma cells (SH-SY5Y) grown to high density (10 ug); Golgi 1 and Golgi 2 - two preparations of bovine brain Golgi membranes (50 ug); Endo F - endoglycosidase F-treated synaptic plasma membranes (25 ug).

SA_1^*: endoglycosidase F product of SA_1

SA_3^*: endoglycosidase F product of SA_2

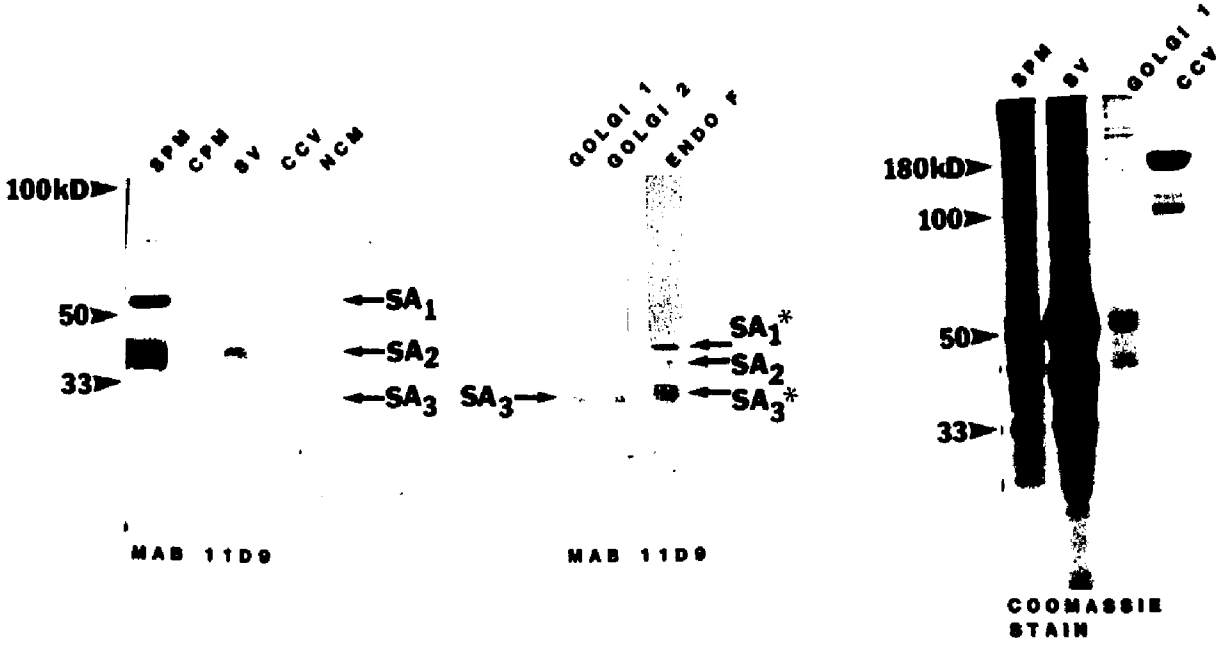

SPM
CPM
SV
CCV
NCM
100kD
50
33
SA1
SA2
SA3
MAB 11D9
GOLGI 1
GOLGI 2
ENDO F
SA3
SA1*
SA2
SA3*
MAB 11D9
SPM
SV
GOLGI 1
CCV
180kD
100
50
33
COOMASSIE
STAIN

Table 4. Relative Abundance of SA Antigens in Different Subcellular Membranes

	Golgi	SPM	CCVs	SVs
SA_1	-	+++	-	-
SA_2	-	++	+	+++
SA_3	+++	-	-	-

Key:

SPM - Synaptic plasma membrane

CCVs - Clathrin coated vesicles

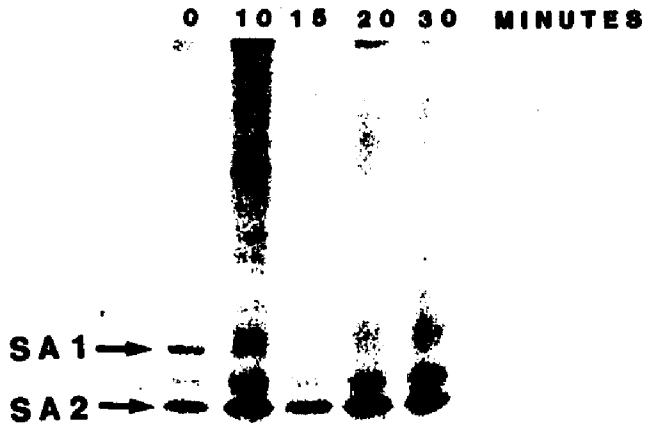
SVs - Synaptic vesicles

30 kD. SA_3 is only faintly detectable in SPM and is not detectable in SV or CCV preparations (Figure 21). SA_3 is most prevalent in Golgi membrane fractions. SA_1 is not detected in SV, CCV, or Golgi fractions, but is prevalent in SPM. SA_2 is not detected in Golgi fractions, is detectable in CCV fractions, and is prevalent in SPM and SV fractions. The distribution of SA proteins among these fractions is summarized in Table 4.

Biosynthetic relationships of SA_1, SA_2, and SA_3: A series of experiments was performed to determine whether the SA proteins were three form of the same antigen or two to three distinct antigens with common epitopes. First, endoglycosidase F digestion of SPM (Figure 21) resulted in the generation of one new band which co-migrated with SA_3, and another which migrated slightly above SA_2. SA_1 was depleted by this treatment, as was most of SA_2. These data indicate that SA_3 may represent a less glycosylated form of SA_2. SA_2, however, is smaller than endoglycosidase F-digested SA_1.

A series of pulse-chase experiments using ^{35}S-methionine was performed with nerve growth factor (NGF)-differentiated PC12 cells (which express large amounts of SA_1 and SA_2, Figure 28). At zero time (15 minute pulse), both SA_1 and SA_2 were detected in the immunoprecipitates

Figure 22. Pulse-chase anlysis of SA_1 and SA_2 biosynthesis. PC12 cells were cultured with nerve growth factor for 10 days. Subsequently, they were incubated with methionine-free medium for 15 minutes, then with methionine-free medium containing 0.25 mCi/ml ^{35}S-methionine for 15 minutes. This is time 0. Cells were then pulsed by incubation in medium containing 1 mM methionine. Immunoprecipitations were performed with mAb S-11D9. Gel was dried under vacuum, and autofluorographed with EnHance (New England Nuclear).


0 10 15 20 30 MINUTES
SA 1
SA 2

(mAb S-11D9) of cell lysates (Figure 22). SA_1 began to disappear five minutes after chasing with cold methionine, and a slight enhancement of SA_2 was observed. SA_1 disappeared almost completely 30 minutes after the addition of cold methionine. These data indicate that SA_1 may be a precursor of SA_2.

In vitro translation of HL60 cell mRNA using a rabbit reticulocyte lysate produced only one polypeptide (48 kD) that was specifically immunoprecipitated by S-11D9 (Figure 23). The molecular mass of this polypeptide is virtually identical to the mass of endoglycosidase F-digested SA_1. This polypeptide apparently represents pre-SA_1, which should be unglycosylated and may contain a leader sequence. Curiously, the addition of canine microsomal membranes (Amersham) had no discernable effect on the apparent mass of the translation product, and only appeared to reduce the efficiency of its translation. Therefore, in order to confirm that SA_1 and SA_2 are transmembrane polypeptides, HL60 cells were surface radioiodinated with lactoperoxidase, lysed, and analyzed by immunoprecipitation with mAb S-11D9. As shown in Figure 24, a prominant band is detected at 56 kD (SA_1) and a less prominant one is detected near 40 kD (SA_2). The relative intensities of these bands corresponds well to the intensities of SA_1 and SA_2 bands in immunoblots of SPM (Figure 21). As noted

Figure 23. Developmental expression of SA proteins during differentiation of HL60 cells. A. Immunoblotting analysis with mAb S-11D9. Cell homogenates were resolved by SDS-PAGE and immunoblotted 0, 1, 3, 5, and 7 days after the addition of DMSO.

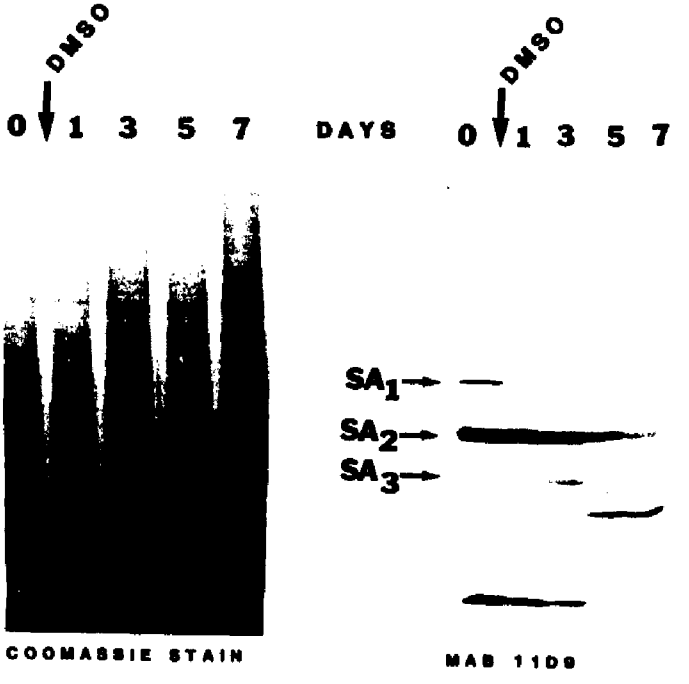

DMSO
0 1 3 5 7
DAYS
DMSO
0 1 3 5 7
SA1
SA2
SA3
COOMASSIE STAIN
MAB 11D9

Figure 23. Developmental expression of SA proteins during differentiation of HL60 cells. B. Reticulocyte lysate translation of total mRNA from differentiating HL60 cells. Total mRNA was purified from HL60 cells 0, 1, 3, 5, and 7 days after the addition of DMSO. Translated proteins were incubated with or without mAb S-11D9 and precipitated by rabbit anti-mouse IgM coupled to Sepharose 4B.

a. Translation mix without added mRNA

b. Translation mix without added mRNA, with microsomal membranes

c. Translation mix with 0 day HL60 mRNA

d. Translation mix with 0 day HL60 mRNA, with microsomal membranes added co-translationally

e. Translation mix with 0 day HL60 mRNA, with microsomal membranes added post-translationally

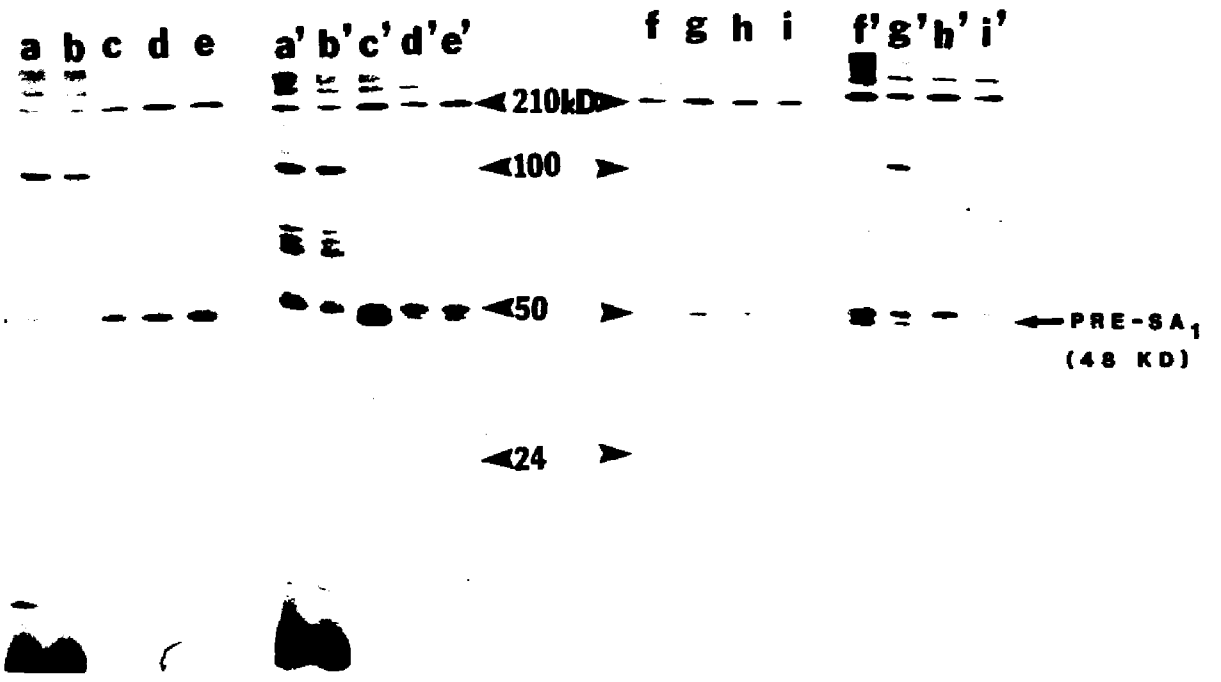
f. Translation mix with 1 day HL60 mRNA

g. Translation mix with 3 day HL60 mRNA

h. Translation mix with 5 day HL60 mRNA

i. Translation mix with 7 day HL60 mRNA

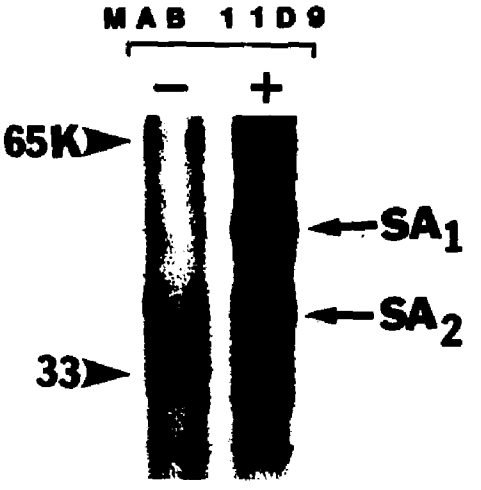
a-i: no mAb S-11D9 added; a'-i': mAb S-11D9 added.


a b c d e a' b' c' d' e' f g h i f' g' h' i'
210kD
100
50
24
PRE-SA1
(48 KD)

above, mAb S-11D9 can be used to immunoprecipitate CCVs, indicating that SA_2 has a domain on the cytoplasmic face of the membrane as well. Since the epitope reactive with S-11D9 apparently lies on this domain, it is reasonable to conclude that SA_1, SA_2, and SA_3 share similar cytoplasmic domains.

HL60 cells differentiate through the myelocytic lineage when cultured in the presence of 1.5% Dimethyl sulfoxide (DMSO). This process alters considerably the distribution and molecular masses of the SA proteins. This process can be observed by immunoblotting whole cell extracts at different times during the course of differentiation (Figure 21). SA_1 disappears as soon as three days after the addition of DMSO, and SA_2 steadily diminishes over a seven day period. SA_3 accumulates and peaks at day 3, then diminishes. A novel form of SA protein steadily accumulates at 26 kD. Another form at 10 kD steadily diminishes. When mRNA is extracted from equivalent time points during the differentiation process, no novel products are translated by the erythrocyte lysates, although the signal for pre-SA_1 steadily decreases (Figure 23). These data contrast the results obtained by immunoblotting cell extracts with mAb S-11D9. The variety of SA forms observed during HL60 cell differentiation must therefore be due to post-translational modification of the

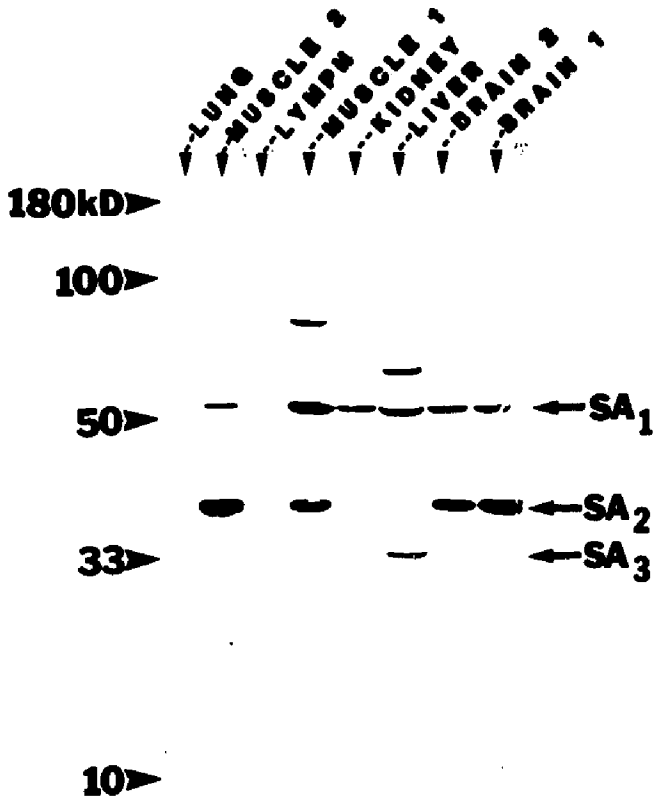
Figure 24. Lactoperoxidase surface radioiodination of HL60 cells. After surface radioiodination, cells were lysed with Triton and immunoprecipitated with mAb S-11D9.


MAB 11D9
−
+
65K
33
SA1
SA2

single 48 kD *in vitro* translation product (putatively pre-SA_1). This is most conspicuous in the case of SA_2, which is the most abundant form present in HL60 cells at any phase of the differentiation process, but lacks a transcript that can be detected by the erythrocyte lysates.

Distribution of SA proteins in different tissues and differentiating cells in culture: Cytodifferentiation and histogenesis often results in the generation cell type-specific pathways of membrane transport. This may include the generation of new membrane organelles, such as synaptic vesicles in neurons, transcytotic vesicles in hepatocytes, or secretory granules in endocrine cells and cells of hematopoetic origin. To determine the quantitative distribution of SA_1, SA_2, and SA_3 in different tissues, several murine tissue samples were homogenized into Laemmli sample buffer containing 100 mM dithiothreital, resolved by SDS-PAGE, and immunoblotted with mAb S-11D9 (Figure 25). Some tissues, such as lung, lymph node and pancreas (not shown), do not contain detectable SA proteins. The observation that pancreas does not produce this polypeptide and that the canine microsomal membranes (which are derived from pancreas) partially inhibit translation of SA transcripts may be significant. Brain tissue and skeletal muscle contain (in order of intensity) SA_2 and SA_1.

Figure 25. Distribution of SA proteins in various tissues. Tissue samples were homogenized in sample buffer, resolved by SDS-PAGE, and immunoblotted with mAb S-11D9. 200 ug total protein was loaded into each lane. Muscle 2 is skeletal, while muscle 1 is cardiac. Brain 1 is frontal cortex, while brain 2 is cerebellum.


LUNG
MUSCLE 2
LYMPH
MUSCLE 1
KIDNEY
LIVER
BRAIN 2
BRAIN 1
180kD
100
50
33
10
SA1
SA2
SA3

Cardiac muscle contains SA_1 and SA_2 and an unidentified 76 kD crossreactive polypetide. Liver contains SA_1 but not SA_2. It does contain SA_3 and another reactive polypeptide of 65 kD molecular mass. The variety of SA proteins observed in these tissues may correlate directly to their participation in different membrane transport systems.

Further examination of muscle tissue by immunofluorescence microscopy revealed that SA proteins are not produced by the muscle cells themselves but rather by the vascular endothelial cells (Figure 26). This observation correlates well with results obtained using cultured C_2 myoblast (C_2M) cells. C_2M cells were grown in culture medium containing 20% fetal bovine serum and 0.5% chick embryo extracts, then induced to differentiate in medium containing 4% horse serum. Differentiating C_2M cells were dissolved in Laemmli sample buffer 0, 1, and 3 days after changing to horse serum (Figure 27). As is shown by immunoblotting with mAb S-11D9, SA protein expression is eliminated during myogenesis.

In contrast to both HL60 cell and C_2M differentiation, PC12 differentiation, induced by NGF, enhances the expression of both SA_1 and SA_2 (Figure 28). An accumulation of SA_2 is observed as soon as 6 hours after the addition of NGF, and continues for 7 days (the longest time tested). An accumulation of SA_1 is first detectable

Figure 26. Immunofluorescence microscopy of a human heart section using mAb S-11D9. Top: immunofluorescence microscopy (fluorescein-conjugated second antibody); bottom: phase contrast microscopy. Brightly staining region is a capillary. This section was derived from formaldehyde-fixed autopsy material. Total magnification, 900 X.

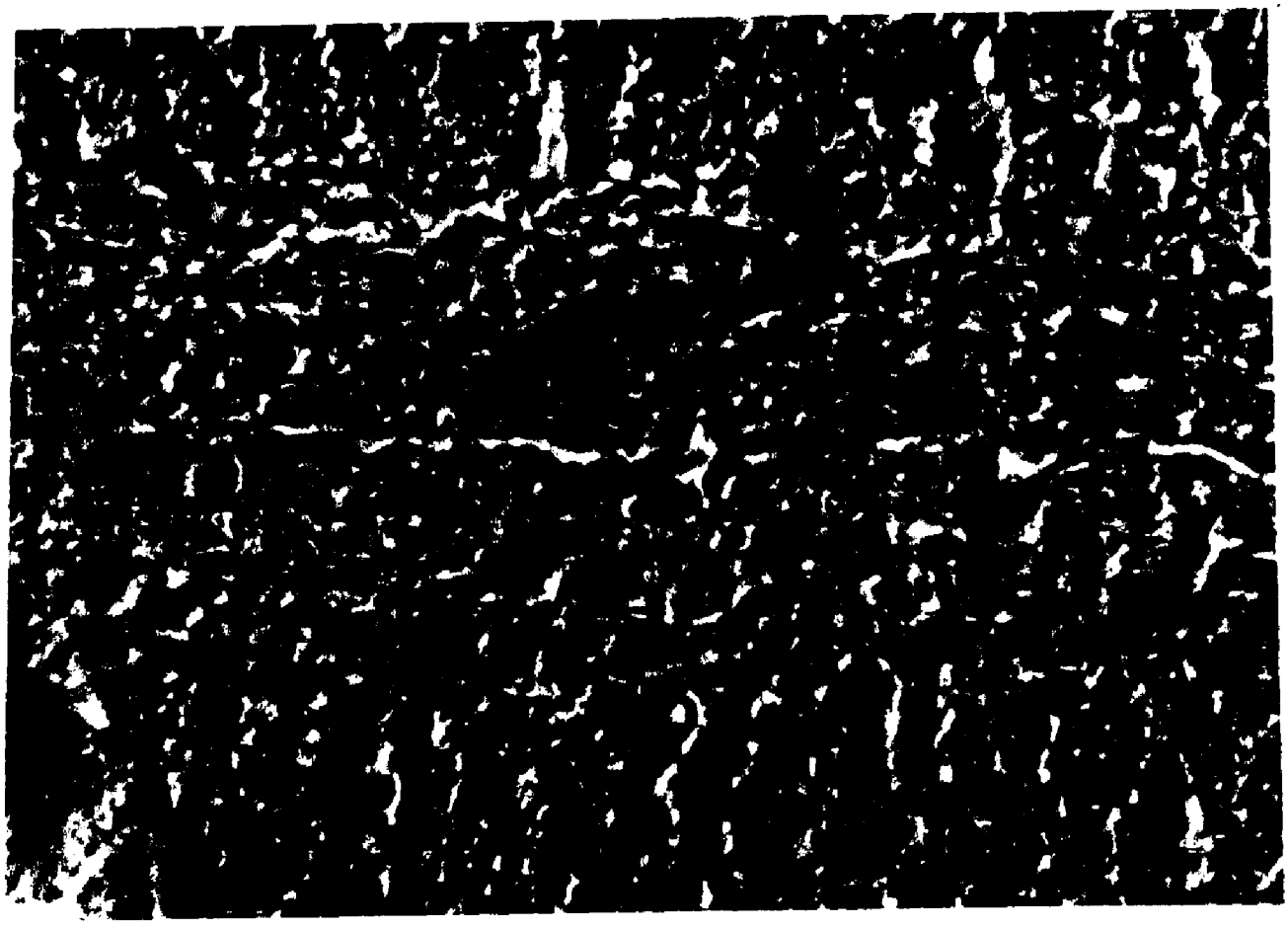
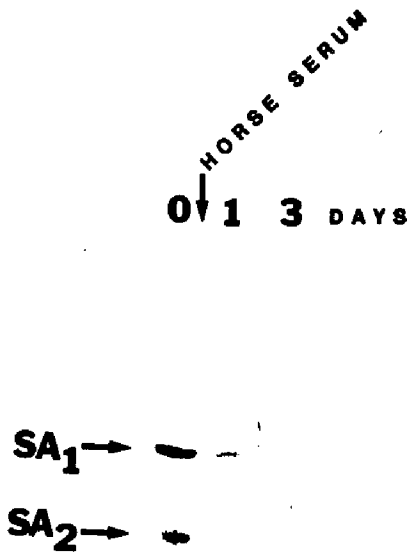


Figure 27. Loss of SA protein expression during C_2 myoblast differentiation. C_2 myoblasts were induced to differentiate in culture medium containing horse serum, homogenized, and analyzed by immunoblotting with mAb S-11D9. 100 ug total protein was loaded into each lane.


HORSE SERUM
0 1 3 DAYS
SA1
SA2

after 24 hours. The reversibility of these changes decreases with time. When NGF is removed after 15 hours, the reduction of SA_2 to basal levels occurs in approximately 15 hours; when NGF is removed after 7 days, the reversal takes several days (Figure 28). Immunoflurescence (using mAb S-11D9) microscopy of PC12 cells diffentiated by NGF for 3 days reveals intense areas of reactivity at the tips of developing neurites. This correlates well with the accumulation of synaptic vesicles containing SA_2 and the accumulation of SA_1 at the synaptic plasma membrane.

Discussion: A pathway resulting in the generation of three forms of SA protein can be deduced from the data presented in this report. Pre-SA_1 is translated as a 48 kD polypeptide and inserted (perhaps co-translationally) into the membrane of the rough endoplamic reticulume. Pre-SA_1 is core glycosylated, transferred to the Golgi and modified further, then transported to the cell surface where it first appears as SA_1. SA_1 is proteolytically cleaved at the plasma membrane prior to or during internalization by CCVs or coated pits. The molecular mass shifts induced by endoglycosidase F digestion of SA_1 and SA_2 are roughly equivalent; therefore, the carbohydrate content of the two forms should be roughly equivalent. The proteolytic

Figure 28. Enhanced expression of SA proteins during nerve growth factor-induced differentiation of PC12 cells. PC12 cells were induced to differentiate by nerve growth factor for the indicated lengths of time. After 15 hours and 7 days, nerve growth factor was removed from some of the cells (-NGF). SA protein expression was assessed by immunoblotting with mAb S-11D9.

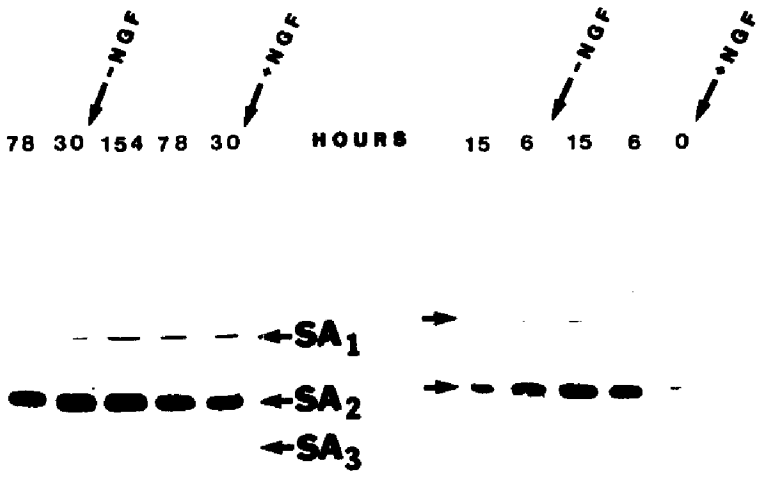
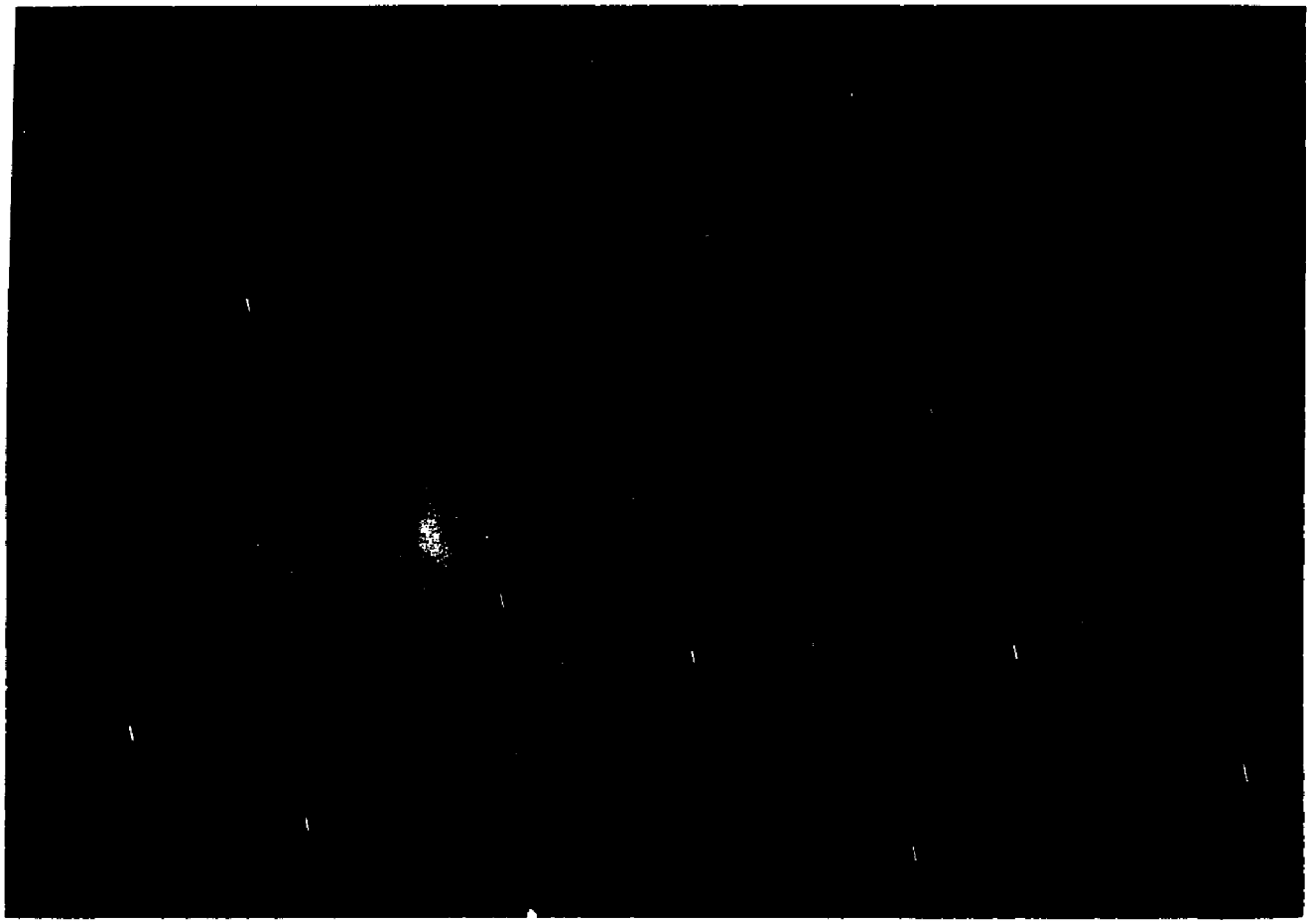

-NGF
+NGF
-NGF
+NGF
78 30 154 78 30
HOURS
15 6 15 6 0
SA1
SA2
SA3

Figure 29. Immunofluorescence microscopy of PC12 cells cultured three days in the presence of nerve growth factor. Cells were grown on coverslips, fixed with paraformaldehyde, treated with Triton, and reacted with mAb S-11D9 (first antibody) and fluorescein-conjugated rabbit anti-mouse IgM (second antibody). Total magnification, 900 X.



cleavage resulting in the generation of SA_2 from SA_1 apparently does not remove any carbohydrate moities, and thus may occur on the cytoplasmic face of the membrane.

As is evidenced by endoglycosidase F digestion of SA_2 *in vitro*, cleavage of the carbohydrate moities from SA_2 may generate SA_3 *in vivo*. Alternatively, SA_3 may be generated from protelytic cleavage of an unglycoslylated form of SA_1. This possibility is supported by the presence of SA_1 and SA_3 in liver in the absence of detectable SA_2. Presumably, SA_3 is cycled to the Golgi where it accumulates prior to being moved to the lysosome for degradation. Alternatively, SA_3 may be reglycosylated to generate SA_2 or an unidentified form of SA protein. The recycling and resialylation of membrane polypeptides has been previously noted (Snider and Rogers, 1985).

One question raised by this scheme is the source of SA_2 in vesicles without obvious endocytic origins, such as synaptic vesicles. One possible source is reglycosylated SA_3, which would be generated from the Golgi and transferred to the synaptic vesicles by the same pathway as their other cargo molecules. There is no direct evidence, however, that SA_2 can be generated from SA_3. An alternative explanation would require that the generation of SA_2 from SA_3 occur in the trans aspect of the Golgi. Recent evidence from Tooze and Tooze (1986) indicates that

some cargo molecules are transported to secretory vesicles in AtT20 cells via CCVs at the trans aspect of the Golgi. On the other hand, SA_2 was observed in the subpopulation of actin$^+$ CCVs (surface vesicles, putatively of endocytic origin); thus, conversion of SA_1 to SA_2 in CCVs occurs in an endocytic pathway. The generation of SA_2 from SA_1 in a secretory pathway may therefore occur in a non-coated compartment.

The presence of novel forms of SA protein in tissues such as liver and in differentiating cultured cells implies that the maintenance of unique secretory and transport organelles requires the generation of novel modification mechanisms to target polypeptides to these organelles. Hepatocytes, for example, are polarized cells that mediate the trancytotic processing of IgA. In this process, molecules are endocytosed on the basolateral side and transferred across the cell to be secreted on the apical side. The uniquely modified SA proteins present in liver may be adapted to allow their participation in this process, or to exclude them from it. The actual polarity of the cells is probably not solely responsible for the alterations observed, since other types of polarized cells (epithelial cells, for example) accumulate SA_1 and SA_2. Further work should reveal the significance of each form of SA protein and the organelles to which they are targeted.

Finally, a heirarchy is apparent in the structural elements which target the SA proteins to various intracellular membranes. SA_1 is not found in Golgi or vesicle membranes, yet it putatively contains all the structural elements present in SA_2 and SA_3. Thus, the "plasma membrane" signal contained by SA_1 dominates over the "vesicle" or "Golgi" signals. Similarly, SA_2 is not found in the Golgi, yet it putatively contains all the structural elements present in SA_3. Thus, the "vesicle" signal of SA_2 dominates over the "Golgi" signal. The signal for transport to the Golgi appears to be only a passive one, resulting from the lack of elements signaling transport to the plasma membrane or to vesicles. The accumulation of incomplete membrane glycoproteins in the Golgi has been observed before by Guan et al (1985), and may reflect a ubiquitous passive mechanism of targeting proteins to the Golgi during synthesis and receptor recycling.

XIV. Characterization of a unique structural protein in neuronal clathrin-coated vesicles

Introduction: Clathrin-coated vesicles are the vehicles of several significant endocytic and secretory processes (reviewed by Fine and Ockleford, 1984; Wileman et al., 1985). Site-directed mutagenesis experiments, however, indicate that the ability to synthesize clathrin is not essential to the survival of yeast (Payne and Schekman, 1985). Since yeast represent particularly simple organisms, these data imply that CCVs are most essential to metabolic processes associated exclusively with the survival of higher organisms. Thus, the evolutionary conservation of the CCV may be attributed to its versatility; that is, CCVs may provide a highly adaptable vesicle prototype which can be readily modified by phenotypically diverse cells to serve varied functions. The participation of CCVs in highly specialized processes such as synaptic membrane recovery in neurons (Heuser and Reese, 1973) and transcytosis in hepatocytes (Mostov and Simister, 1985) clearly supports this hypothesis.

One way CCVs could be adapted to the specific needs of different types of cells is by genetic or post-translation modification of the structure of clathrin.

Both biochemical and immunochemical studies have indicated so far that clathrin is highly conserved between different tissues and species (Croze et al., 1982; Rothman et al., 1980). Still, this possibility can be conclusively evaluated only when sequence data for clathrin becomes available. Alternatively, modifications may occur in CCV accessory proteins. One possible example is the greater size of clathrin light chains derived from neuronal tissues compared to those derived from non-neuronal sources (Kirchhausen et al., 1983). No functional significance has been associated with this difference so far. Biochemical heterogeneity has also been noted in the composition of assembly polypeptides isolated from brain, and this has been associated with the geometry of the vesicles generated by each form (Pearse and Robinson, 1984). In addition, immunochemical studies have revealed diversity between the assembly polypeptides present in the perinuclear cisternae and those present near the plasma membrane (Robinson and Pearse, 1986).

Another way CCVs could be adapted to cell-type specific functions is by the integration of novel vesicle component. We present here what may be the first example of this type of modification. Neurons are highly polar cells with sophisticated mechanism for regulating secretion and membrane traffic. A new neuronal CCV component is

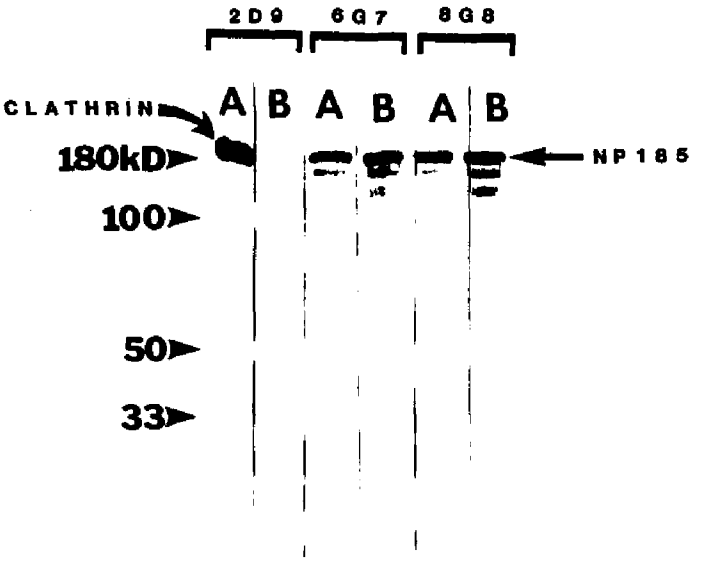
Figure 30. Two dimensional immunoblotting of clathrin-coated vescles with mAbs S-8G8 and 2D9. Radioiodinated CCVs (250,000 cpm) and unlabelled CCVs (100 ug) were resolved by two dimensional gel electrophoresis, and autoradiographed or immunoblotted with mAbs S-8G8 (anti-NP185) and 2D9 (anti-clathrin heavy chain). Labelled spots include: a and a*, NP185; b, 110 kD assembly polypeptide; c, tubulin; d, CAP_1; e, CAP_2. Each square gel represents a pH range of 6 (base) to 4 (acid). The interface between stacking and resolving SDS-PAGE gels is indicated (i).


SDS-PAGE
ACID
MAB 209
MAB 208
a
b
c
d
e
AUTORADIOGRAPHY
BASE
I E F
I
180kD
100
50
33

described here (NP185). This has not been observed previously because its mobility during SDS-PAGE is virtually identical to that of clathrin. In the course of developing mAbs to other CCV proteins, two hybridoma clones were selected that react specifically with NP185. Apart from its size, NP185 shares little functional or biochemical homology with clathrin. By virtue of two distinct sites for binding tubulin, NP185 appears to be involved in regulating the interaction of neuronal CCVs with the cytoskeleton. In addition, NP185 binds the CCV assembly complex polypeptides, indicating a possible role in regulating the sites and extent of CCV assembly.

NP185 is not clathrin: Monoclonal antibodies (S-8G8 and S-6G7) to NP185 were produced from the fusion of Sp2/0 Ag 14 myeloma cells to the spleen cells of a Balb/C mouse immunized with heat dennatured CCV proteins. Clones were initially screened with an ELISA using whole CCVs as antigen. Positive clones were subcloned by limiting dilution and subsequently screened by immunoblotting. Both S-8G8 and S-6G7 are IgG1, kappa, but each reacts with distinct sites on NP185 as determined by sandwich radioimmune assay using radioiodinated antibodies and NP185 extracted from CCVs (data not shown). Monoclonal antibody 2D9 (IgM, kappa) was generated from the fusion of Sp2/0

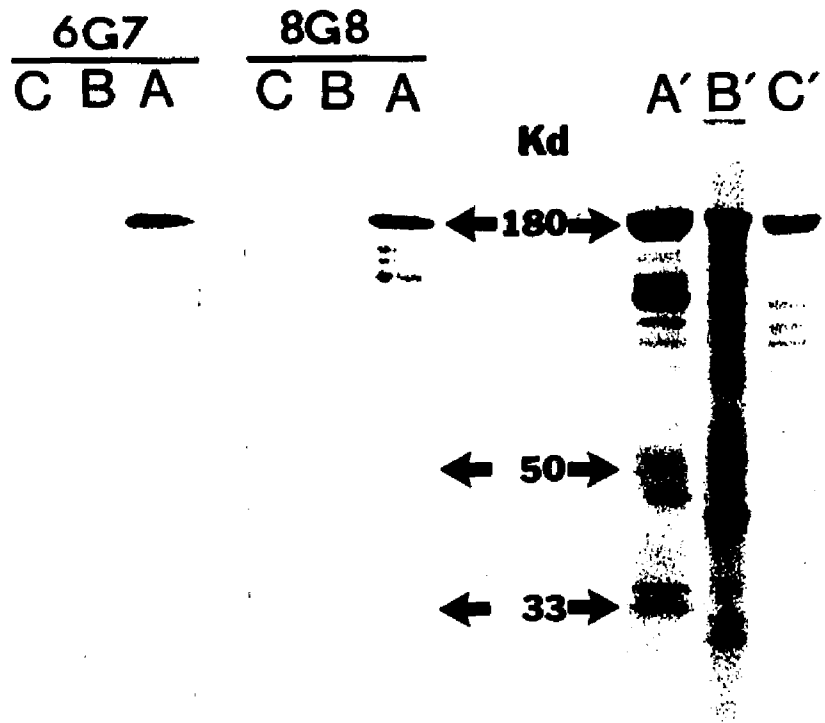
Figure 31. Immunoblotting of clathrin-coated vesicles with mAbs 2D9, S-6G7, and S-8G8 in the absence (A) or presence (B) of 100 ug/ml purified clathrin.


2D9
6G7
8G8
A
B
A
B
A
B
CLATHRIN
180kD
100
50
33
NP185

Ag14 myeloma cells with the spleen cells of a Balb/C mouse immunized with chymotryptically digested clathrin cages. This mAb reacts with the 180 kD clathrin heavy chain in purified preparations and whole cell extracts, and does not react with any other CCV proteins. Monoclonal antibody 2D9 reacts with clathrin derived from several tissues and species, as evidenced by immunoblotting of purified CCV preparations and immunofluoresence analysis of cultured cells (not shown). Formaldehyde fixation of samples adversely affects the reactivity of mAb 2D9, but has no affect on the reactivity of S-8G8 and S-6G7.

Monoclonal antibodies S-8G8 and S-6G7 react with an abundant CCV polypeptide (185 kD) that migrates with clathrin during SDS-PAGE. Immunofluorescence analysis of several fibroblast, epithelial, and myoblast cell lines from several different species revealed no obvious reactivity. On the other hand, intense punctate reactivity was observed in the processes of neuronally derived cell lines. This implies that the reactive antigen may be either a modified clathrin or another protein entirely. Resolution of the two polypeptides by two dimensional gel electrophoresis proved difficult, since clathrin could not be focussed (as has been observed previously, Kelly et al., 1983). When CCV proteins were resolved by two dimensional electrophoresis and immunoblotted with mAb S-8G8, a smeared

Figure 32. Immunoblot analysis of NP185 in bovine brain clathrin-coated vesicles (A), bovine adrenal clathrin coated vesicles (B), and chymotryptically digested brain clathrin-coated vesicles (C). 40 ug protein was loaded in each lane. A', B', C': Coomassie stain.

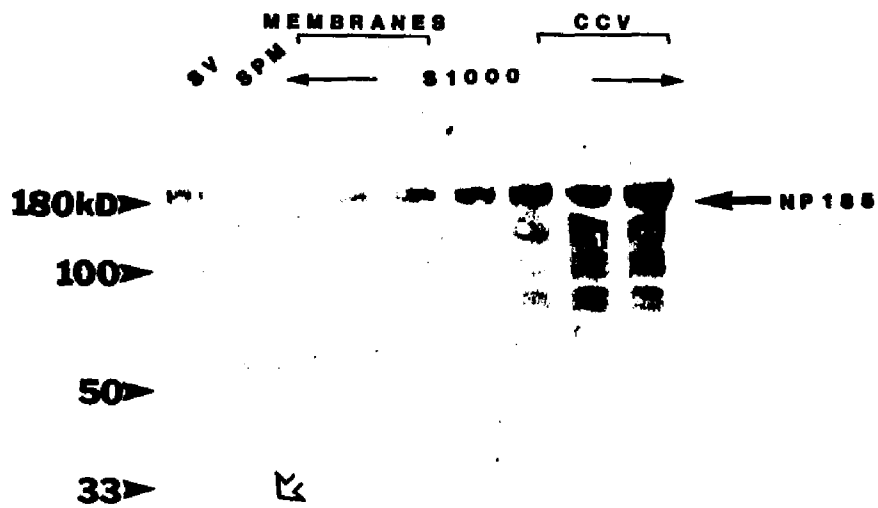

6G7
C B A
8G8
C B A
A′ B′ C′
Kd
180
50
33

spot was observed at pH 6.0. No reactivity was observed when an identical gel was immunoblotted with mAb 2D9 (Figure 30). Clathrin apparently did not even enter the isoelectric focussing gel, while NP185 focussed at a pH near 6.0.

Two other experiments were performed to distinguish NP185 from clathrin. In the first, clathrin was purified from CCVs by extraction with 0.5M Tris followed by Sepharose 4B chromatography, and one cycle of assembly-disassembly. These preparations contained no NP185 as assessed by immunblot (most of the NP185 remains membrane-bound during Tris extraction; not shown). Clathrin-coated vesicles were then resolved by SDS-PAGE, electroblotted to nitrocellulose and reacted with mAbs S-8G8, S-6G7, or anti-clathrin mAb 2D9 either in the presence or absence of 100 ug/ml purified clathrin. As shown in Figure 31, clathrin effectively competes out mAb 2D9 but not mAbs S-8G8 or S-6G7.

In the second experiment, CCVs were partially digested with chymotrypsin, then pelleted, and analyzed by immunoblotting with mAbs S-6G7 and S-8G8. This treatment has previously been shown to remove the clathrin light chains without damaging the clathrin heavy chain or coat structure (Lisanti et al., 1982). The digested pellets were analyzed by SDS-PAGE and Coomassie stain, which

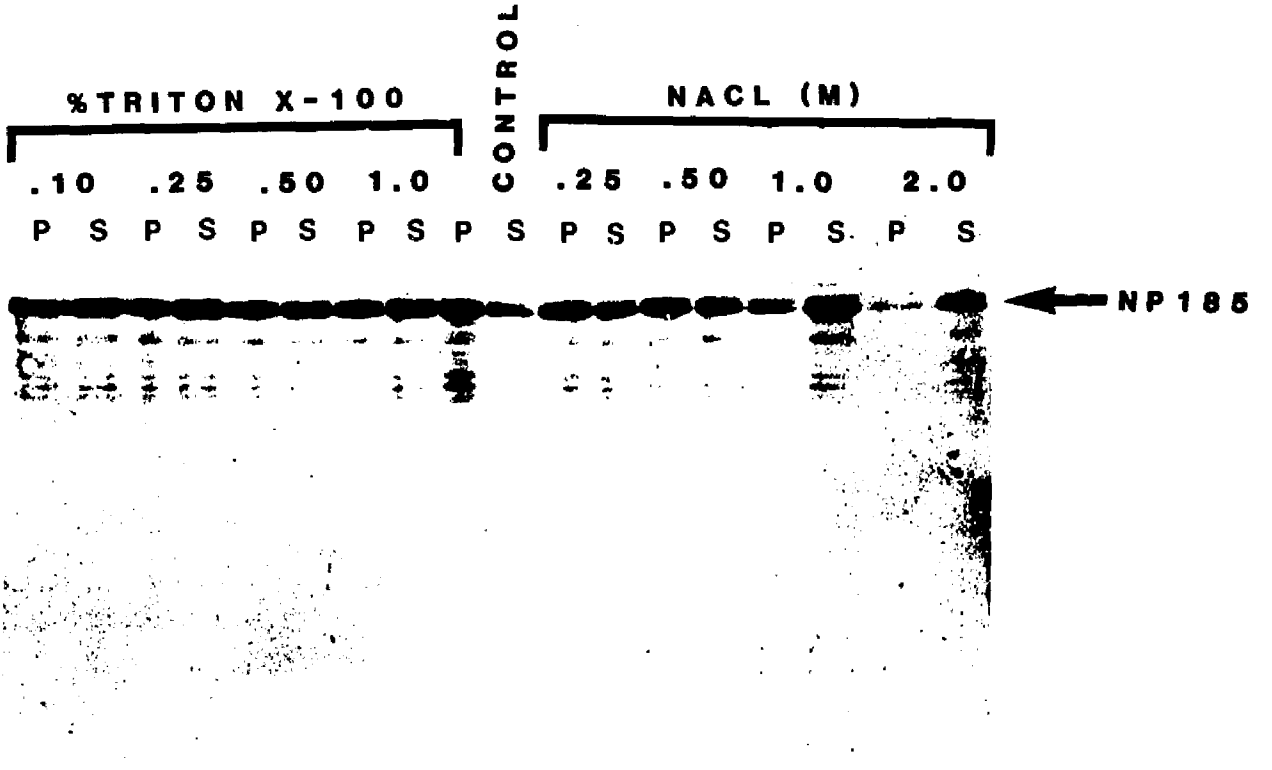
Figure 33. Immunoblot analysis of NP185 content in synaptic vesicles (SV), synaptic plasma membrane (SPM), and S-1000 column fractions. 50 ug protein was loaded in SV and SPM lanes. Equal volumes were loaded of column fractions. Open arrow indicates an unidentified band in the SPM fraction that also appears when no first antibody is used. Monoclonal antibody S-8G8 was used for this immunoblot.


MEMBRANES
CCV
S1000
180kD
100
50
33
NP165

revealed that several of the vesicle proteins were digested, including the light chains. Consistent with previous results, the clathrin heavy chain was unaltered. When the digested CCVs were immunoblotted with either S-8G8 or S-6G7, however, no reactivity was observed (Figure 32). NP185 is bound to the vesicle in a way that renders it exquisitely sensitive to chymotrypsin, and this clearly distinguishes it from clathrin. Also shown in Figure 32 is the near lack of NP185 reactivity observed in CCVs derived from adrenal gland. Some reactivity was occasionally observed, and this corresponded to an increased incidence of the higher molecular mass neuronal CAPs as well (not shown). This indicated that NP185 in adrenal gland is probably contributed by neuronal tissues.

NP185 is not a transmembrane protein: Clathrin-coated vesicles were prepared as described by Schook and Puzskin (1985). In the final step, the crude CCVs are chromatographed through an S-1000 column to separate them from uncoated membranes. To determine whether this procedure also enriches for NP185, consecutive column fractions were resolved by SDS-PAGE and immunoblotted with mAb S-8G8. As shown in Figure 32, NP185 is concentrated in the CCV peak fractions and nearly absent from the membrane peak. In agreement with this, a preparation of purified

Figure 34. Extraction of clathrin-coated vesicles with varying concentrations of Triton X-100 or sodium chloride. Extracted CCVs (50 ug) were spun at 100,000 xg, and pellets (P) and supernantants (S) were analyzed for NP185 content by immunoblotting with mAb S-8G8. Control lane represents unextracted CCVs.

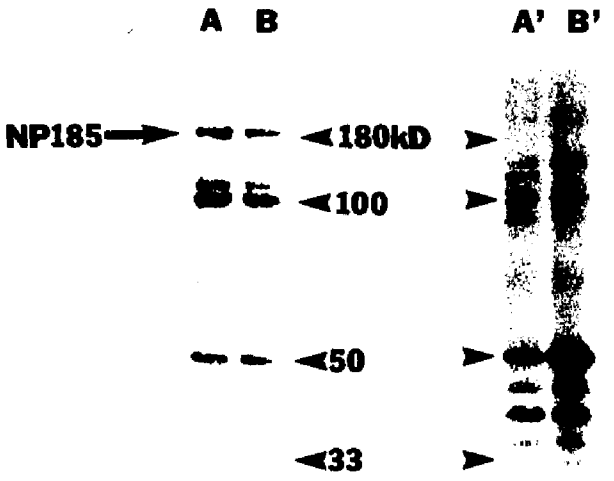

%TRITON X-100
CONTROL
NACL (M)
.10
.25
.50
1.0
.25
.50
1.0
2.0
P S P S P S P S P S P S P S P S P S
NP185

synaptic plasma membranes also contained no detectable NP185 (Figure 33). Synaptic vesicles, on the other hand, contain some NP185, but significantly less than CCVs. As will be shown below, NP185 can be extracted from CCVs, immunoprecipitated, and analyzed for NP185 content by Coomassie staining. Using such a preparation as a standard, immunoblotting analysis indicates that there are 10 to 15 NP185 molecules per CCV, and .5 to 1 per synaptic vesicle.

To determine whether NP185 is bound to the cytoplasmic face of the vesicle or if it is a transmembrane cargo molecule, differential extraction experiments using various concentrations of either a non-ionic detergent (Triton X-100) or sodium chloride were performed. As shown in Figure 34, concentrations of Triton between 0.1% and 1.0% did not efficiently extract all of the NP185 from CCVs. The NP185 that was extracted by Triton probably results from fragmentation of the CCVs. Concentrations of sodium chloride of 1M or greater efficiently extracted NP185 from the vesicle. These data indicate that NP185 is not a transmembrane cargo molecule, but is associated instead with the cytoplasmic face of the CCV.

NP185 forms a complex with a fraction of the assembly

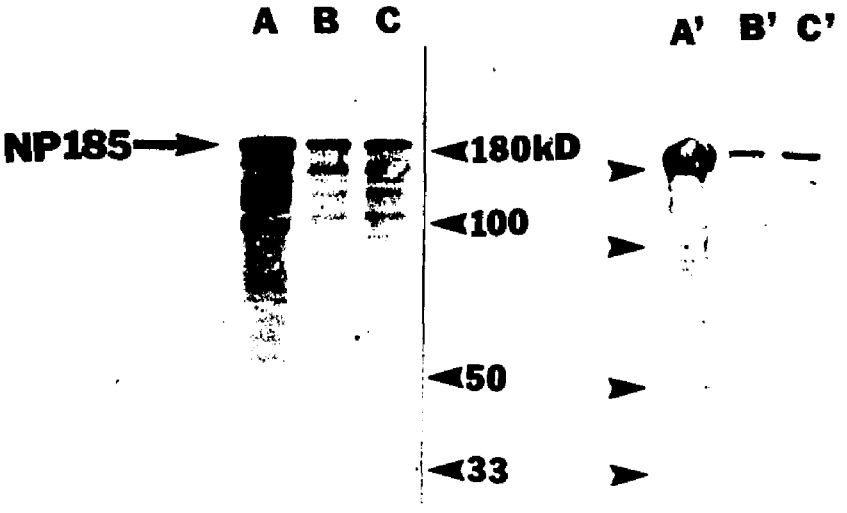
Figure 35. Presence of associated assembly complex polypeptides and kinase activity in immunoprecipitates of clathrin-coated vesicle derived NP185. NP185 was immunoprecipited from 0.9 M sodium chloride extracts of clathrin coated vesicles and incubated with gamma-^{32}P-labelled ATP. Precipitates were washed twice with .25M sodium chloride and once with 20mM HEPES pH 7.0 (A), or twice with 20 mM HEPES .25M sodium chloride pH 7.0 and once with 0.5 M Tris pH 7.0 (B). A and B: Coomassie stain; A' and B': autoradiography.


A
B
A'
B'
NP185
180kD
100
50
33

polypeptides: When NP185 is extracted from the CCV by .9M NaCl, diluted to .25M NaCl with Hepes pH 7.0, and immunoprecipitated by mAbs S-8G8 and S-6G7 covalently linked to Sepharose 4B, it appears as a complex with the 100 kD and 50 kD assembly complex polypeptides (Figure 35). The immunoprecipitations are performed in and washed twice with .25M NaCl; therefore, NP185 and the assembly polypeptides remain associated in solutions of higher than physiological ionic strength. The complex is also stable to washes by .5M Tris (Figure 35). The lower affinity of the antibody-antigen complex prohibits testing the stability of the NP185-assembly polypeptide complex at higher concentrations of NaCl; thus, it is not known whether the complex is removed directly from the vesicle or whether it forms after dilution.

NP185 was extracted from CCVs by 0.9M NaCl, immunoprecipitated by either mAb S-6G7 or S-8G8, and analyzed for NP185 or clathrin content by immunoblotting. As shown in Figure 36, NP185-assembly polypeptide complexes immunoprecipitated by either mAb S-8G8 or S-6G7 contain a small amount of tightly bound clathrin. This clathrin remains bound after two .25M NaCl washes, but is partly removed by 0.5M Tris (Figure 36). This result indicates that NP185, the assembly polypeptides, and a small amount of clathrin can form a trimolecular complex. Additional

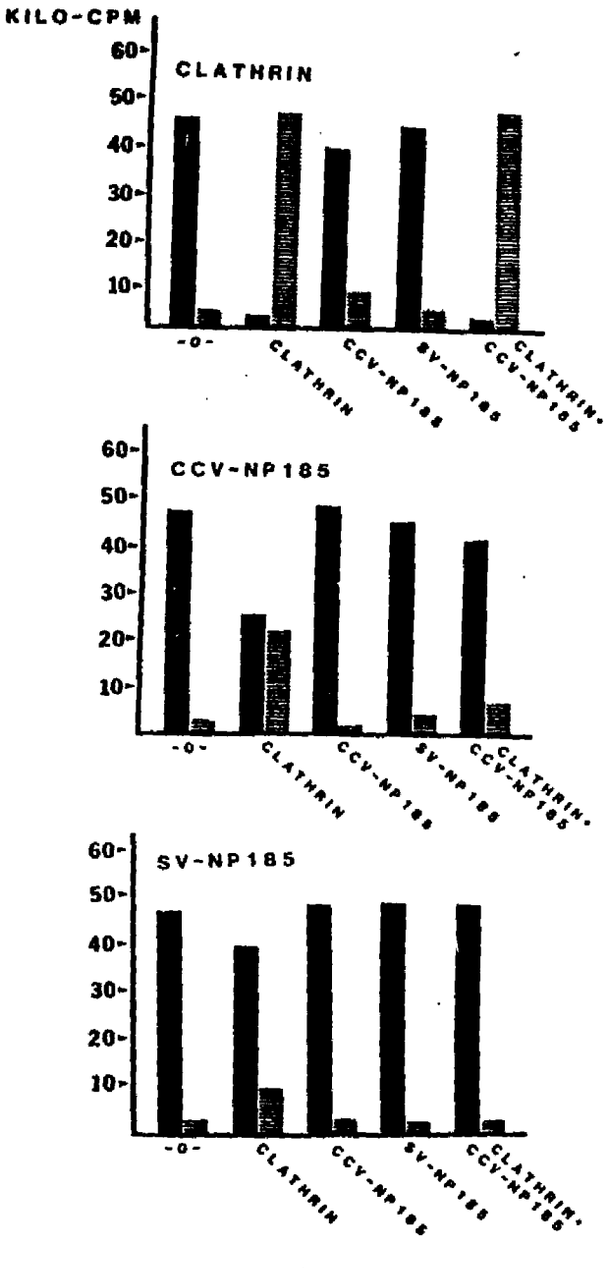
Figure 36. Analysis of the clathrin and NP185 content of NP185 immunoprecipitates by immunoblotting with mAb 2D9 (A, B, and C; anti-clathrin) and mAb S-8G8 (A', B', and C'). A: 50 ug clathrin coated vesicles; B and C: Immunoprecipitated NP185 derived from clathrin-coated vesicles. Precipitates were washed twice with 20 mM HEPES .25 M sodium chloride pH 7.0 (C), followed by a wash with 0.5 M Tris pH 7.0 (B).


A
B
C
A'
B'
C'
NP185
180kD
100
50
33

data will be presented below to show that this complex is composed of NP185 bound to assembly polypeptides, which bind the clathrin heavy chain molecules. As expected, NP185 devived from purified bovine synaptic vesicles (SV) by 0.9 M NaCl extraction did not contain either clathrin or assembly polypeptides, as determined by immunoblotting with mAb 2D9 or a rabbit anti-100 kD antiserum (kind gift of Dr. B. M. F. Pearse; not shown). The 100 kD assembly polypeptide in CCV-NP185 complexes reacted with this anti-serum as determined by both immunoblotting and ELISA (not shown).

A functional assay that distinguishes NP185 from clathrin: NP185 can be functionally distinguished from clathrin by the ability of clathrin to self-assemble into cages at pH 6.5 (Blitz et al., 1977; Keen et al., 1979). Clathrin cages were radioiodinated, isolated by centrifugation, and dissociated in 0.5M Tris. NP185 was extracted from CCVs and SVs by 0.9M NaCl, affinity purified by mAb S-8G8, and radioiodinated. The preparations were then assayed for reassembly by dialysis into 0.1M MES buffer, 2.5 mM $MgCl_2$ pH 6.5. After centrifugation at 100,000 xg assembled material is found in the pellet while unassembled material remains in the supernatant. Purified unlabelled clathrin (200 ug/ml) was added to some

Figure 37. Reassembly analysis of clathrin, clathrin-coated vesicle derived NP185 (CCV-NP185), and synaptic vesicle derived NP185 (SV-NP185). Clathrin, CCV-NP185, and SV-NP185 were radioiodinated, dialyzed into reassembly buffer, and pelleted at 100,000 xg. 50,000 cpm of starting material was used per assay, and both supernatants (black bars) and pellets (grey bars) were counted. Labelled samples were incubated alone (-0-), or with 200 ug/ml cold clathrin (clathrin), 200 ug/ml cold CCV-NP185 (CCV-NP185), 100 ug/ml cold SV-NP185 (SV-NP185), or 200 ug/ml cold clathrin and 200 ug/ml cold CCV-NP185 (clathrin-CCV-NP185).

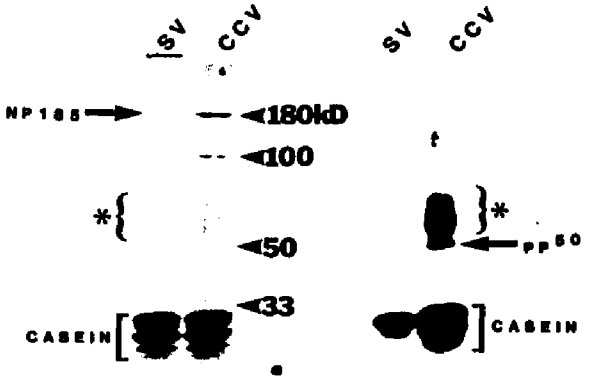

KILO-CPM
CLATHRIN
CCV-NP185
SV-NP185
60
50
40
30
20
10
-0-
CLATHRIN
CCV-NP185
SV-NP185
CLATHRIN+ CCV-NP185

samples. None of the samples reassembled spontaneously without the addition of unlabelled clathrin. This is undoubtedly attributable to the low concetrations of protein present (less than 1 ug/ml). The addition of unlabelled CCV-NP185 (200 ug/ml) or SV-NP185 (100 ug/ml) also did not induce detectable assembly. When unlabelled clathrin was added to the radiolabelled clathrin sample, however, assembly was observed, as is shown by the accumulation of label in the pellet (Figure 37). Much less incorporation of label into the pellet was observed with CCV-NP185 (NP-185 extracted from CCVs), and virtually none was detected with SV-185 (NP-185 extracted from synaptic vesicles). The incorporation of some CCV-NP185 into the pellets of those samples to which unlabelled clathrin had been added was probably due to the association of assembly polypeptides (bound to CCV-NP185) with the assembling clathrin. To test this hypothesis, an unlabelled 200 ug/ml CCV-NP185 was also added to the mixture. If CCV-NP185 is integrated into the assembling cage, then the addition of unlabelled CCV-NP185 should have no effect on the accumulation of label in the pellet. If, on the other hand, CCV-NP185 is only associated with the assembling cage by the assembly polypeptides, then the addition of unlabelled CCV-NP185 should act competitively and inhibit the accumulation of label in the pellet. As is shown in

Figure 37, the latter assumption was correct, and unlabelled CCV-NP185 had an inhibitory effect on the accumulation of label in the pellet. Since no incorporation of label into the pellet was observed when clathrin was added to SV-NP185, this binding must be due to the assembly polypeptides present on CCV-NP185. Although equivalent masses of CCV-NP185 and clathrin added to the reassembly mixture successfully inhibited labelled CCV-NP185 incorporation, the unlabelled CCV-NP185 was well below the molar equivalence of assembly complex binding sites (Robinson and Pearse, 1984). The binding of CCV-185-bound assembly polypeptides to cages must be restricted to either a select subset of these binding sites, or sterically limited to binding only a fraction of the sites in each cage.

Clathrin-coated vesicle NP185 contains kinase activities: The CCV assembly complex has an associated kinase activity which in brain phosphorylates the 50 kD polypeptide. This activity is independent of Ca^{2+}/calmodulin and cAMP (Pauloin and Jolles, 1984). The CCV-NP185-bound assembly polypeptides also contain this activity, although it represents only a fraction of the total activity in the vesicle (Figure 35 and Figure 44). Interestingly, a single 0.5M Tris wash of the

Figure 38. Casein kinase activity is associated with NP185 derived from synaptic vesicles (SV) and clathrin-coated vesicle (CCV). NP185 extracted from either source was immunoprecipitated by mAb S-8G8, and incubated in phosphorylation buffer with gamma-^{32}P-labelled ATP. 50 ug casein was added to each sample. The area marked by an asterisk is produced by contaminating polypeptides in the casein preparation.



COOMASSIE STAIN AUTORADIOGRAPHY

immunoprecipitated CCV-NP185 removed no bands that could be detected by Coomassie stain of the SDS-PAGE resolved proteins. Autoradiography of this gel, however, revealed that the 50 kD associated kinase activity was partly inhibited. Two explanations are possible: 1) the kinase activity is inhibited by Tris; or 2) the kinase actvity is dissociated by Tris. The latter possibility contrasts evidence from Keen and Black (1986) that this activity represents an autophosphorylation reaction.

Clathrin-coated vesicles have also been shown to contain an associated casein kinase II that, in the presence of polybasic compounds, phosphorylates CAP_2 (Schook and Puszkin, 1985; Bar-Zvi and Branton, 1986). This kinase activity can be extracted from the vesicle by 1M NaCl (similarly to NP185). In order to determine whether any casein kinase II activity is associated with NP185, 0.9M NaCl extracts were derived from CCVs and SVs, diluted 20 mM HEPES and immunoprecipitated with mAb S-8G8 covalently-coupled to Sepharose 4B. Polylysine was added to the immunoprecipitated material to stimulate the casein kinase, and casein was added as substrate. As shown in Figure 38, both CCV-NP185 and SV-NP185 have tightly complexed casein kinase II activity. SV-185 lacks the 50 kD assembly polypeptide kinase activity, indicating that neither the 100 kD nor the 50 kD subunits of the assembly

complex are present. The NP185-bound casein kinase II activity and the enzyme activity remaining in solution after extraction are analyzed further in a later section.

NP185 is bound to vesicle tubulin: NP185 is associated with both SVs and CCVs, yet is not an integral membrane polypeptide. Therefore, NP185 could be associated with another protein element of both vesicles. A possible characteristic of such a protein would be its presence in both types of vesicles. With this in mind, a competitive assay was devised to determine whether NP185 is attached to CCVs by an association with tubulin. Tubulin has been shown by Kelly et al. (1983) to be a major component of brain CCVs. CCVs were radioiodinated, then immunoprecipitated by mAb S-8G8 in the presence of various concentrations of purified clathrin, CAPs, or tubulin. As shown in Figure 39, clathrin and CAPs had no competitive effect on the efficiency of CCV immunoprecipitation by S-8G8. Tubulin, on the other hand, competitively inhibited CCV immunoprecipitation in a dose dependent manner. This occurred despite the absence of any detectable reactivity of mAb S-8G8 with purified tubulin preparations (not shown). Therefore, tubulin must competitively inhibit NP185 binding to the vesicle and not antibody binding to NP185. To confirm this interpretation, the

Figure 39. Competitive analysis of NP185-mediated immunoprecipitation of clathrin-coated vesicles. Radioiodinated clathrin-coated vesicles were immunoprecipitated by mAb S-8G8 in the presence of varying concentrations of clathrin, CAPs, and tubulin. 100,000 cpm of starting material was used for each experiment, and 1/10 of the pelleted material was counted.

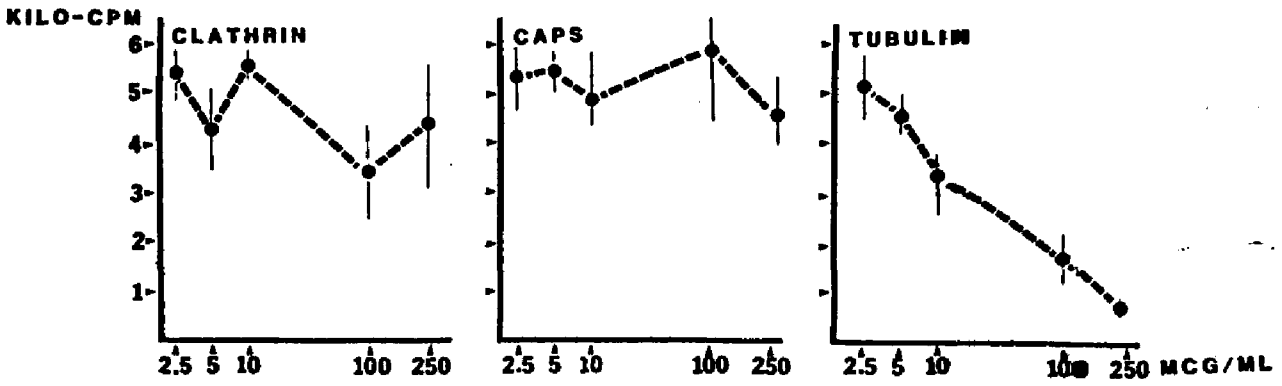
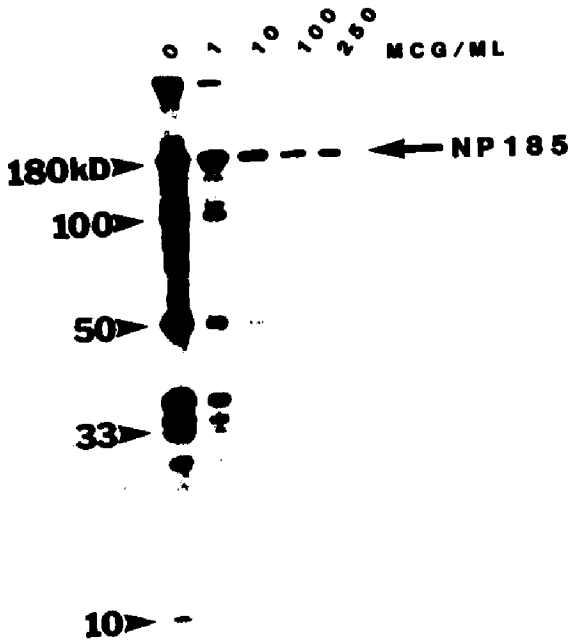

KILO-CPM
CLATHRIN
CAPS
TUBULIN
6
5
4
3
2
1
2.5 5 10 100 250
2.5 5 10 100 250
2.5 5 10 100 250
MCG/ML

Figure 40. Analysis by SDS-PAGE of the precipitates competed with tubulin in Figure 39. The remaining 90% of each precipitated sample was loaded into the lanes as indicated. This gel was exposed to film with a Cronex lightening plus intensifying screen for 16 hours.


0
1
10
100
250
MCG/ML
NP185
180kD
100
50
33
10

immunoprecipitates were analyzed by SDS-PAGE. If NP185 is being competed off the vesicle by tubulin, then it should remain attached to the antibody and be present in the precipitate. As is shown in Figure 40, NP185 does remain attached to the pellet, while the other CCVs proteins remain attached to the dissociated CCVs. This is most evident in the last concentration point, where only NP185 continues to be precipitated. The dissociated NP185 is precipitated as a complex with tubulin, although it is not labelled and therefore not detected in the autoradiograph. It is particularly noteworthy that no other CCV proteins are co-precipitated with NP185 in this experiment. In particular, the assembly complex proteins which co-precipitate with NP185 from 0.9M NaCl extracts are conspicuously absent from the tubulin-extracted NP185. This indicates that the assembly complex binding site on NP185 is competitively inhibited by tubulin.

In order to determine whether NP185 can bind both tubulin and assembly complex polypeptides simultaneously, anti-tubulin mAb T-2H9 was coupled to Sepharose 4B, incubated with various amounts of tubulin, spun out, then incubated with CCV-NP185. The coated beads were subsequently spun out, and the supernatants analyzed for the absorption of NP185 and assembly complex protein by SDS-PAGE. As shown in Figure 41, as the amount of tubulin

Figure 41. Tubulin-mediated immunoprecipitation of NP185-assembly polypeptides complex. Sepharose 4B covalently-coupled mAb T-2H9 (anti-tubulin) was preincubated with various concentrations of tubulin (indicated in ug), pelleted and washed, then incubated with clathrin-coated vesicle-derived NP185. After a 4 hour incubation at 4 degrees C with agitation, the samples were pelleted and supernantants were assayed for depletion of NP185 (185 kD) and assembly polypeptides (100-110 kD).

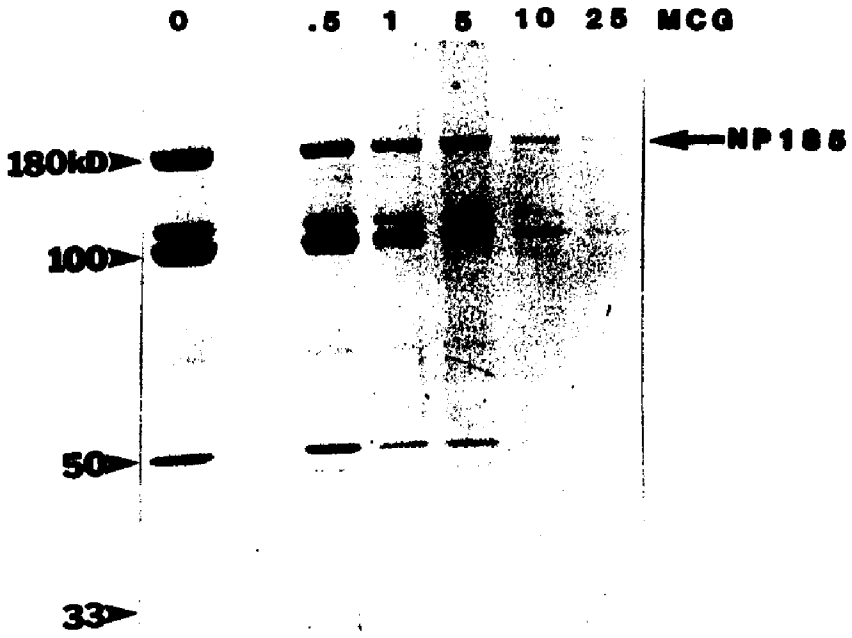
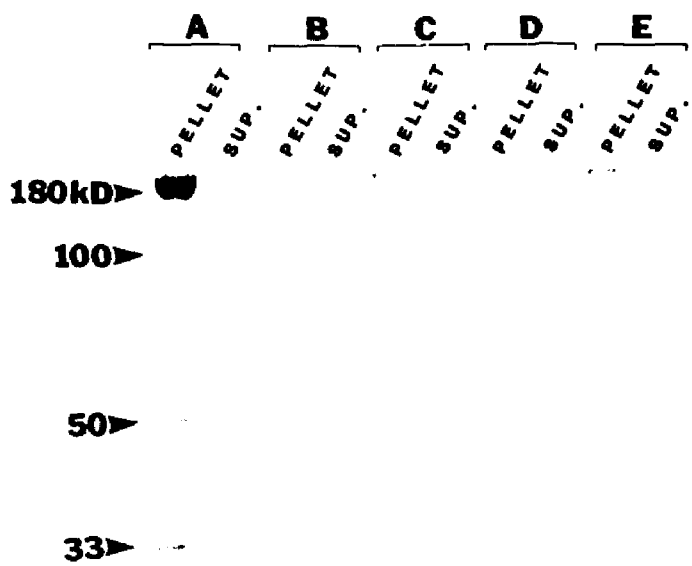

0
.5
1
5
10
25
MCG
180kD
100
50
33
NP185

Figure 42. NP185-mediated precipitation of clathrin-coated vesicles by tubulin. Tubulin was covalently coupled to Sepharose 4B, and incubated with whole clathrin-coated vesicles (CCVs) (A); 0.9 M sodium chloride extracted CCVs (B); 0.9 M sodium chloride extracted CCVs reconstituted with dialyzed 0.9 M sodium chloride extract (C); 0.9 M sodium chloride extracted CCVs reconstituted with dialyzed 0.9 M sodium chloride extracts depleted by immunoprecipitation with either mAb S-8G8 (anti-NP185) (D) or mAb C-10B2 (anti-CAP_2) (E).


A
B
C
D
E
PELLET
SUP.
180kD
100
50
33

bound increased, so did the absorption of NP185 and assembly complex. This indicated that NP185 can bind assembly complex and tubulin simultaneously. Thus, NP185 has at least two tubulin binding sites, one of which is competitively bound by the assembly polypeptides in CCVs.

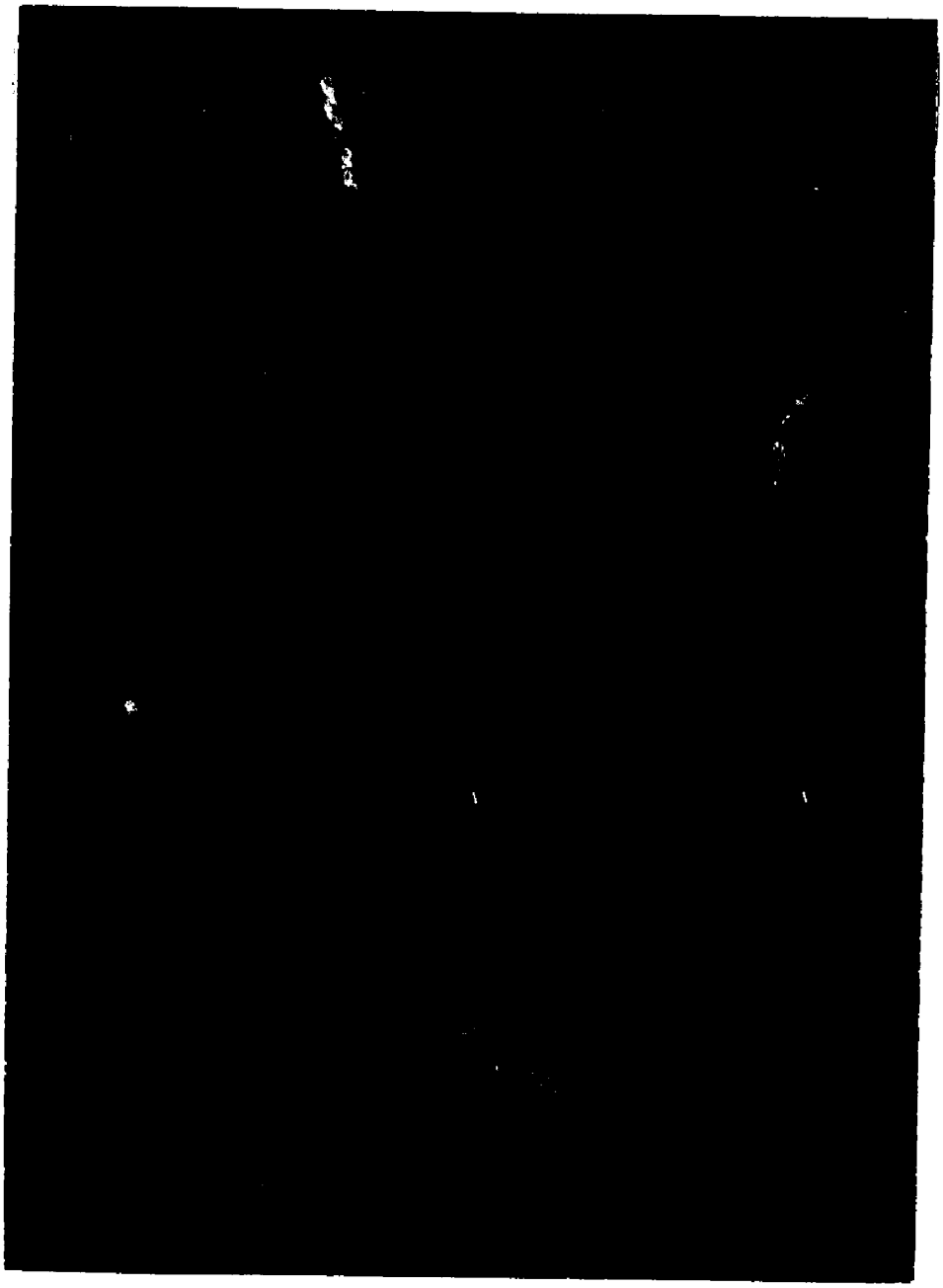
Imhof et al. (1983) have shown that CCVs are frequently associated with isolated brain microtubules. In order to determine whether NP185 can mediate the attachment of CCVs to tubulin, purified tubulin was coupled covalently to Sepharose, and used to directly precipitate CCVs and 0.9M NaCl treated CCVs. As shown in Figure 42, the tubulin-coupled beads efficiently precipitate CCVs, but do not precipitate 0.9M NaCl treated CCVs. When treated CCVs are reconstituted with dialyzed NaCl extracts, they are once again precipitated by the tubulin coupled beads. If the NaCl extracts are absorbed by mAb S-8G8, they no longer mediate the precipitation of the NaCl treated CCVs. If the NaCl extracts are absorbed by mAb C-10B2 (anti-CAP) they retain the ability to mediate precipitation. These results indicate that exogenous tubulin binding by CCVs is mediated by NP185. The results also indicate that NP185 directly mediates CCV binding to microtubules.

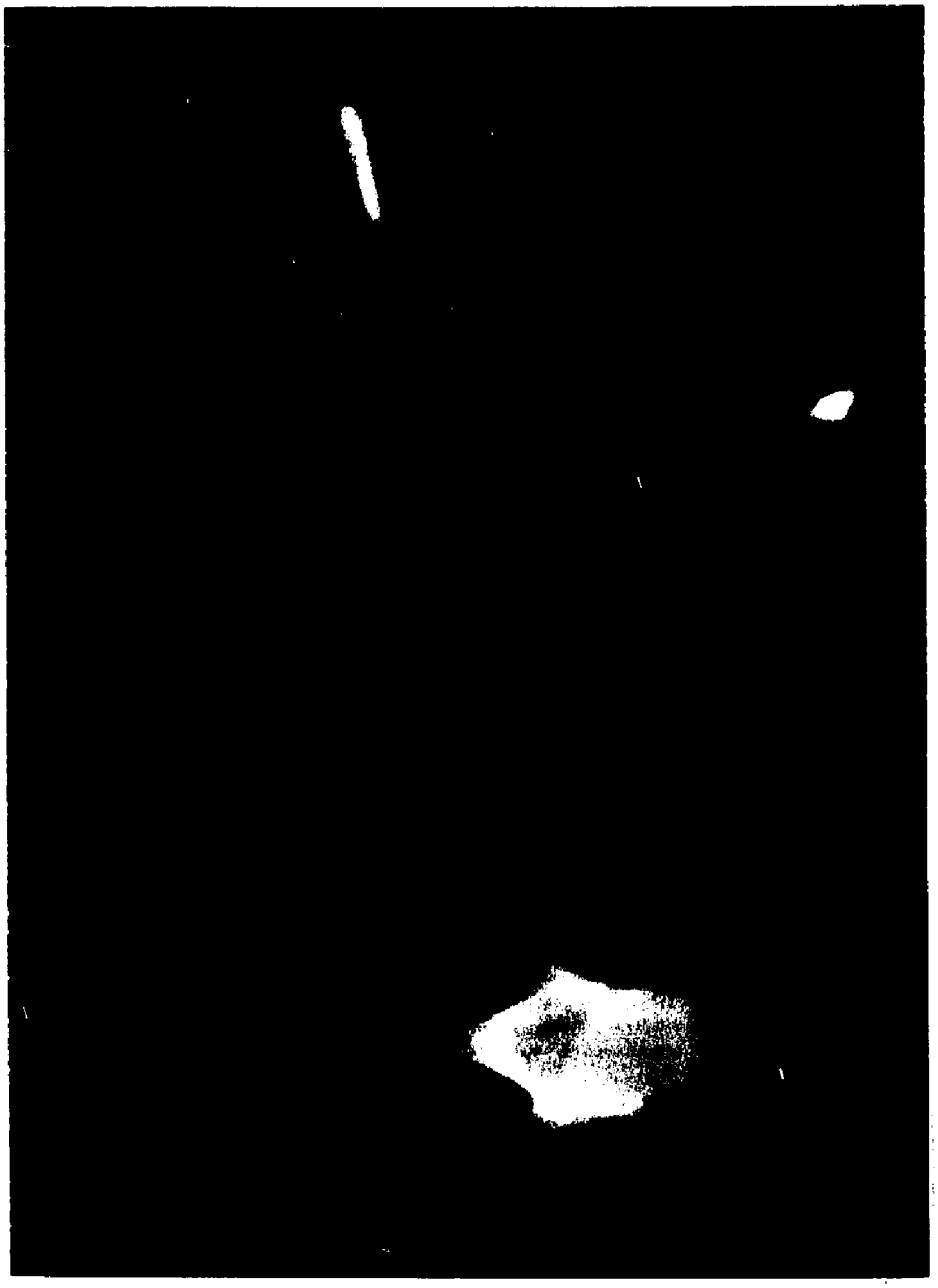
Expression of NP185 by PC12 cells: As was indicated above, NP185 is found in immunologically detectable amounts

only in cells of neuronal origin. This is illustrated well by PC12 cells, which differentiate from chromaffin cells into cholinergic neuronal-like cells when cultured in the presence of nerve growth factor (NGF; Greene and Tischler, 1976). Immunofluorescence microscopy of day 7 differentiated PC12 cells with mAb S-8G8 revealed that NP185 is distributed in a punctate manner throughout the developing neurites and in the growth cones (Figure 43). The expression of NP185 during PC12 differentiation was examined by immunoblotting with mAb S-8G8 (Figure 44). Little or no NP185 expression was detected in PC12 cells grown in the absence of NGF, but as soon as 12 hours after the addition of NGF NP185 was detected. By 7 days, a significant amount of NP185 had accumulated in the differentiated PC12 cells. Two forms of NP185 (differing slightly in their mobilities during SDS-PAGE) are present in PC12 cells, but only one is prevalent in purified bovine brain CCVs and SVs. The expression of NP185 in differentiated PC12 cells is reversed when NGF is removed from the medium. Interestingly, the higher mobility form of NP185 accumulates slower in differentiating PC12 cells and is lost sooner after NGF is removed from the culture medium.

Casein kinase II associated with NP185 phosphorylates

Figure 43. Immunofluorescence microscopy of PC12 cells cultured for 7 days in the presence of nerve growth factor. Cells were fixed with 3.7% paraformaldehyde and treated with 0.1% Triton X-100 before being incubated with primary antibody. Rhodamine-conjugated rabbit anti-mouse IgG second antibody was used. Total magnification: 900X.





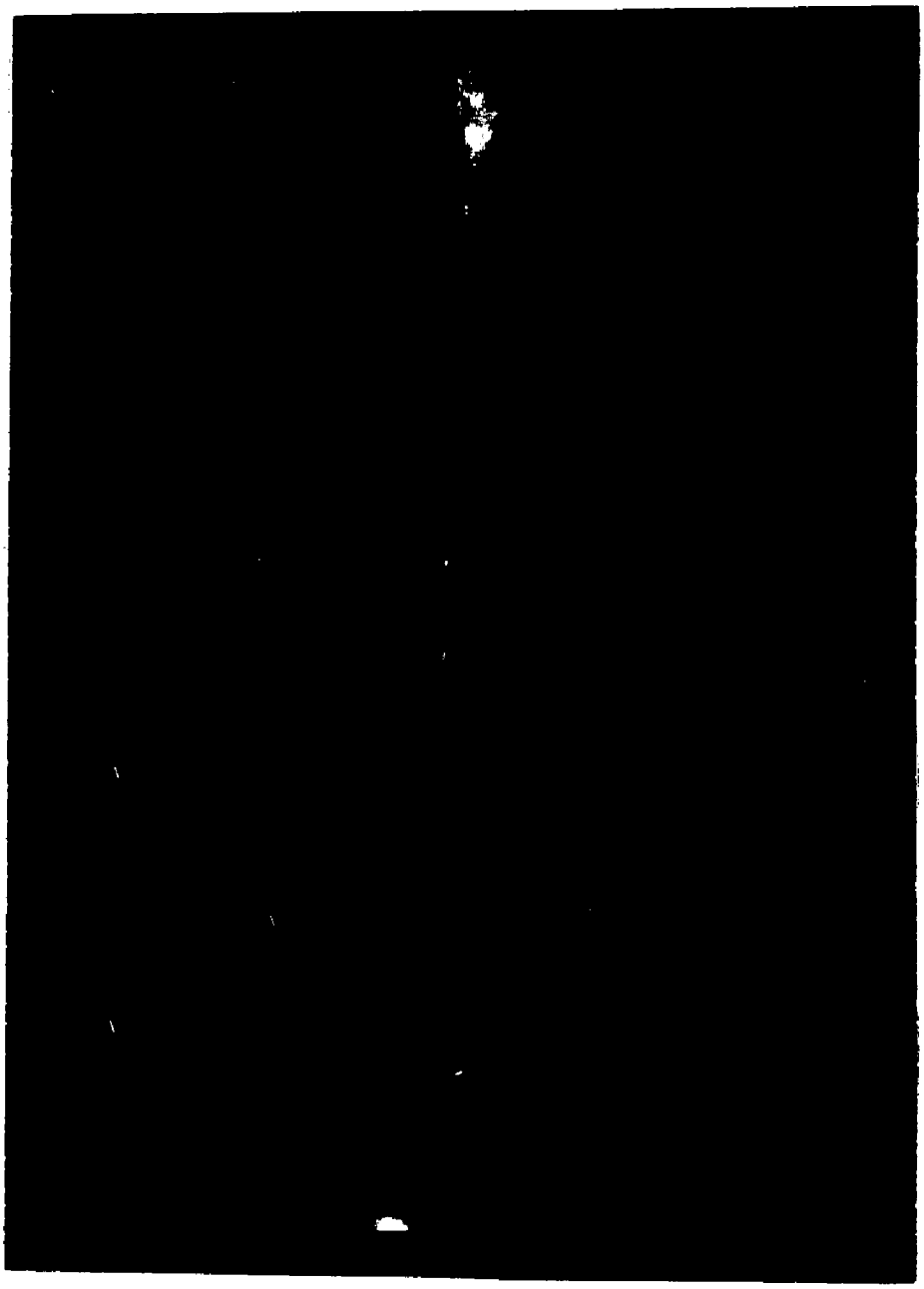
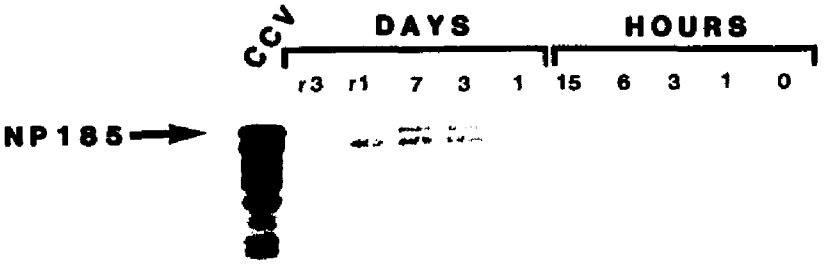


Figure 44. Expression of NP185 during nerve growth factor-induced differentiation of PC12 cells as detected by immunoblotting with mAb S-8G8. Time of culturing with 50 ng/ml nerve growth factor is shown; r1 and r3 indicate cells cultured 7 days with nerve growth factor then without nerve growth factor for 1 and 3 days respectively.

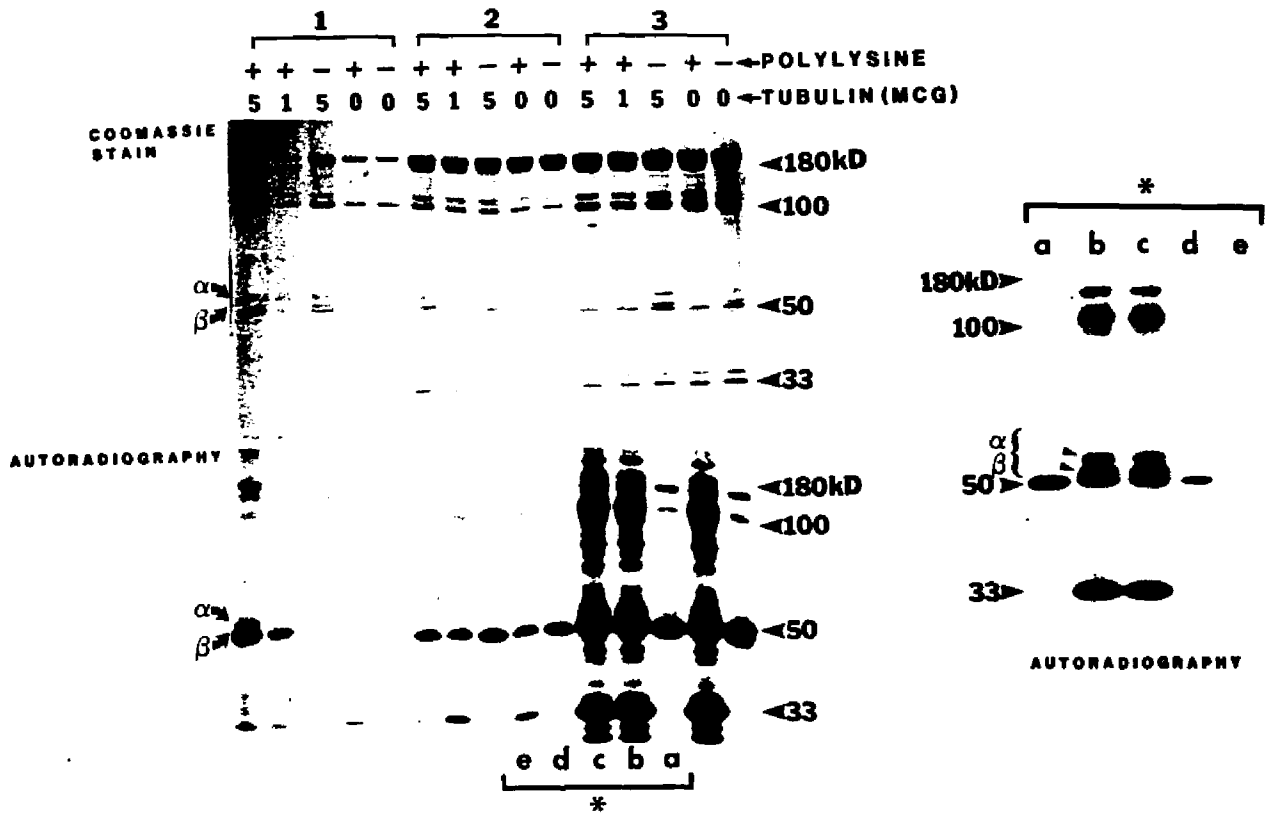

CCV
DAYS
HOURS
r3
r1
7
3
1
15
6
3
1
0
NP185

tubulin: Casein kinase II can be extracted from CCVs by extraction with 0.9M NaCl. Virtually all of the kinase activity is released from the vesicle by this treatment. As was shown above, some of the enzyme is associated with NP185, which is also extracted by this treatment. Since NP185 binds tubulin, experiments were performed to determine whether NP185-bound casein kinase II phosphorylates tubulin.

Casein kinase II activity in CCVs is stimulated by polylysine. When CCVs are treated with 10 ug/ml polylysine in the presence of gamma-^{32}P-labelled -ATP or GTP several polypeptides are labelled, most prominantly CAP_2 (Schook and Puszkin, 1985). Other polypeptides are labelled as well, including a doublet at 51 and 53 kD that co-migrates with vesicle tubulin (Figure 45). The endogenous 50 kD assembly complex kinase activity may be slightly inhibited by polylysine, although this may be due to the depletion of available ATP by the activated casein kinase II.

When CCVs are extracted with 100mM MES buffer containing 0.9M NaCl, virtually all the casein kinase activity is lost from the vesicle pellet (Figure 45). These results are consistent with others (Bar-Zvi and Branton, 1986). A fraction of the 50 kD assembly polypeptide kinase is depleted as well. This is reflected in both the intensity of the Coomassie stain of the 100 kD

Figure 45. Analysis of the distribution of kinase activities in whole clathrin-coated vesicles (3), 0.9 M sodium chloride extracted clathrin-coated vesicles (2), and mAb S-8G8 immunoprecipitates of sodium chloride extracts (1). Each lane represents the material derived from 50 ug of clathrin-coated vesicles. Kinase conditions are described in Methods. The asterisk shows an area of the autoradiographed gel that was exposed for less time and enlarged to better show the tubulin bands. As is revealed by comparing (1) and (2), the 50 kD kinase activity is found mostly in the pellet (2) after sodium chloride extraction, while the the kinase activity directed at tubulin is found mostly in the immunoprecipitates (1). This is true despite the presence of most of the vesicle-associated tubulin in the pellets (2, Coomassie stain). Phosphorylation of tubulin is observed in the immunoprecipitates only when tubulin is added exogenously. Exogenously added tubulin is not, however, phosphorylated when added to the pellet (2, autoradiograph). Endogenous tubulin is clearly phosphorylated in the whole vesicle in the presence of polylysine, although exogenously added tubulin does not enhance the signal (asterisk gel).


1
2
3
+ + − + − + + − + − + + − + −
5 1 5 0 0 5 1 5 0 0 5 1 5 0 0
POLYLYSINE
TUBULIN (MCG)
COOMASSIE STAIN
180kD
100
50
33
AUTORADIOGRAPHY
180kD
100
50
33
e d c b a
*
a b c d e
180kD
100
50
33
AUTORADIOGRAPHY

and 50 kD assembly polypeptides, and in the diminished incorporation of phosphate into the 50 kD polypeptide (Figure 45). The loss of casein kinase II activity from the salt extracted CCVs is observed despite the presence of all the CAPs and tubulin in this fraction.

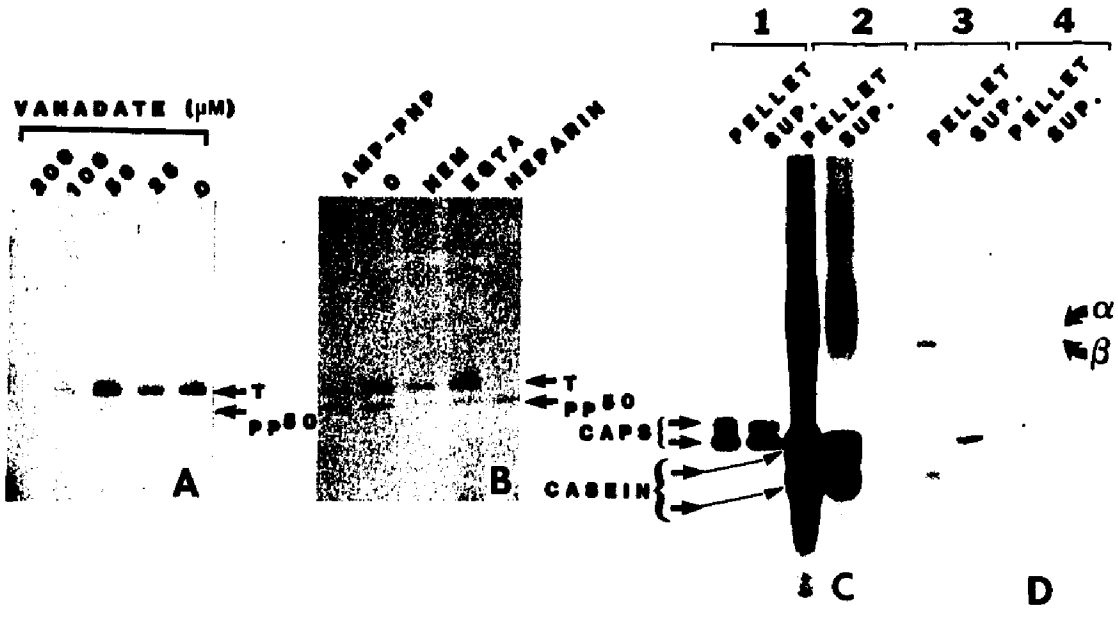
The supernatants of the 0.9 M NaCl extracts were diluted 1:4 with 20mM HEPES pH 7.0 and analyzed after immunoprecipitation with S-8G8. As shown before, when extracted from CCVs, NP185 immunoprecipitates as a complex with one molar equivalent of the assembly polypeptides. The extracted assembly complex retains some of the kinase activity associated with the 50 kD polypeptide (Figure 45). When polylysine is added to the reaction mixture, phosphorylation of the 50 kD protein is reduced, and several trace components appear to be labelled. When purified tubulin is added to a reaction mixture lacking polylysine no detectable phosphate is incorporated into either the alpha or beta forms. On the other hand, when immunoprecipitated NP185 is incubated with purified tubulin and polylysine (10 ug/ml) in a phosphorylation reaction mixture, considerable incorporation of phosphate into tubulin is observed. Tubulin alone at this concentration in the presence of polylysine is not phosphorylated (Figure 45). The phosphorylation of tubulin in the presence of polylysine is observed in the whole CCV and in

immunoprecipitates of 0.9 M NaCl extracts when exogenous tubulin is added, but not in the vesicle pellets of CCVs extracted by 0.9 M NaCl. This is observed despite the retention of nearly all the vesicle-bound tubulin by the sodium chloride-extracted vesicles. Interestingly, exogenously added tubulin does not accentuate the signal of phosphorylated tubulin in whole CCVs (Figure 45). Vesicle-bound tubulin is apparently phosphorylated preferentially until the enzyme is extracted from the vesicle.

Not all of the casein kinase II activity extracted from the vesicle is associated with NP185. Comparison of the kinase activity in S-8G8 immunoprecipitates with the activity remaining in the depleted supernantants indicated that considerable activity remains unprecipitated (Figure 46). The enzyme activity immunoprecipitated by S-8G8 and the activity remaining in the supernatant both phosphorylate CAP_2, casein, and tubulin in the presence of polylysine. Both are inhibited by 1 ug/ml heparin, AMP-PNP (not shown for supernatant enzyme), vanadate, and cold GTP (Figure 46). Thus, the kinase activities are due to biochemically indistinguishable enzymes.

A second kinase activity directed at tubulin becomes prevalent when concentrations of tubulin in the reaction mixtures becomes higher than 500 ug/ml. This activity is not associated with NP185. Type II Ca^{2+}/calmodulin-

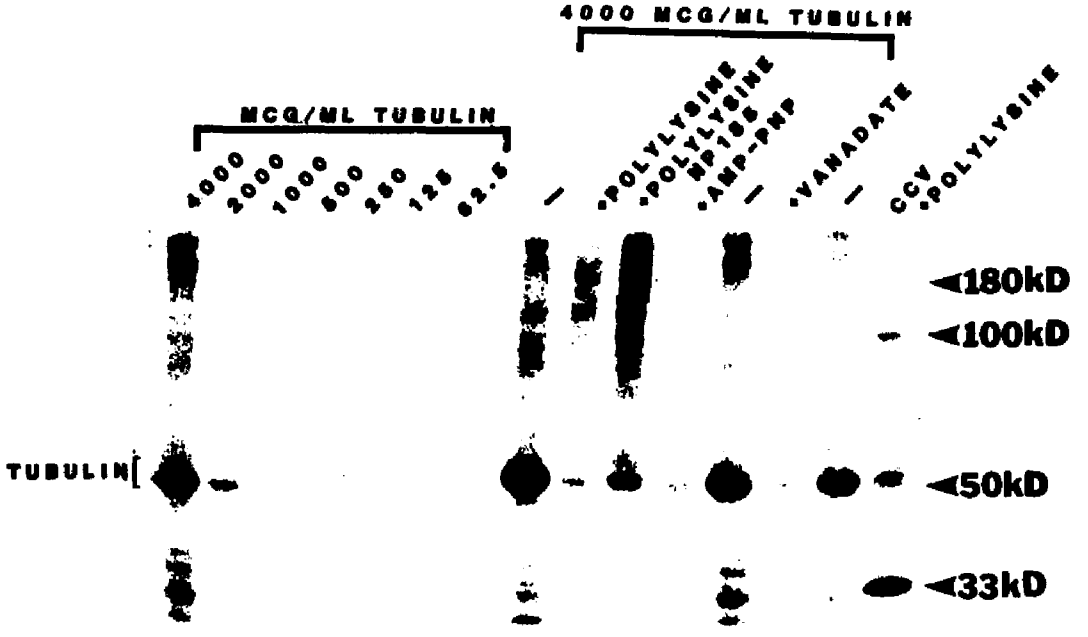
Figure 46. Characterization of NP185-associated kinase activity as casein kinase II. NP185 was dissociated from clathrin-coated vesicles with 0.9 M sodium chloride, diluted 1:4 with 20 mM HEPES pH 7.0 and immunoprecipitated with mAb S-8G8. Each lane contains enzyme derived from 50 ug clathrin-coated vesicles. Pellets and supernatants were assayed for enzyme activity with 1 ug CAPs (1), 10 ug casein (2) , and 1 ug tubulin (3, 4, A, and B) as substrate. 1 ug/ml of heparin was added to sample 4. A: Phosphorylation of 1 ug of tubulin in the presence of 10 ug/ml polylysine and varying concentrations of vanadate. B: AMP-PNP (5'-adenylyl-imidodiphosphate;1 mM); N-ethylmaleimide (0.1 mM); EGTA (1 mM); heparin (1 ug/ml) were added to a phosphorylation mixture containing 1 ug of tubulin.


VANADATE (μM)
200
100
50
25
0
T
PpSO
A
AMP-PNP
0
NEM
EGTA
HEPARIN
T
PpSO
B
1
2
3
4
PELLET
SUP.
PELLET
SUP.
PELLET
SUP.
PELLET
SUP.
CAPS
CASEIN
C
D
α
β

dependent kinase (Cal kinase) is the most abundant enzyme in the brain (Erondu and Kennedy, 1985) and a considerable amount co-purifies with brain microtubules (Larson et al., 1985). One of the substrates of this enzyme is tubulin (Larson et al., op Cit.). The tubulin used in these experiments (kind gift of Dr. R. Liem) was purified from rat brain in a manner previously shown to retain enzyme activity. Recent work by Miller and Kennedy (1986) has shown that at high concentrations the Cal kinase autophosphorylates, and thereby looses its dependence on Ca^{2+}/calmodulin. Thus, activation of the enzyme is observed at concentrations of tubulin higher than 500 ug/ml (Figure 47). Activation by Ca^{2+}/calmodulin is observed at lower concentrations of tubulin (Figure 47).

The concentration dependent activity of the Cal kinase is inhibited by polylysine, AMP-PNP, and vanadate (Figure 47). The concentration dependence of this activity in the absence of Ca^{2}+/calmodulin and its inhibition by polylysine provide a reliable means of generating tubulin phosphorylated exclusively by either the casein kinase or the Cal kinase. At tubulin concentrations less than 100 mcg/ml in the presence of polylysine the Cal kinase is virtually inactive. The casein kinase is active under these conditions, being stimulated by polylysine and independent of the concentration of tubulin (Figure 48).

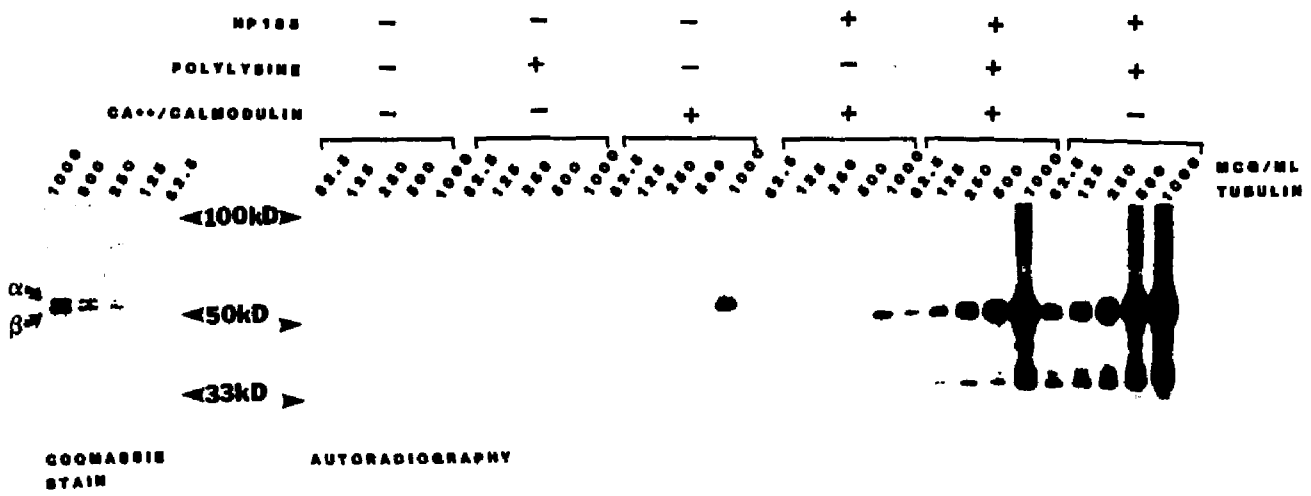
Figure 47. Demonstration of concentration dependence of the endogenous Ca^{2+}/calmodulin-dependent kinase of rat brain tubulin. Tubulin was incubated at various concentrations with gamma-^{32}P-labelled ATP (.002 mCi; .02 mM final concentration), then analyzed by SDS-PAGE. Concentration dependence of the enzyme activity is apparent as no phosphorylation is detected below .5 mg/ml. 4 mg/ml tubulin was also incubated with 10 ug/ml polylysine (+polylysine), 10 ug/ml polylysine and NP185 precipitated from 50 ug/ml clathrin-coated vesicle (+polylysine NP185), 1 mM AMP-PNP (+AMP-PNP), or 0.2 mM vanadate (+vanadate).


4000 MCG/ML TUBULIN
MCG/ML TUBULIN
4000
2000
1000
500
250
125
62.5
—
+POLYLYSINE
+POLYLYSINE XP18E
+AMP-PNP
—
+VANADATE
—
CCV +POLYLYSINE
180kD
100kD
TUBULIN
50kD
33kD

Characterization of this enzyme activity in Figure 46 was therefore carried out under these conditions, which resulted in a relatively weak signal in the tubulin band. In the absence of polylysine the casein kinase does not phosphorylate tubulin (Figure 45), and at concentrations of 500 ug/ml or more tubulin is phosphorylated efficiently by the Cal kinase in the absence of Ca^{2+}/calmodulin.

Phosphorylation of tubulin modulates binding to NP185: NP185 derived from either CCV or synaptic vesicle preparations binds tubulin. CCV-derived NP185 also binds one molar equivalent of the assembly complex. Previous experiments indicated that tubulin and the assembly complex proteins can bind NP185 simultaneously. On the other hand, NP185 competed off the CCV by purified tubulin contains no assembly complex, indicating that binding of more than one tubulin dimer to CCV-NP185 necessitates dissociation of the assembly complex. In summary, NP185 can bind two tubulin dimers, and while SV-NP185 has both sites available, one site on CCV-NP185 is less accessible due to the presence of bound assembly complex proteins. The mechanism by which the assembly complex renders one tubulin binding site less accessible is unclear, although it is ultimately reversible by excess tubulin. It may be concluded that the assembly complex and one tubulin binding site are situated very

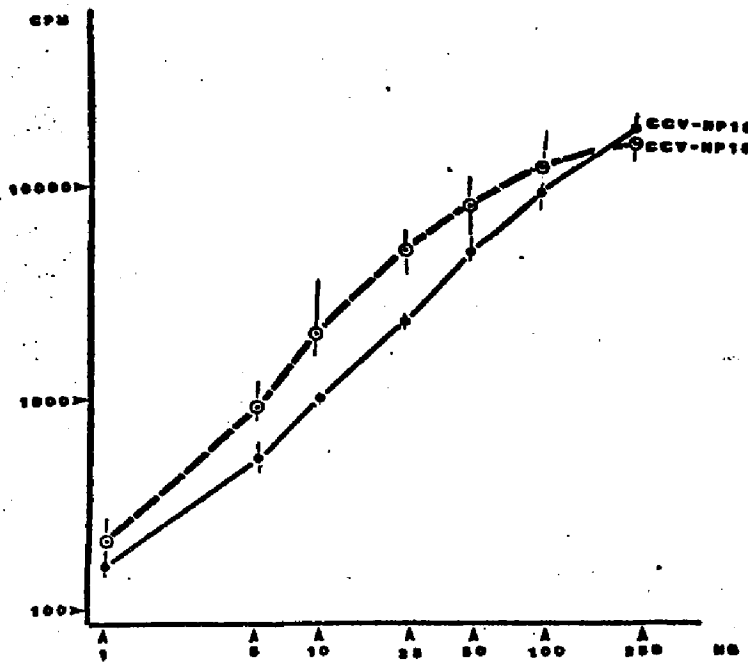
Figure 48. Distinguishing casein kinase II phosphorylation from Ca^{2+}/calmodulin-dependent kinase phosphorylation of tubulin. Phosphorylation reaction were carried out with various concentrations of tubulin in the presence of NP185, polylysine, and/or Ca^{2+}/calmodulin. As is evident from the data in Figure 47, phosphorylation by the Ca^{2+}/calmodulin-dependent kinase is not detected at concentrations of tubulin of 0.5 mg/ml or below in the absence of Ca^{2+}/calmodulin. This observation is also supported by this set of experiments. In contrast, phosphorylation of tubulin by the casein kinase is relatively efficient at lower concentrations of tubulin.

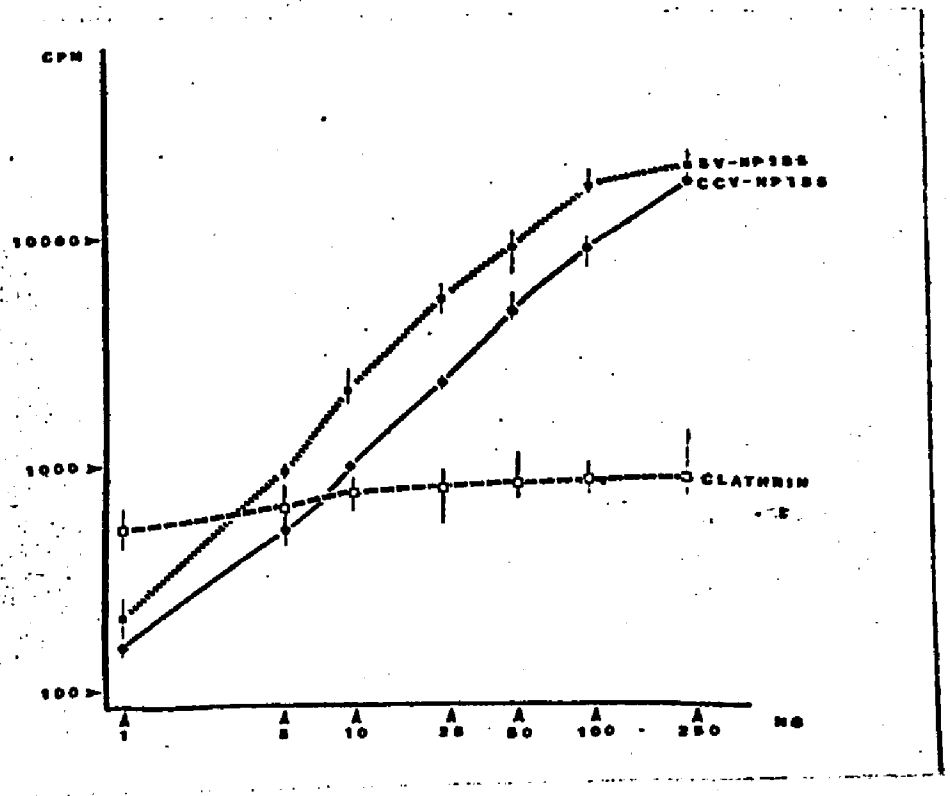

MP188
POLYLYSINE
CA++/CALMODULIN
MCG/ML TUBULIN
<100kD>
<50kD
<33kD
COOMASSIE STAIN
AUTORADIOGRAPHY

close to each other on NP185. Alternatively, assembly complex proteins and tubulin may allosterically alter distinct sites on NP185.

To decipher the parameters of each tubulin binding site on NP185, purified tubulin was radioiodinated and immunoprecipitated by NP185 bound to a mixture of mAbs S-8G8 and S-6G7 covalently coupled to Sepharose 4B. Bound NP185 was quantitated by Coomassie staining of the dissociated proteins resolved by SDS-PAGE. The intensity of the NP185 band was compared to clathrin standards. Dilutions were generated by diluting a stock of NP185-coated antibody-coupled beads into uncoated antibody-coupled beads. Two conditions were used when binding labelled tubulin to NP185: non-equilibrium conditions, in which NP185 prebound to antibody-coupled beads was incubated at 4 degrees centigrade for 20 to 30 minutes with ligand (labelled tubulin of constant protein concentration and specific activity was incubated with varying amounts of NP185). The total binding plots varied very little when either non-equilibrium or equilibrium conditions were used with SV-NP185 (Figure 49). The total binding plots of CCV-NP185, on the other hand, varied considerably with the binding conditions (Figure 49). Under equilibrium conditions, both CCV-NP185 and SV-NP185 bound 2 molar equivalents of tubulin dimer as long as at least a 6-fold

Figure 49. Tubulin binding as a function of NP185 protein concentration. Values (ng) represent total NP185 protein in a 0.1 ml reaction mix. Specific activity of radioiodinated tubulin was adjusted to 1.8×10^5 cpm/ug by the addition of cold tubulin. SV-NP185: NP185 protein derived from synaptic vesicles, non-equilibrium conditions; CCV-NP185: NP185 protein derived from clathrin-coated vesicles, non-equilibrium conditions; CCV-NP185*: NP185 protein derived from clathrin-coated vesicles, equilibrium conditions; clathrin: purified clathrin, non-equilibrium conditions, precipitated by mAb 2D9 covalently coupled to Sepharose 4B. Binding conditions are described in the text.


CPM
10000
1000
100
CCV-HP188
CCV-HP188'
1
5
10
25
50
100
250
NG


CPM
10000
1000
100
SV-HP1B5
CCV-HP1B5
CLATHRIN
1
5
10
25
50
100
250
NG

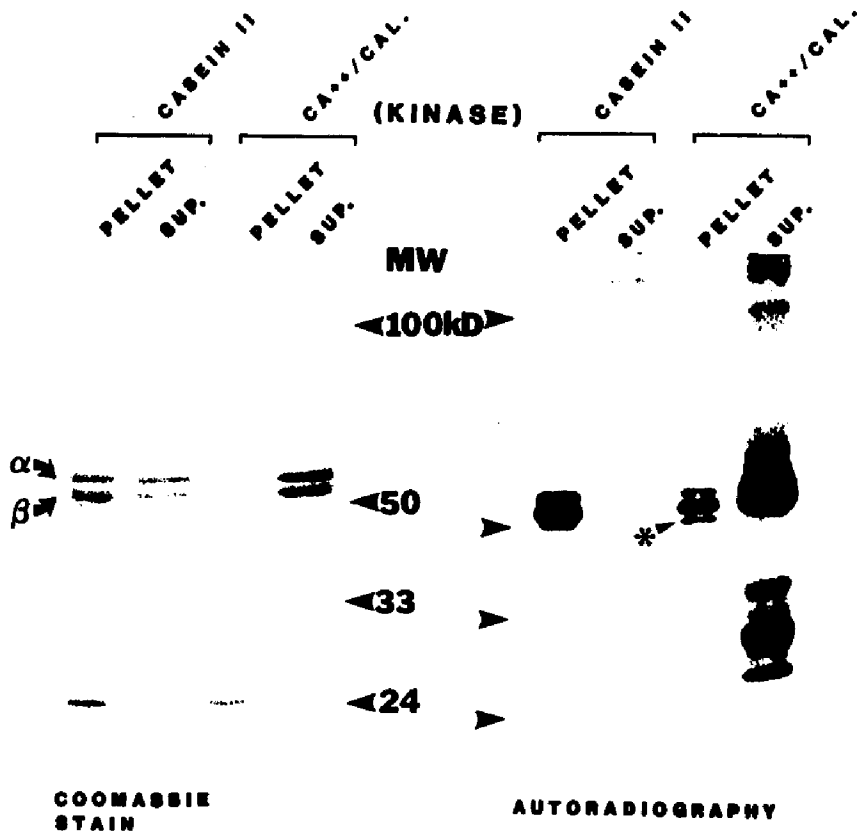
excess was present. Under non-equilibrium conditions, however, CCV-NP185 bound only slightly over 1 molar equivalent of tubulin, while SV-NP185 still bound two molar equivalents. These data indicate that the presence of assembly polypeptides reduces the rate of tubulin binding to one site on NP185. This is clearly evident from the lack of a kinetic differential in SV-NP185. Therefore, by varying the conditions of binding two tubulin binding sites can be distinguished on CCV-NP185: an unhindered site (NP185 T-site), which is unaffected by the presence of assembly complex polypeptides; and a hindered site (NP185 A-site), whose binding activity is contingent on dissociation of the assemply complex.

To initially assess whether phosphorylation of tubulin by the Cal or Cas (casein kinase II) kinase affects its binding to NP185, immunoprecipitation experiments were performed with mixtures of phosphorylated and unphophosporylated tubulin (unphosphorylated tubulin is meant to refer to tubulin that was extracted from rat brain, but not phosphorylated <u>in vitro</u>). CCV-NP185 was prebound to mAb S-8G8-coupled beads, and titered to precipitate 10 ug out of a solution containing 20 ug of unphosphorylated tubulin. Non-equilibrium binding conditions were used. 10 ug of unphosphorylated tubulin was then mixed with 10 ug of tubulin phosphorylated by

either the Cas or Cal kinase, and precipitated under identical conditions. The pellets and supernatants were then analyzed by SDS-PAGE. Under these conditions, Cas kinase-phosphorylated tubulin bound more efficiently to CCV-NP185 than unphosphorylated tubulin, as is evidenced by comparing the intensity of tubulin phosphorylation in the supernatant and pellet lanes (Figure 50). In contrast, Cal kinase-phosphorylated tubulin does not bind as efficiently as unphosphorylated tubulin. This is shown by the presence of most of the phosphorylated tubulin in the supernatant of these precipitations (Figure 50). Since non-equilibrium binding conditions and CCV-NP185 were used in both cases, these are binding characteristics of the NP185 T-site.

To further characterize each of the two tubulin binding sites of NP185, CCV- and SV-NP185 were analyzed by competitive binding studies with radioiodinated unphosphorylated tubulin and cold tubulin, Cal kinase-phosphorylated tubulin, and Cas kinase-phosphorylated tubulin. Preparations of either SV-NP185 or CCV-NP185 bound to S-6G7- and S-8G8-coupled beads were incubated with labelled tubulin without or with varying amount of competitor. The assays were performed under two sets of conditions: non-equilibrium conditions, in which the incubation is continued for 20 to 30 minutes at 4 degrees C; and equilibrium conditions, in which the incubation is

Figure 50. Differential immunoprecipitation of phosphorylated tubulin mediated by clathrin-coated vesicle derived NP185. Monoclonal antibodies S-6G7 and S-8G8 were coupled to Sepharose 4B and reacted with clathrin-coated vesicle derived NP185. The coated beads were then titered to precipitate 10 ug of unphosphorylated tubulin from a reaction mixture containing 20 ug total tubulin (under non-equilibrium conditions, described in text). Phosphorylated tubulin was then mixed 1:1 with unphosphorylated tubulin and the precipitations were performed with 20 ug of this mix. Supernatants and pellets were analyzed by SDS-PAGE and autoradiography. Casein kinase II phosphorylated tubulin was precipitated more efficiently while Ca^{2+}/calmodulin-dependent kinase phosphorylated tubulin was precipitated less efficiently than unphosphorylated tubulin. An asterisk indicates the 50 kD assembly complex protein associated with NP185 in clathrin-coated vesicles. This polypeptide has an associated kinase activity and must have been phosphorylated with residual ATP in the Ca^{2+}/calmodulin dependent-kinase phosphorylated tubulin. The 185 kD NP185 band did not migrate far enough into this gel to be detected.


CASEIN II
Ca++/CAL.
(KINASE)
CASEIN II
Ca++/CAL.
PELLET
SUP.
PELLET
SUP.
PELLET
SUP.
PELLET
SUP.
MW
100kD
α
β
50
33
24
*
COOMASSIE STAIN
AUTORADIOGRAPHY

continued for 4 hours at 4 degrees C. In all cases unphosphorylated tubulin was used as the labelled ligand. Data was expressed as a percentage of total bound labelled tubulin in the absence of competitor, then subtracted from 100% to yield a value reflecting relative inhibition. The data were fitted to either one or two site equilibrium binding equations (see Methods) by the Prophet system.

In the first analysis, SV-NP185 and CCV-NP185 were analyzed using unphosphorylated tubulin as both the labelled and competitive ligands. As was indicated above, SV-NP185 behaves similarly under both non-equilibrium and equilibrium conditions. Therefore, all data pertaining to SV-NP185 was derived under non-equilibrium conditions. Using unphosphorylated tubulin, the binding curves generated for CCV-NP185 (both non-equilibrium and equilibrium) and SV-NP185 were indistiguishable (Appendix I). As shown in Table 5, these curves fit more significantly to a single site binding model than to a two site binding model. The data reveal a single binding site with an apparent K_d near 2.0×10^{-7} M (Table 6). Since SV-NP185 and CCV-NP185 under equilibrium conditions bind two molecules of tubulin, the affinities of each site for unphosphorylated tubulin must be similar. This conclusion is supported by results obtained using CCV-NP185 under non-equilibrium conditions. As indicated above, non-

equilibrium binding conditions favor the T-site on CCV-NP185 due to the presence of assembly complex polypeptides. When these data were fitted to the single site model, an apparent K_d of approximately 2.0×10^{-7} M (Table 6) was also calculated for the NP185 T-site. Together the data indicate that both the A- and T-site of NP185 have apparent K_ds for unphosphorylated tubulin near 2.0×10^{-7} M.

In the second analysis, casein kinase phosphorylated tubulin was used as the competitive ligand and unphosphorylated tubulin was used as the labelled ligand. When CCV-NP185 was analyzed under non-quilibrium conditions, the T-site bound Cas kinase-phosphorylated tubulin with an apparent K_d of $1.7 \pm .1 \times 10^{-8}$ M (K_d ±standard error). The data fit best a single-site binding model. This relative K_d value reflects a 10-fold higher affinity of the T-site for Cas kinase-phosphorylated tubulin than for unphosphorylated tubulin. The two site binding model provided the best statistical fit of the data generated from SV-NP185 or CCV-NP185 under equilibrium conditions. The high affinity sites comprised 49.9% ±2.9% of the total binding sites, with a relative K_d of $1.4 \pm .1 \times 10^{-8}$ M. The low affinity site had an apparent K_d $3.9 \pm .2 \times 10^{-7}$ M, 2-fold lower than its apparent K_d for unphosphorylated tubulin. Since the high affinity site was

Table 5. Statistical Fits of Tubulin Binding to NP185.

Source	Binding	Binding Model Used: Tubulin	Tubulin Cal P		Tubulin Cas P
CCV	Non-equ.	one site	two site (.001)	/one site (.016, .081, .0001)/(.001)	one site (.001)
CCV	Equilibrium	one site	two site (.001)	(.001, .003, .003)	two-site (.0001,.002,.001)
SV	Non-equ.	one site	two site (.0001)	(.0001, .001, .001)	two site (.001, .001, .001)

Numbers in parentheses indicate statistical significance of the following parameters:

One site - apparent K_d

Two site - % high affinity sites, apparent K_d of high affinity site, apparent K_d of low affinity site (respectively).

Key: CCV - Clathrin coated vesicle

SV - Synaptic vesicle

Tubulin Cal P - Tubulin phosphorylated by Ca^{2+}/calmodulin-dependant kinase.

Tubulin Cas P - Tubulin phosphorylated by casein kinase II.

Non-equ. - non-equilibrium binding conditions

Table 6. Parameters of Statistical Fits to NP185-Tubulin Binding

	CCV - NP185 (equilibrium)			CCV - NP185 (non-equ)			SV - NP185 (non-equ)		
	% H	$K_dH(M)$ $\times10^{-7}$	$K_dL(M)$ $\times10^{-7}$	% H	$K_dH(M)$ $\times10^{-7}$	$K_dL(M)$ $\times10^{-7}$	% H	$K_dH(M)$ $\times10^{-7}$	$K_dL(M)$ $\times10^{-7}$
Tubulin	100	2.0 (±.1)	-	100	2.0 (±.1)	-	100	1.9 (±.1)	-
Tubulin Cal P	48.1 (±8.5)	.76 (±.17)	8.0 (±1.8)	100	6.0 (±.3)	-	48.1 (±5.6)	0.66 (±.17)	11 (±2)
				10.4 (±3.3)	.48 (±.23)	7.7 (±.5)			
Tubulin Cas P	47.5 (±4.6)	.13 (±.03)	3.7 (±.7)	100	.17 (±.01)	-	49.9 (±2.9)	0.14 (±.1)	3.9 (±.2)

Key:

% H - percent high affinity sites

$K_dH(M)$ - apparent K_d of high affinity site (moles)

$K_dL(M)$ - apparent K_d of low affinity site (moles)

Tubulin Cal P - Tubulin phosphorylated by the Ca^{2+} calmodulin kinase.

Tubulin Cas P - Tubulin phosphorylated by the casein kinase II.

Non-equ - Non-equilibrium binding conditions (in text)

shown by the analysis done under non-equilibrium conditions to be the T-site, the A-site is represented by the low affinity site. Therefore, the A-site has a 2-fold lower apparent affinity for Cas kinase-phophorylated tubulin than for unphosphorylated tubulin, while the T-site has a 10-fold higher affinity. Similar results were obtained using SV-NP185 (Table 6).

In the third analysis, Cal kinase-phosphorylated tubulin was used as the competing ligand and unphosphorylated tubulin was used as the labelled ligand. When CCV-NP185 was analyzed under non-equilibrium conditions, the T-site bound Cal kinase-phosphorylated tubulin with an apparent K_d of 6.0 ±.3 x 10^{-7} M when fitted to a single site model. Interestingly, these data could also be fitted to a two site binding model in a statistically significant manner, perhaps due to the low affinity of the T-site for Cal kinase-phosphorylated tubulin. In this analysis, the high affinity sites comprise 10.4% ±3.3% of the total binding sites, with an apparent K_d of 4.8 ±2.3 x 10^{-8} M. The low affinity site, with the majority of available sites under kinetic conditions, had an apparent K_d of 7.7 ±.5 x 10^{-7} M. Both analyses indicate that the T-site has a 3- to 4-fold lower affinity for Cal kinase-phosphorylated tubulin than for unphosphorylated tubulin.

When CCV-NP185 was analyzed under equilibrium conditions with Cal kinase-phosphorylated tubulin, the two site model provided the best statisitical fit of the data. In CCV-NP185, high affinity sites comprised 48.1% ±5.6% of the total binding sites, with an apparent K_d of 6.6 ±1.7 x 10^{-8} M. This is nearly a 4-fold higher affinity than this site has for unphosphorylated tubulin. The low affinity site had an apparent K_d of 1.1 ±.2 x 10^{-6} M, which is represents an even lower affinity than was calculated under non-equilibrium conditions. In summary, the T-site, which was identified by non-equilibrium analysis has 3- to 5- fold lower affinity for Cal kinase-phosphorylated tubulin than it has for unphosphorylated tubulin. In contrast, the A-site has 4-fold higher affinity for Cal kinase-phosphorylated tubulin than it has for unphosphorylated tubulin.

Discussion: A summary of the apparent K_d values of the two tubulin binding sites on NP185 is presented in Table 7. The data have shown that two distinct sites are present on NP185 that bind tubulin. When NP185 is extracted from CCVs, it immunoprecipitates as a complex containing one molar equivalent of assembly polypeptides. SV-NP185 lacks the assembly polypeptides. The presence of assembly polypeptides on CCV-NP185 acts competitively to

Table 7. Summary of Apparent Affinities of the A and T Tubulin Binding Sites of NP185.

Site	Tubulin	Tubulin Cal P	Tubulin Cas P
A	+	++	-
T	+	-	+++

+++	apparent $K_d < 5.0 \times 10^{-8}M$
++	$5.1 \times 10^{-8}M <$ apparent $K_d < 1 \times 10^{-7}M$
+	$1.1 \times 10^{-7}M <$ apparent $K_d < 3.0 \times 10^{-7M}$
-	apparent $K_d >$ 3.1 x 10

inhibit the binding of tubulin to one site on NP185. This site is therefore called the A-site. The other tubulin binding site is unaffected by the presence of assembly complex proteins, and is called the T-site.

The presence of assembly polypeptides on CCV-derived NP185 kinetically hinders the binding of tubulin to the A-site. This characteristic permits the two sites to be distinguished by competitive tubulin binding assays. Two sets of conditions were used for these assays: non-equilibrium conditions, which favor binding to the T-site, and equilibrium conditions, which favor neither site. Competitive assays were first performed using kinetic conditions, and an apparent K_d value is determined for the T-site by fitting the data to a single site binding curve. The experiments were then performed under equilibrium conditions, and the data were fitted to a two site binding model. If the fit is statistically significant this yields two apparent K_d values, one of which approximates the value observed using kinetic conditions (T-site value), while the other differs significantly when Cal or Cas kinase phosphorylated-tubulin are used as competing ligands. By this type of analysis, the two tubulin binding site of NP185 were analyzed independently.

The two sites are virtually indistinguishable using

unphosphorylated tubulin as the labelled and competing ligand. Using non-equilibrium binding conditions, however, it was possible to ascertain the differing relative affinites of Cal and Cas kinase-phosphorylated tubulin for the T-site of CCV-NP185. When equilibrium binding conditions were used and the data fitted to a two site binding model, the differing relative affinities of Cal and Cas-kinase phosphorylated tubulin for the A-site became apparent. The results indicate that the T-site has a 3- to 5-fold lower and the A-site a 3- to 5-fold higher relative affinity for Cal kinase-phosphorylated tubulin than for unphosphorylated tubulin. Alternatively, the T-site can be interpretted as having a relative affinity for Cal kinase-phosphorylated tubulin that is at least 10-fold lower than the A-site (with unphosphorylated tubulin used as the labelled ligand). The results also indicate that the T-site has a 10-fold higher and the A-site a 2-fold lower apparent affinity for Cas kinase phosphorylated-tubulin. Alternatively, the T-site can be interpretted as having a 20-fold higher apparent affinity for Cas kinase-phosphorylated tubulin (with unphosphorylated tubulin used as the labelled ligand). Together with the observation that tubulin already bound to NP185 can be readily phosphorylated by either kinase, these data indicate that associated kinase activities may modulate the binding of

NP185 to microtubules or tubulin associated with various organelles.

Phosphorylation of tubulin may affect its binding to NP185 by inducing either site-specific or allosteric affects. Whether the A-site or T-site of NP185 bind the same site or different sites on tubulin also remain to be determined. Although the apparent affinities of the A- and T- sites for unphosphorylated tubulin are nearly the same, this does not directly indicate that they bind the same site on tubulin. Indeed, it will be an interesting biophysical problem to elucidate how the A-site is inhibited from binding tubulin when assembly complex is bound to NP185, and whether the assembly complex has a private binding site or if it shares one with tubulin. The latter possibility implies some structural homology between the assembly polypeptides and tubulin.

The functional significance of binding between the A- and T- sites of NP185 and tubulin are far from solved, but a few interesting observations can be made. When an anti-tubulin mAb (IgG1) is incubated with CCVs overnight extensive aggregation of the vesicles is observed. This is due to crossklinking of tubulin molecules between different vesicles by the two tubulin binding sites on the anti-tubulin immunoglobulin molecules. Since NP185 is even larger than an IgG1 molecule it is surprising that a

similar phenomenon is not observed in preparations of purified brain CCVs. That is, NP185 does not crosslink CCVs despite its abundance and dimeric structure. A recent report (Hargeaves et al., 1986) has indicated that Cal kinase-phosphorylated tubulin has a high affinity for phosphatidylcholine vesicles while unphosphorylated tubulin is preferentially incorporated into microtubules. This implies that vesicle-bound tubulin is already phosphorylated by the Cal kinase. Since the T-site has a very low apparent affinity for Cal kinase-phosphorylated tubulin, crosslinking of the vesicle by NP185 is not be observed. The A-site, on the other hand, has a high affinity for Cal kinase-phosphorylated tubulin, and this end of the molecule is probably bound to vesicle tubulin. This implies that the T-site is actually extended away from the vesicle rather than bound to it by endogenous tubulin. This may account for its unusual sensitivity to protease treatment.

The Cas kinase activity associated with NP185 preferentially phosphorylates tubulin on the vesicle. This is shown in Figure 45, where the addition of tubulin to CCVs in a phosphorylation mixture containing polylysine does not enhance the intensity of phosphorylation in the tubulin bands. When the enzyme is extracted from the vesicle, however, it readily phosphorylates exogenously

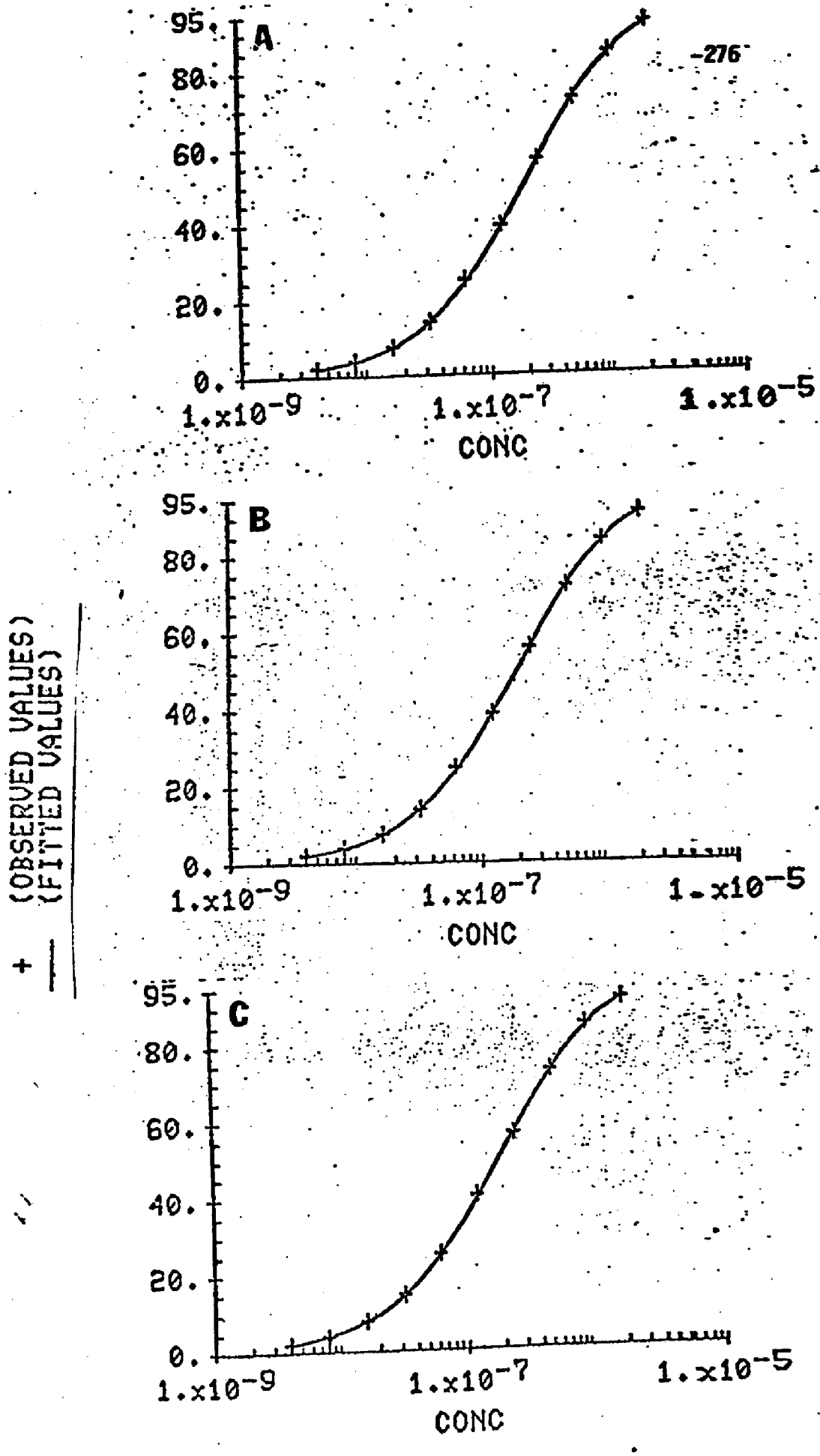
added tubulin. Cas kinase does not phosphorylate tubulin at all in the absence of polylysine; thus, its activity may not be relevant to NP185 binding except under special conditions when an equivalent intracellular stimulator is present. In these cases, phosphorylation of vesicle tubulin would lower its affinity for the A-site bound to it, and increase its affinity for the T-site 10-fold. Since the T-site is normally facing away from the vesicle, phosphorylation of vesicle tubulin by the Casein kinase would cause CCV-bound NP185 to reverse its polarity. This could have several effects on the CCV: 1. it may induce disassembly, since the A-site could carry assembly complex molecules to the outside of the vesicle when it flips; 2. it may result in movement of the vesicle; 3. it may allow interaction of NP185 with novel proteins which bind near the A-site similarly to the assembly polypeptides.

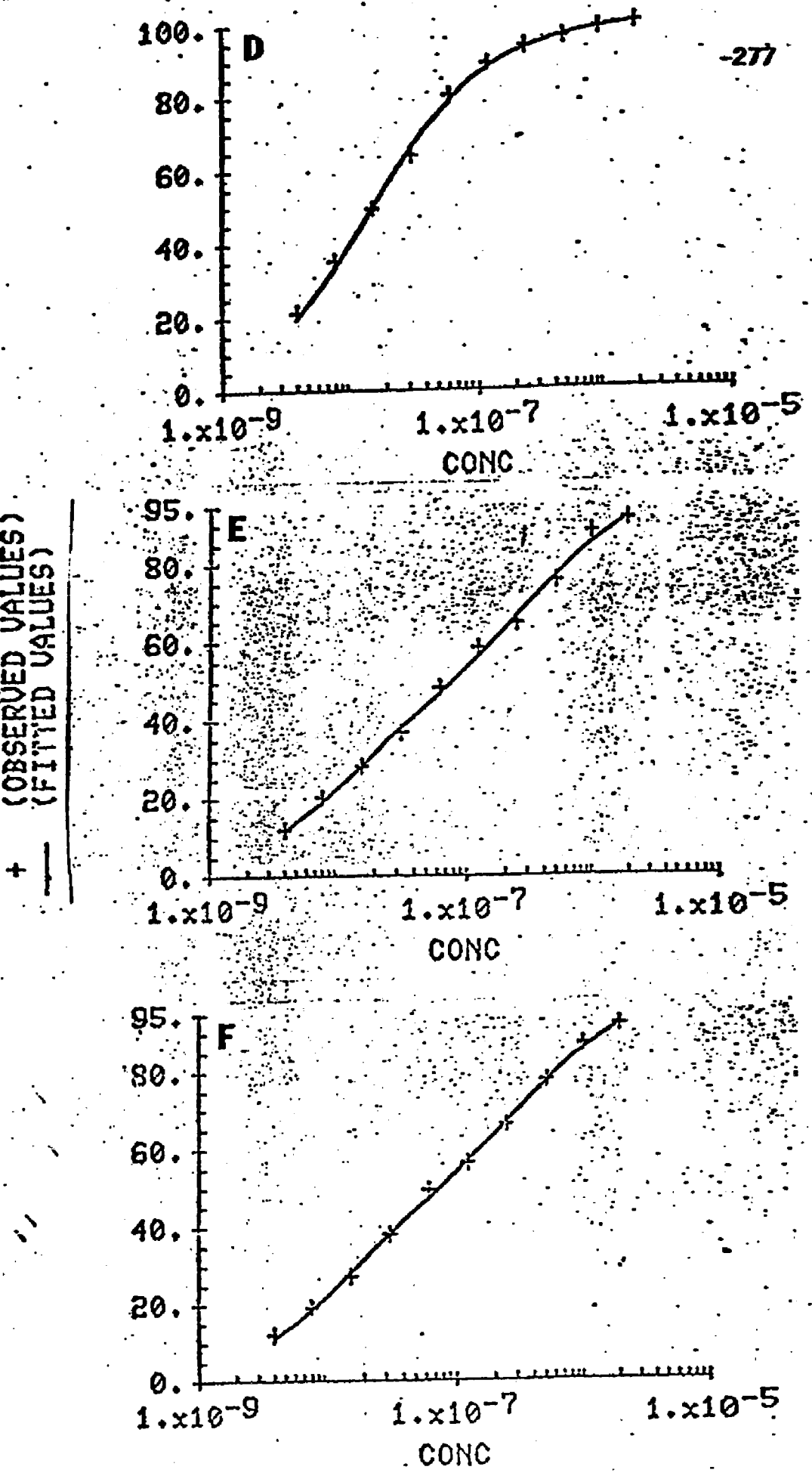
Note added in proof: The possibility that NP185 is a microtubule associated protein (MAP) cannot be ignored. Huber et al. (1985) have identified a 180 kD brain protein (MAP_3) that is associated with microtubules, is protease sensitive, and appears as a doublet during SDS-PAGE. MAP_3, however, was not detected in any brain fractions other than microtubules. Murofushi et al. (1986) recently identified a 190 kD MAP that binds 2.3 moles of tubulin. This

protein, however, was detected in bovine brain, pituitary gland, liver, kidney, adrenal medulla, adrenal cortex, and several cultured cell lines. Immunofluorescence analyses have shown that MAP_3 and 190 kD-MAP are distributed coincidently with microtubules. If NP185 is to be considered a MAP, then perhaps it should be called V-MAP (vesicle MAP).

APPENDIX

Plots of NP185-tubulin competitive binding assays fitted by the PROPHET. Unphosphorylated tubulin was used as the labelled ligand in all cases. A, B, C: Competing ligand: unphosphorylated tubulin; D, E, F: Competing ligand: casein kinase phosphorylated tubulin; G, H, I: Competing ligand: Ca^{2+}/calmodulin-dependent kinase phosphorylated tubulin. A, D, G: Non-equilibrium conditions, clathrin-coated vesicle derived NP185; B, E, H: Equilibrium conditions, clathrin-coated vesicle derived NP185; C, F, I: Non-equilibrium conditions, synaptic vesicle derived NP185.


A
-276
95.
80.
60.
40.
20.
0.
1.x10-9
1.x10-7
1.x10-5
CONC
B
95.
80.
60.
40.
20.
0.
1.x10-9
1.x10-7
1.x10-5
CONC
C
95.
80.
60.
40.
20.
0.
1.x10-9
1.x10-7
1.x10-5
CONC
+ — (OBSERVED VALUES) / (FITTED VALUES)


D
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80.
60.
40.
20.
0.
1.x10-9
1.x10-7
1.x10-5
CONC
E
95.
80.
60.
40.
20.
0.
1.x10-9
1.x10-7
1.x10-5
CONC
F
95.
80.
60.
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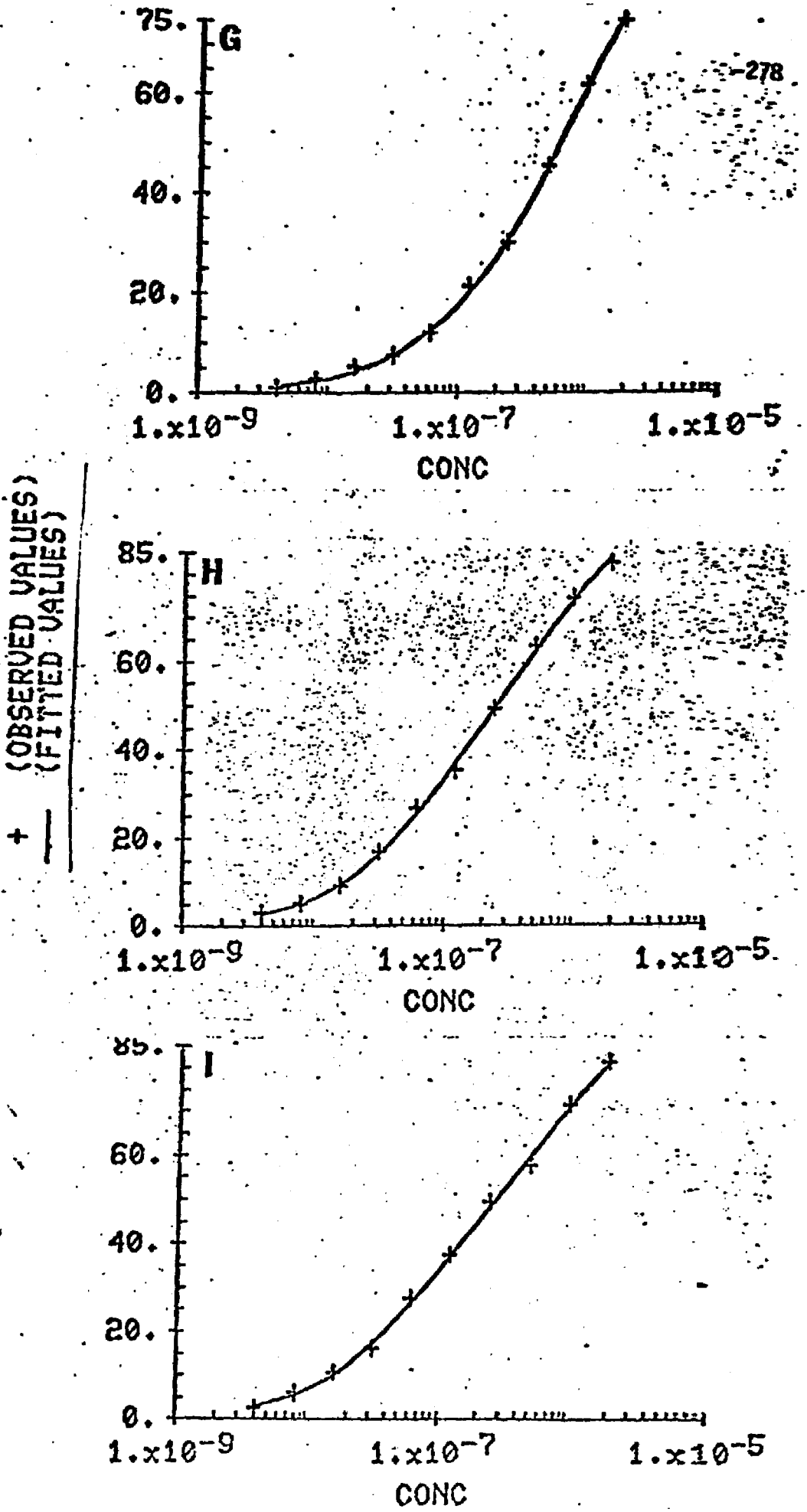
20.
0.
1.x10-9
1.x10-7
1.x10-5
CONC
+ — (OBSERVED VALUES) / (FITTED VALUES)


G
-278
75.
60.
40.
20.
0.
1.x10-9
1.x10-7
1.x10-5
CONC
H
85.
60.
40.
20.
0.
1.x10-9
1.x10-7
1.x10-5
CONC
I
85.
60.
40.
20.
0.
1.x10-9
1.x10-7
1.x10-5
CONC
+ — (OBSERVED VALUES) / (FITTED VALUES)

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