Identification of Three Coated Vesicle Components as α- and β-Tubulin Linked to a Phosphorylated 50,000-dalton Polypeptide

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ABSTRACT Coated vesicles are involved in the intracellular transport of membrane proteins between a variety of membrane compartments. The coats of bovine brain coated vesicles contain at least six polypeptides in addition to an 180,000-dalton polypeptide called clathrin. In this report we show that the 54,000- and 56,000-dalton coated vesicle polypeptides are α- and β-tubulin, determined by immunoblotting and two-dimensional gel electrophoresis. An affinity-purified tubulin antiserum can precipitate coated vesicles. The tubulin polypeptides are tightly associated with a 50,000-dalton coated vesicle polypeptide, which is phosphorylated. The phosphorylated 50,000-dalton polypeptide appears to be related to brain microtubule-associated tau proteins since it can be specifically immunoprecipitated by an affinity-purified antiserum directed against these proteins. In addition, gel filtration experiments indicate that at least a fraction of the 50,000-dalton polypeptide may associate with the 100,000-dalton coated vesicle polypeptide. Since brain is a tissue rich in tubulins, liver coated vesicles were analyzed for the presence of α- and β-tubulin. Like brain coated vesicles, liver coated vesicles also contain an endogenous kinase activity, which phosphorylates polypeptides of the same molecular weights and isoelectric points as the brain coated vesicle 50,000-dalton, tau-like polypeptide, and α- and β-tubulin. The phosphorylated 50,000-dalton polypeptide may link the membrane and contents of coated vesicles with components of the cytoskeleton.

The mechanisms by which membrane and secreted proteins are sorted and targeted to specific intracellular destinations are for the most part not understood. Coated vesicles are thought to mediate the transport of membrane proteins between various membrane compartments within the cell, for example between the endoplasmic reticulum and the Golgi apparatus and between the Golgi apparatus and the plasma membrane. They are also involved in the internalization of selected plasma membrane components during receptor-mediated endocytosis (12, 30). Isolated bovine brain coated vesicles contain ~100 copies (10) of a 180,000-dalton polypeptide called clathrin (28). Two proteins of 33,000 and 36,000 daltons are tightly bound to clathrin (16, 29) and comprise an assembly unit of the coat structure ("triskelion") (17, 40). Several other polypeptides have been shown to copurify with clathrin during various separation procedures (31, 34). The properties and precise functions of each of the coated vesicle polypeptides are only beginning to be understood. A 50,000-dalton polypeptide appears to be phosphorylated by a cAMP- and Ca^{++}-independent protein kinase activity associated with coated vesicles (14, 26). Little is known about the 54,000- and 56,000-dalton polypeptides or about a series of polypeptides between 100,000 and 150,000 daltons, which are present in coated vesicle preparations at stoichiometries comparable with those of the 33,000- and 36,000-dalton polypeptides (31).

In this report we identify the 54,000- and 56,000-dalton coated vesicle polypeptides as α- and β-tubulin. As would be expected, these two polypeptides are not unique to coated vesicles (31), yet they maintain a constant molar ratio relative to clathrin through several purification procedures and require harsh treatments for their dissociation from the coated vesicle structure.

We have confirmed the report of Pauloin et al. (26) and Kadota et al. (14) that a 50,000-dalton coated vesicle polypeptide can be phosphorylated by a kinase activity present in

THE JOURNAL OF CELL BIOLOGY · VOLUME 97 JULY 1983 40-47

bovine brain coated vesicle fractions, and have shown that the kinase activity remains after purification of coated vesicles by controlled pore glass column chromatography. We detect several additional phosphorylated polypeptides including α- and β-tubulin, and have made use of this endogenous phosphorylation activity to selectively label bovine brain coated vesicle components. An affinity-purified tubulin antiserum can precipitate in vitro-phosphorylated or iodinated coated vesicles. The tubulin appears to be associated with the 50,000-dalton coated vesicle polypeptide, since the 50,000-dalton polypeptide is immunoprecipitated by a tubulin-specific antiserum in concert with the tubulin polypeptides, even after complete disruption of coated vesicles. The 50,000-dalton, phosphorylated coated vesicle polypeptide is antigenically related but distinct from a microtubule-associated tau protein.

MATERIALS AND METHODS

Coated Vesicle Isolation: Coated vesicles were isolated from fresh bovine brains by our modification (31) of the Pearse procedure (27). Controlled pore glass column chromatography was carried out on a 200-ml CPG-10-2000 column. Bovine liver coated vesicles were isolated from 150 g of tissue essentially by a modification (4) of the procedure of Blitz et al. (1). Because of the high membrane content of liver cells it was necessary to use 15 tubes for the first sucrose gradients and 8 tubes for the second sucrose gradients for 150 g of tissue. The third sucrose gradients (four tubes) were continuous as originally described by Pearse (27).

Gel Electrophoresis: SDS PAGE was carried out according to Laemmli (20). When necessary, gel samples were concentrated by bringing the sample to a final concentration of 0.1 mg/ml deoxycholate and precipitating the sample with an equal volume of 10% trichloroacetic acid (TCA). After addition of 20 μl of sample buffer to the precipitate, samples were neutralized by adding 4 μl of 0.1 M NaOH before loading onto the gel. Gels were calibrated with proteins of known molecular weight: phosphorylase, 94,000; bovine serum albumin (BSA), 68,000; rabbit immunoglobulin heavy chain, 50,000; ovalbumin, 43,500; alcohol dehydrogenase, 37,000; and carbonic anhydrase, 30,000. Two-dimensional gel electrophoresis was done as described by O'Farrell (23), using pH range 5–8 ampholines (LKB Instruments, Inc., Gaithersberg, MD) instead of pH range 5–7 ampholines, and equilibrating the isoelectric focusing gel for 30 min. Isoelectric focusing gels were not prerun; second dimension gels were 8% polyacrylamide.

Immunoblotting: SDS polyacrylamide gels were transferred to unmodified nitrocellulose filters using an E-C "electroblot" apparatus (E-C Apparatus Corp., St. Petersburg, FL) set overnight at 6.5 V/cm. Filters (40 cm^2) were processed according to the procedure of Burnette (3) except that antibody incubations were carried out in folded Parafilm containers in volumes of 3 ml. Immune complexes were detected by using 10^6 cpm of an Iodogen (Pierce Chemical Co., Rockford, IL) iodinated immunoglobulin (Ig) G fraction of goat anti-rabbit Ig antiserum (Cappel Laboratories, Cochransville, PA). Kodak X-O-Mat-AR film was used for autoradiography.

Preparation of Affinity-purified Tau Protein Antiserum: Tubulin and microtubule-associated proteins were purified by the Weingarten et al. modification (41) of the Shelanski et al. procedure (36). Microtubule-associated proteins were fractionated away from tubulin by virtue of their heat stability (13); tau proteins, soluble after boiling, were further purified by excision of individual Coomassie Blue-stained polypeptide bands from SDS polyacrylamide gels. Approximately 0.2 mg of the gel-purified tau proteins was injected into each of three New Zealand white rabbits, which were then boosted after 4 and 6 wk. Two rabbits generated sera of high titer.

A tau protein affinity column was prepared as follows. Tau proteins were purified by the procedure of Cleveland et al. (6) followed by SDS gel electrophoresis and excision of individual Coomassie Blue -stained polypeptide bands. The eluted proteins were coupled to cyanogen bromide activated-Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, NJ).

Rabbit anti-tau sera were pre-adsorbed by passage over a column of gel-excised microtubule-associated protein (MAP) 1 and MAP 2 proteins coupled to Sepharose 4B, to remove antibodies that might react with any proteolytic fragments of these proteins. Sera were then passed over the tau-Sepharose column, which was washed successively with 50 mM Tris/Cl, pH 7.4, 1 M guanidine HCl, 50 mM Tris/Cl, pH 7.4, and then with a buffer containing 4.5 M $MgCl_2$, 2.5 mg/ml BSA, and 50 mM Tris/Cl, pH 7.4, as described (21). $MgCl_2$-eluted antibodies were dialyzed immediately into PBS (0.15 M NaCl, 20 mM Na phosphate, pH 7.5) and then into PBS containing 30% glycerol, in which they were stored at −20°C. The affinity-purified tau antibodies reacted exclusively with tau proteins when tested against total microtubule proteins by immunoprecipitation or immunoblotting procedures.

Phosphorylation and Immunoprecipitation: Phosphorylation reactions were carried out according to the procedure of Pauloin et al. (26) in volumes of 15–200 μl, using [γ-^{32}P]ATP (Amersham Corp., Arlington, Heights, IL) at 1,000 cpm/pmol sp act. Phosphorylation products were analyzed by SDS polyacrylamide gels or were quantitated as follows. Samples were brought to a final concentration of 1% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, Sigma Chemical Co., St. Louis, MO) to facilitate TCA precipitation, and 1 ml of cold 10% TCA was added. After 5 min on ice, samples were filtered through Whatman GF/C filters, rinsed with 20 ml of 10% TCA and 5 ml of 95% ethanol, dried, and counted in a toluene-based scintillation fluid. Under these conditions, incorporation of ^{32}P was linear for at least 30 min at 37°C, up to a final coated vesicle protein concentration of 0.5 mg/ml.

For immunoprecipitations, antisera were added directly to phosphorylation reaction mixes and incubated for 1 h at room temperature on a rotator. No attempt was made to terminate phosphorylation reactions before antisera addition. 25 μl of a 10% suspension of fixed, twice prewashed, *Staphylococcus aureus* cells (Pansorb, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) was added; incubations were continued for 15 more min at 20°C, and cells were pelleted at 12,500 *g* for 3 min in an Eppendorf microfuge. Pellets were washed three times in wash buffer (1% Nonidet P-40, 0.1% SDS, 10 mM Na phosphate, 0.4 M NaCl, pH 7.4) and then once in distilled water before addition of 20 μl of gel electrophoresis sample buffer. Samples were incubated at 100°C for 3 min, spun for 3 min in the microfuge, and resulting supernates were loaded onto a gel. Autoradiography of dried gels was carried out at −70°C with a Cronex Lightning Plus intensifying screen.

Coated Vesicle Iodination: Coated vesicles (0.1 mg) were iodinated using Iodogen in a volume of 55 μl with 0.5 mCi Na-^{125}I (Amersham Corp.) according to directions provided by the supplier. Unincorporated ^{125}I was separated from the coated vesicles by passage of the sample over a Sepharose 4B column prepared in PBS containing 0.1% BSA. Immunoprecipitation of ^{125}I-coated vesicles was done as described above for ^{32}P-labeled coated vesicles. To obtain matched controls, equivalent amounts of nonimmune and affinity-purified tubulin Ig were used. Equivalent Ig concentrations of the two sera were determined by a solid-phase radioimmune assay as follows: 10 consecutive twofold dilutions of the antisera were prepared in PBS, beginning with a 1:40 dilution. The 50-μl samples were pipetted into a polyvinyl 96-well, flexible microtiter U-well plate (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, VA) and allowed to bind for 1 h at room temperature. Wells were washed by flooding with PBS containing 0.1% BSA three times. 50 μl of an Iodogen-iodinated, IgG fraction of goat anti-rabbit Ig antiserum was added and allowed to bind for 40 min. Wells were washed as before, cut out, and then counted in a Beckman 4000 gamma counter (Beckman Instruments, Inc., Mountain View, CA). The initial slopes of the binding curves were directly proportional to the relative Ig concentrations, as confirmed by SDS gel electrophoresis and Coomassie Blue staining.

Gel Filtration of Tris-Cl Released Polypeptides: Approximately 6 mg of coated vesicles from the third sucrose gradient (27) was brought to a final concentration of 0.5 M Tris/Cl, pH 7.5, and centrifuged at 100,000 *g* for 60 min. The released polypeptides in the supernate were concentrated by addition of crystalline ammonium sulfate to 70% saturation and centrifugation at 12,500 *g* for 15 min. The precipitated polypeptides were resuspended in 1 ml of 0.5 M Tris/Cl and loaded onto a 50-ml Ultrogel Aca22 (LKB Instruments, Inc.) sizing column prepared in the same buffer. 1-ml fractions were collected and aliquots of each sample were analyzed by SDS PAGE. Coomassie Blue-stained gel bands were quantified by densitometry using a Hoeffer gel scanner; tracings were cut out and weighed to obtain relative amounts of specific polypeptides in column fractions. When necessary, several different volumes of a given sample were analyzed to ensure proportionality of the staining response.

Other: Protein concentrations were determined by Amido Schwarz staining (35) or by the procedure of Bradford (2) using reagents from Bio-Rad Laboratories (Richmond, CA). Coated vesicle antiserum was generously provided by Dr. R. G. W. Anderson, University of Texas Health Science Center, Dallas.

RESULTS

Identification of the 54,000 and 56,000-dalton Polypeptides as Tubulins

We have recently shown that fractionation of coated vesicles according to size by permeation chromatography can be used to identify polypeptide components of coated vesicles

(31). The majority of the coated vesicles elute as a single included peak from a controlled pore glass (CPG) column. Several polypeptides chromatograph at a constant stoichiometry relative to clathrin and are present in the coated vesicle peak and not in other fractions. By these criteria, we have argued that the 33,000- and 36,000-dalton clathrin-binding polypeptides, a 50,000-dalton polypeptide and a series of polypeptides in the molecular weight range of 100,000 to 150,000 are unique to coated vesicles (31). Two other prominent polypeptides of 54,000 and 56,000 daltons co-purify with clathrin on CPG columns but are not unique to coated vesicles: they are also present in the contaminating material excluded from the column (31). Fig. 1 shows the polypeptide composition of bovine brain coated vesicles included (Fig. 1 *a*) or contaminating membranes excluded (Fig. 1 *b*) from a CPG column.

The molecular weights and distribution in brain membranes of the 54,000- and 56,000-dalton polypeptides suggested that they might be tubulin. To investigate this possibility, the ability of the 54,000- and 56,000-dalton polypeptides to bind affinity-purified anti-α and anti-β-tubulin antiserum (8) was tested by the procedure of immunoblotting (3). Briefly, coated vesicle polypeptides were separated by SDS PAGE, electrophoretically transferred to nitrocellulose sheets, and incubated with the appropriate primary and secondary antibodies; immune complexes were detected by autoradiography of the filters. Two polypeptides of molecular weights near 55,000 present in both the included and excluded fractions reacted with this antiserum (Fig. 2 *a*). Incubation of duplicate filters in secondary antibody alone resulted in no detectable binding to included coated vesicle or excluded polypeptides; only the rabbit Ig present in the molecular weight standards bound the radiolabeled goat anti-rabbit Ig probe (Fig. 2 *b*).

Since the pattern of antibody binding was the same for both included and excluded material, it seemed likely that the antiserum recognized the 54,000- and 56,000-dalton polypeptides, which are not unique to coated vesicle-containing fractions. To be certain that the major 54,000- and 56,000-dalton polypeptides detected by Coomassie Blue staining were in fact the tubulins, the presence of Coomassie Blue-staining α- and β-tubulin in both included and excluded material was verified by an independent method. Figure 3 shows a two-dimensional gel of CPG column purified bovine brain coated vesicles. Both the included and excluded materials contain comparable amounts of the circled set of spots in Fig. 3*A* (31). As can be seen in Fig. 3, *B–D*, the molecular weights, pattern, and isoelectric points of these spots are identical to those for brain α- and β-tubulin, confirming that the 54,000- and 56,000-dalton polypeptides found in both included and excluded volume fractions are α- and β-tubulin.

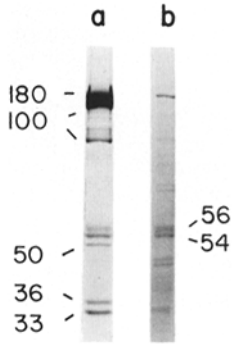

FIGURE 1 SDS PAGE of CPG column fractions. (*a*), bovine brain coated vesicles included in the column; (*b*), contaminating membranes excluded from the column. The numbers at left (× 1,000) correspond to molecular weights of polypeptides unique to coated vesicles; the numbers at right (× 1,000) correspond to molecular weights of polypeptides that are found in both included and excluded fractions. The gel was stained with Coomassie Blue.

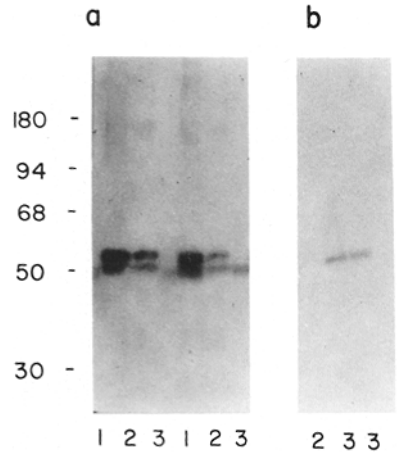

FIGURE 2 Immunoblot analysis of bovine brain coated vesicles. Samples of included material (*1*), excluded material (*2*), or molecular weight standards containing rabbit Ig (*3*) were separated on an 8.5% polyacrylamide gel and transferred electrophoretically onto unmodified nitrocellulose. Filters were incubated with (*a*) or without (*b*) an affinity-purified tubulin antiserum.

Because tubulin comprises a large fraction of the total protein in brain homogenates, its presence in coated vesicle fractions could easily have been artifactual. It was therefore important to determine the strength of the interaction between tubulin and the coated vesicle structure. In initial experiments, various reagents were tested for their ability to dissociate tubulin from coated vesicles. After treatment, coated vesicles were pelleted under standard conditions (1 h, 100,000 *g*), and the resultant supernates were assayed for the presence of the 54,000- and 56,000-dalton polypeptides by gel electrophoresis. Treatment with neither 1% CHAPS nor treatment with a mixture of 0.1% SDS and 1% Nonidet P-40 released detectable tubulin polypeptides. By this criterion, the tubulin was not binding to the lipid bilayer alone or trapped within membrane vesicles. 1 M Tris/Cl, pH 7.4, which re-

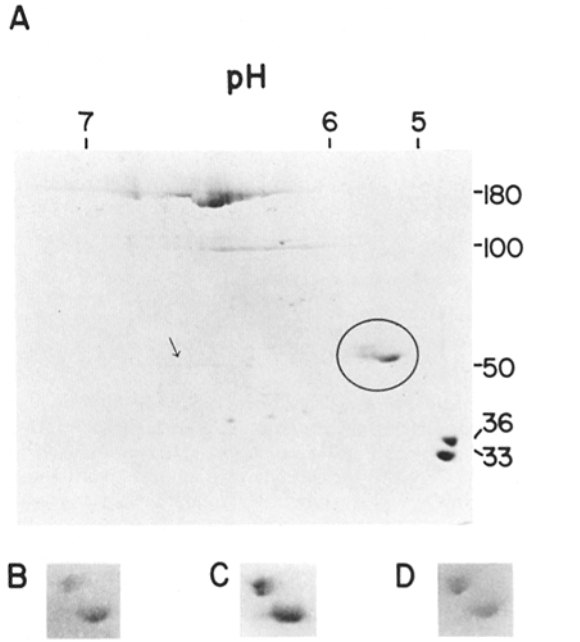

FIGURE 3 Coomassie Blue-stained, two-dimensional gels of CPG column-purified bovine brain coated vesicles (*A* and *B*), purified brain tubulins (*D*), or a mixture of samples *B* and *D* (C). The circled set of spots in *A* correspond to α- and β-tubulin; the arrow indicates the position of the 50,000-dalton coated vesicle polypeptide. Only portions of the gels are shown in *B–D*. The pH gradient was determined by slicing a duplicate isoelectric focusing gel into 0.5-cm slices, soaking each slice in 1 ml of H_2O, and measuring the pH of the resulting solutions.

moves ~50% of the triskelions (15) and 10 mM dithiothreitol (DTT) were ineffective in releasing tubulin. All of the tubulin was susceptible to trypsin degradation and was released with the other coated vesicle polypeptides after treatment with 6 M urea (data not shown). If tubulin had been released in a form that would sediment in 1 h at 100,000 *g*, we would not have detected its release. To minimize tubulin aggregation, these experiments were carried out at 4°C and at low protein concentrations, conditions that should favor tubulin depolymerization.

Tubulin Antiserum Can Precipitate Coated Vesicles

To determine whether the tubulin was directly associated with coated vesicles, the ability of an affinity-purified tubulin antiserum (8) to precipitate coated vesicles was tested. Intact coated vesicles, radiolabeled with ^{125}I, were incubated with various amounts of tubulin antiserum; immune complexes were precipitated by the addition of fixed *S. aureus* cells, and precipitates were extensively washed before analysis by SDS gel electrophoresis and autoradiography. The immunoprecipitation of coated vesicles was quantitated by densitometry of the ^{125}I-clathrin polypeptide on the autoradiograms. As can be seen in Fig. 4, the precipitation of ^{125}I-labeled coated vesicles was directly proportional to the amount of tubulin antiserum added, and was specific in that nonimmune serum precipitated only small amounts of radiolabeled clathrin. This indicated that at least some of the tubulin was directly associated with coated vesicles under the conditions of the immunoprecipitation.

Phosphorylation of Coated Vesicle Polypeptides

It has recently been reported that preparations of bovine brain coated vesicles contain a cAMP- and Ca^{++}-independent kinase activity (14, 26). Pauloin and co-workers (26) found that incubation of [γ-^{32}P]ATP with agarose gel-purified (34) coated vesicles resulted in the phosphorylation of a 50,000-dalton band, which they suggested might be the 50,000-dalton polypeptide unique to coated vesicles (31). A 50,000-dalton polypeptide is also phosphorylated in bovine brain coated vesicles isolated by permeation chromatography, and appears to co-purify with clathrin during size fractionation. Fig. 5 shows a CPG column profile of bovine brain coated vesicles; individual fractions were incubated with [γ-^{32}P]ATP, as described by Pauloin et al. (26), and analyzed by SDS PAGE. While the 50,000-dalton band is the most predominantly labeled, several other distinct polypeptides of molecular weights 35,000, 54,000, 56,000, 72,000, 104,000, and 140,000 show minor labeling, particularly in CPG column fractions. The autoradiogram in Fig. 5 is overexposed to permit visualization of the minor labeled polypeptides. Kadota and coworkers (14) also detected multiple phosphorylated polypeptides after in vitro phosphorylation. Two-dimensional gel electrophoresis of phosphorylated coated vesicles confirmed

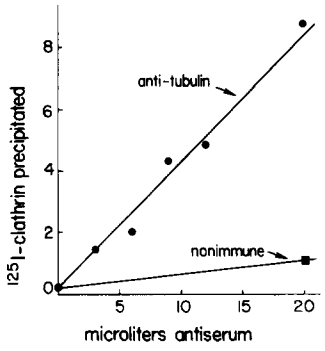

FIGURE 4 Immunoprecipitation of ^{125}I-labeled, intact brain coated vesicles with an affinity-purified tubulin antiserum. The amount of ^{125}I-clathrin precipitated is presented in arbitrary units, and was determined by using densitometry of autoradiograms after gel electrophoresis and autoradiography of each sample. ●, microliters of affinity-purified tubulin antibodies; ■, microliters of diluted nonimmune rabbit serum. The nonimmune serum was diluted 1:2,000 to achieve an equivalent concentration of immunoglobulin per milliliter, relative to the affinity-purified antiserum, as described in Materials and Methods.

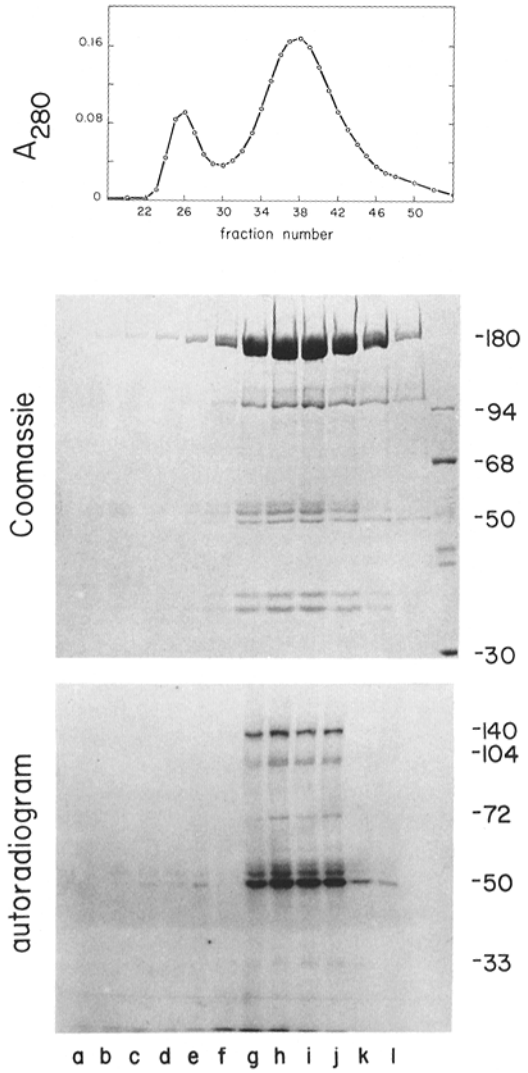

FIGURE 5 Phosphorylated proteins co-purify with clathrin during permeation chromatography. Approximately 4 mg of bovine brain coated vesicles obtained from the third sucrose gradient of the Pearse (27) purification procedure was loaded onto a 150-ml CPG-10-2000 column. 0.2 ml of individual fractions was analyzed by PAGE and Coomassie Blue staining (*center* panel) or was concentrated by centrifugation in a Beckman airfuge at 100,000 *g* for 60 min and phosphorylated by incubation with [γ-^{32}P]ATP (*lower* panel). Lanes *a–l* correspond to column fractions 22, 24, 26, 28, 30, 32, 35, 37, 39, 41, 44, and 47. The far right lane of the Coomassie Blue-stained gel (center panel) contains molecular weight markers described in Materials and Methods.

that the phosphorylated 50,000-, 54,000-, and 56,000-dalton polypeptides are identical to the prominent coated vesicle 50,000-dalton and tubulin polypeptides detected by Coomassie Blue staining (see Figs. 3 and 9). No phosphorylation of clathrin or the 33,000- and 36,000-dalton clathrin binding proteins was detected.

The 50,000-dalton Polypeptide Is Precipitated with the Tubulins after Coated Vesicle Disruption

We have shown that the tubulin antiserum can precipitate ^{125}I-labeled coated vesicles, using clathrin as a marker (Fig. 4). The phosphorylation activity endogenous to coated vesicles provides a sensitive means to confirm this result and to detect the presence of other specific coated vesicle components in immunoprecipitates. We have used this sensitive detection method to test whether tubulin antisera could also immunoprecipitate the phosphorylated 50,000-dalton and tubulin polypeptides in intact or disrupted coated vesicles. After incubating ^{32}P-labeled coated vesicles with antisera, immune complexes were precipitated as before, and analyzed by SDS gel electrophoresis and autoradiography. As can be seen in Fig. 6*a*, affinity-purified tubulin antibodies (8) precipitated a large amount of the 50,000-dalton, unique coated vesicle polypeptide in addition to labeled α- and β-tubulin. A Coomassie Blue-staining polypeptide of 180,000 daltons (clathrin) was detected in the immune complexes but not in the nonimmune controls (not shown). The relative proportion of labeled 50,000-dalton and tubulin polypeptides precipitated by the tubulin antiserum was comparable with that precipitated by an anti-coated vesicle antiserum (Fig. 6*c*), further confirming that the tubulin antiserum was able to precipitate coated vesicle components. The tubulin must have been associated with the coated vesicles to result in both tubulin and coated vesicle immunoprecipitation by both of these antisera. It is unlikely that the radiolabeled 50,000-dalton polypeptide was itself recognized by the tubulin antiserum, since the 50,000-dalton polypeptide did not display tubulin antibody binding in our immunoblotting experiments (Fig. 2*a*). These data further suggest that tubulin is strongly associated with isolated coated vesicles.

Microtubules could associate with coated vesicles by nonspecific sticking. To reduce such sticking in the above experiment, immunoprecipitates were washed with 0.1% SDS and 1% Nonidet P-40, under conditions that do not disrupt the clathrin coat. We tested further the strength of the association of the 50,000-dalton polypeptide with the tubulin polypeptides by first incubating the coated vesicles with 0.5% SDS and 1% Nonidet P-40, diluting the mixture, and then incubating it with tubulin antiserum. 0.5% SDS/1% Nonidet P-40 treatment dissociates virtually all of the clathrin and 33,000- and 36,000-dalton clathrin-binding polypeptides from the residual vesicle structure; this detergent mixture probably acts as a mild denaturing agent at these concentrations. As shown in Fig. 6*d*, while the tubulins comprise a larger fraction of the precipitated material, the 50,000-dalton polypeptide is still concomitantly precipitated after this harsh treatment. This experiment once again confirms that the 54,000- and 56,000-dalton polypeptides that become phosphorylated are α- and β-tubulin since they are enriched when precipitated by specific antisera under increasingly stringent reaction conditions. The tubulin molecules must be tightly associated with the 50,000-dalton polypeptide to result in co-precipitation even under these conditions.

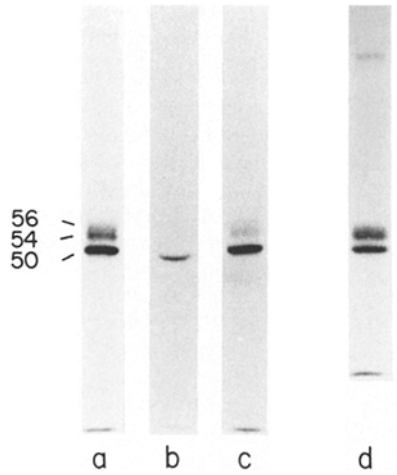

FIGURE 6 Immunoprecipitation of ^{32}P-coated vesicles with various antisera. 100 μg of sucrose gradient 3 coated vesicles (27) was phosphorylated in a volume of 60 μl for 30 min at 37°C. In an attempt to reduce background, samples were pretreated with 50 μl of a 10% suspension of *S. aureus* cells for 10 min at room temperature; samples were centrifuged for 2 min in an Eppendorf microfuge to remove the added *S. aureus* cells. 60 μl of PBS containing 1% BSA was added to the pretreated sample; portions were then incubated with (*a*) 20 μl of affinity-purified tubulin antibodies (6), (*b*) 20 μl of nonimmune rabbit serum to detect nonspecific binding, or (*c*) 1.25 μl of coated vesicle antiserum. The immunoglobulin protein concentrations in samples (*a*) and (*c*) are not equivalent; however, sample (*b*) contained the most immunoglobulin. Lane *d*: 20 μl of 1% SDS/2% Nonidet P-40 was added to 20 μl of ^{32}P-coated vesicles. This mixture was brought to a volume of 0.24 ml with PBS containing 0.05% Nonidet P-40 and 0.25% BSA. 20 μl of affinity-purified tubulin antiserum was added and the sample was processed as described above. All *S. aureus* cell suspensions were washed two times in wash buffer before use. Electrophoresis and autoradiography were carried out as described in Materials and Methods.

Tau Antiserum Recognizes the Phosphorylated 50,000-dalton Polypeptide

Since the 50,000-dalton polypeptide can associate with tubulin, it might resemble one of the previously described microtubule-associated proteins. The first candidates examined were the tau proteins, a family of closely related proteins that range in molecular weight from 55,000 to 62,000 (6, 7, 41). These proteins co-assemble with microtubules and can induce assembly of purified tubulin into microtubules in vitro; they have also been shown to be associated with microtubules in vivo (9, 24). Fig. 7 shows an immunoprecipitation of phosphorylated coated vesicles after denaturation in 1% SDS and boiling, using an affinity-purified tau protein antiserum. While nonimmune serum failed to precipitate any significant amount of radioactivity (Fig. 7*b*), the tau antibodies specifically precipitated the phosphorylated 50,000-dalton polypeptide, in addition to small amounts of two slightly larger polypeptides (Fig. 7*a*). The two faint bands that migrate slower than the 50,000-dalton polypeptide probably represent other minor tau proteins, since their mobilities are somewhat different from those of α- and β-tubulin.

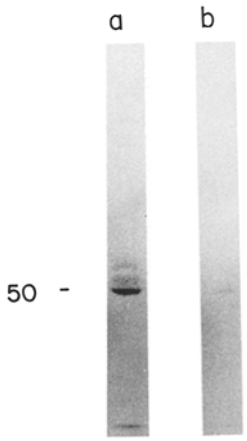

FIGURE 7 Immunoprecipitation of ^{32}P-coated vesicles with an affinity-purified tau antiserum. 15 μl of PBS containing 2% SDS was added to 15 μl of ^{32}P-coated vesicles, and the mixture was boiled for 2 min at 100°C, diluted fivefold in wash buffer, and processed essentially as described in Fig. 6. (*a*) 40 μl of affinity-purified tau protein antiserum; (*b*) 0.5 μl of nonimmune rabbit serum. Samples *a* and *b* contained equivalent amounts of immunoglobulin as determined by SDS gel electrophoresis and Coomassie Blue staining.

The 50,000-dalton Polypeptide May Also Associate with the 100,000-Dalton Coated Vesicle Components

We have preliminary evidence that the 50,000-dalton protein may associate not just with tubulin but with the 100,000-dalton components of coated vesicles, as well. We observe that while Tris/Cl treatment releases primarily triskelions (16, 40) a small fraction of the total 50,000- and 100,000-dalton polypeptides is also released from coated vesicles. Tubulin is not released (see above). Fig. 8 shows a profile obtained after chromatographic fractionation of Tris/Cl-released coated vesicle polypeptides on an Ultrogel sizing column. A peak of the 50,000-dalton polypeptide co-chromatographs with the 100,000-dalton polypeptide at a position consistent with an aggregate molecular weight, since the 50,000-dalton polypeptide elutes ahead of a higher-molecular-weight protein (68,000) present in the sample. The tau-like polypeptide may therefore be attached in the coated vesicles to the 100,000-dalton polypeptide. Evidence has already been presented (39) that the 100,000-dalton polypeptide may anchor triskelions to coated vesicles. Because none of the tubulin is released by Tris/Cl treatment, the small fraction of 50,000-dalton polypeptides that is released is presumably not bound to tubulin.

Several polypeptides appear to streak across the two-dimensional gel shown in Fig. 3*A*, including clathrin, polypeptides in the 100,000 molecular weight range, and a band at 50,000 daltons, while the 33,000-dalton, 36,000-dalton, and tubulin polypeptides are completely dissociated and migrate to distinct isoelectric points. Multiple forms of clathrin, 50,000- and 100,000-dalton polypeptides might explain the streaking. Alternatively, aggregates containing variable amounts of these polypeptides may be incompletely dissociated in the 9 M urea and 1% Nonidet P-40 used in the first-dimension isoelectric focusing, consistent with a 50,000/100,000 dalton complex as described above, which perhaps associates with clathrin under these dissociation conditions. Cross-linking experiments will be necessary to confirm this point.

Liver Coated Vesicles Also Phosphorylate Endogenous 50,000-dalton and Tubulin Polypeptides

Brain is particularly rich in α- and β-tubulin, thus it was of interest to investigate whether coated vesicles from another tissue possessed analogous tubulin and 50,000-dalton polypeptides, in addition to an endogenous kinase activity. To address this question, liver coated vesicles were incubated with [γ-^{32}P]ATP under the same phosphorylation conditions used for brain coated vesicles, and the reaction mixture was analyzed by two-dimensional gel electrophoresis. As can be seen in Fig. 9, liver coated vesicles do indeed possess an endogenous kinase activity. Perhaps more surprising was the finding that the major phosphorylated polypeptides were identical in molecular weights and isoelectric points to the phosphorylated brain 50,000-dalton and tubulin polypeptides (Fig. 9). Thus, in addition to phosphorylation activity, the presence of a 50,000-dalton polypeptide and α- and β-tubulin were common to both brain and liver coated vesicles.

DISCUSSION

Proteins with molecular weights in the range of 50,000–55,000 have been reported in all brain coated vesicle preparations (see references 1, 15, 29, 31, 34, 42). We have identified the 54,000- and 56,000-dalton coated vesicle polypeptides as α- and β-tubulin, using an affinity-purified antiserum in conjunction with immunoblotting and by two-dimensional gel electrophoresis. Several types of experiments indicate that the

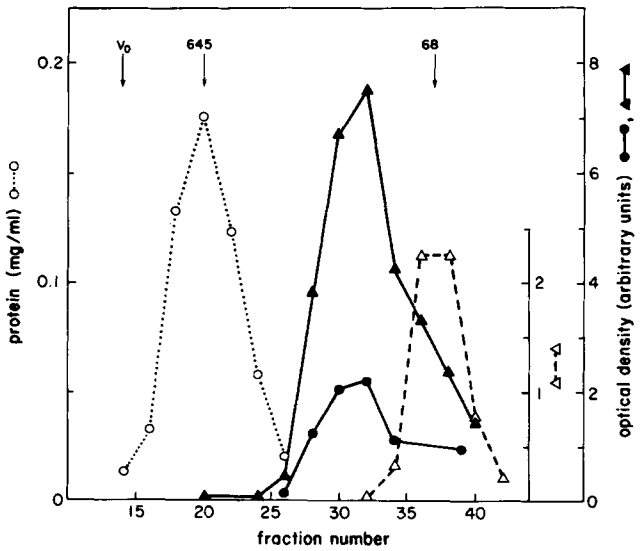

FIGURE 8 Gel filtration of Tris/Cl-released coated vesicle polypeptides. Gel filtration and elution profile quantitation were carried out as described in Materials and Methods. The integrated optical densities are shown in arbitrary units: ▲, 100,000-dalton polypeptide; ●, 50,000-dalton polypeptide; Δ, 68,000-dalton polypeptide present in the sample. The profile for marker triskelions run on a parallel column is also shown (○ 645,000 daltons). V_0 equals void volume.

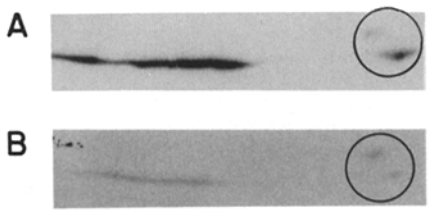

FIGURE 9 Two-dimensional gel electrophoresis of ^{32}P-labeled bovine brain (*A*) and bovine liver (*B*) coated vesicles. Phosphorylation, two-dimensional gel electrophoresis, and autoradiography were carried out as described in Materials and Methods. The acidic end of the gel is shown at the right. Spots corresponding to α- and β-tubulin are circled (see also Figs. 3 and 5).

tubulin is strongly associated with the coated vesicles. We have found that α- and β-tubulin co-purify with clathrin during permeation chromatography (31) and also during electrophoresis in a Ficoll gradient (not shown). Although tubulin has been reported to bind to various membrane preparations (see references 37 and 43) and phospholipid vesicles (5, 19), tubulin cannot be released from the coated vesicles by 1% CHAPS, a zwitterionic detergent, or by treatment with 1% Nonidet P-40/0.1% SDS. It is therefore unlikely that the tubulin is interacting nonspecifically with the lipid bilayer. All of the tubulin is susceptible to digestion by trypsin and thus is not trapped within the membranes of the coated vesicles. While a large fraction of the clathrin and the 33,000- and 36,000-dalton clathrin binding proteins are released by 1 M Tris/Cl, pH 7.4, (15, 17), none of the tubulin is released, indicating that the tubulin is not bound to or trapped by triskelions. Although tubulin contains multiple free sulfhydryl groups (see reference 22) which might have formed disulfide bonds to free sulfhydryl groups on coated vesicles, 10 mM DTT did not release any of the tubulin polypeptides from the coated vesicles as determined by a sedimentation assay. Hence, tubulin appears to form a strong noncovalent association with coated vesicles.

Affinity-purified tubulin antiserum precipitated ^{125}I-labeled coated vesicles, using clathrin as a marker, under conditions where nonimmune serum did not (Fig. 4), indicating some association under the conditions of our immuneprecipitation. Tubulin antiserum precipitated the 50,000-dalton, unique coated vesicle polypeptide even after complete disruption of the coated vesicles with 0.5% SDS/1% Nonidet P-40. Because coated vesicles are disrupted by this detergent treatment, it appears that the tubulin polypeptides interact either directly with the 50,000-dalton polypeptide or with some other unlabeled component tightly bound to the 50,000-dalton polypeptide.

Several lines of evidence suggest that the 50,000-dalton phosphorylated polypeptide is a true coated vesicle component. The 50,000-dalton polypeptide has been shown to co-purify with clathrin during permeation chromatography (31), agarose gel electrophoresis (34), and electrophoresis in Ficoll gradients (S. Pfeffer, unpublished results), and is present in coated vesicles isolated by sucrose (29), metrizamide (26), and D_2O/Ficoll (S. Pfeffer, unpublished results) equilibrium density centrifugation. It is unique to coated vesicles in that it is detected only in fractions containing coated vesicles, after initial purification steps (31). Furthermore, a fraction of it co-chromatographs with the 100,000-dalton coated vesicle polypeptides after coated vesicle disruption (Fig. 8).

The 50,000-dalton phosphorylated polypeptide that is tightly associated with α- and β-tubulin is recognized by an affinity-purified tau protein antiserum. The major tau proteins are a family of four microtubule-associated proteins with molecular weights ranging from ~55,000 to 62,000. Other tau proteins of 50,000 and 53,000 daltons have recently been identified (D. G. Drubin and M. W. Kirschner, manuscript in preparation). However, the 50,000-dalton coated vesicle polypeptide is distinct from the authentic 50,000-dalton tau protein since a comparable amount of the 50,000-dalton coated vesicle polypeptide binds less tau antiserum than the 50,000-dalton tau protein as detected by a solid-phase radioimmunoassay or immunoblotting procedures (not shown). It is most likely that the coated vesicle 50,000-dalton polypeptide is a relative of the tau protein family, perhaps by virtue of a conserved tubulin-binding domain. It is highly unlikely that coated vesicles are contaminated with random microtubules since in that case at least four tau species would be expected to be present.

Densitometer scans of Coomassie Blue-stained gels indicate that CPG column purified bovine brain coated vesicles contain ~22 and 28 molecules of α- and β-tubulin, respectively, and ~18 molecules of the 50,000-dalton polypeptide/100 clathrin molecules. This amount of α- and β-tubulin may be less or more than that associated with coated vesicles physiologically. We also do not know whether all brain coated vesicles contain these polypeptides. It will be important to localize the coated vesicle-associated tubulin and 50,000-dalton polypeptides by electron microscopy in conjunction with specific antisera.

We have obtained evidence that a kinase phosphorylates the 50,000-dalton polypeptide that is unique to bovine brain coated vesicles, as suggested by Pauloin et al. (26). Phosphorylation is another feature common to both the 50,000-dalton polypeptide and the family of tau proteins (7, 24). We also observed minor labeling of several distinct polypeptides of molecular weights 140,000, 104,000, 72,000, α- and β-tubulin, and very light labeling of two polypeptides near 35,000 daltons. We have also detected kinase activity in preparations of bovine liver coated vesicles; interestingly, the major phosphorylated polypeptides have the same molecular weights and isoelectric points as the 50,000-dalton polypeptide and tubulins detected in brain coated vesicles (Fig. 9). Because of the identity of the major phosphorylated proteins, it is possible that the kinase activity associated with coated vesicles is involved in regulating their microtubule association.

Tubulin appears to be linked to a 50,000-dalton, tau-like, phosphorylated coated vesicle polypeptide, which may itself be anchored to coated vesicles by association with the 100,000-dalton polypeptides. Since the 100,000-dalton, 50,000-dalton, and tubulin polypeptides appear to remain associated under conditions that remove at least 50% of the triskelions, it is reasonable to suggest that the coated vesicle coat consists both of clathrin triskelions and of a coat substructure of 50,000 and 100,000-dalton polypeptides, to which the triskelions bind.

While microtubules do not appear to be involved in adsorptive pinocytosis (38), microtubule-disrupting agents do interfere with the process of secretion in many cell types, at some point before exocytosis (see references 25, 32, 33). Coated vesicles are thought to mediate such secretory events as the transport of certain proteins from the endoplasmic reticulum to the Golgi apparatus, and transport from the Golgi apparatus to the plasma membrane. Franke and coworkers have detected the association of casein-containing coated vesicles with microtubules in lactating rat mammary epithelial cells (11). Our identification of a coated vesicle-tubulin linkage suggests that proteins in the coat might interact with microtubules during the intracellular transport processes carried out by these organelles.

Special thanks are extended to Dr. Marc W. Kirschner for his enthusiastic support, stimulating discussions, and critical reading of this manuscript. We thank Leslie Spector for preparation of this manuscript.

This research was supported by research grants NS 09878 and NS 15927 to R.B.K. from the National Institutes of Health. S.R.P. and D.G.D. were predoctoral trainees of the National Institutes of Health (grant 5 T32 CA09270).

Received for publication 6 January 1983, and in revised form 9 March 1983.

REFERENCES

1. Blitz, A. L., R. E. Fine, and P. A. Toselli. 1977. Evidence that coated vesicles isolated from brain are calcium-sequestering organelles resembling sarcoplasmic reticulum. *J. Cell Biol.* 75:135–147.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
3. Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195–203.
4. Campbell, C. H., R. E. Fine, J. Squicciarini, and L. H. Rome. 1983. Coated vesicles from rat liver contain cryptic mannose-6-phosphate receptors. *J. Biol. Chem.* 258:2628–2633.
5. Caron, J., and R. D. Berlin. 1979. Interaction of microtubule proteins with phospholipid vesicles. *J. Cell Biol.* 81:665–671.
6. Cleveland, D. W., S. Y. Hwo, and M. W. Kirschner. 1977. Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin. *J. Mol. Biol.* 116:207–225.
7. Cleveland, D. W., S. Y. Hwo, and M. W. Kirschner. 1977. Physical and chemical properties of purified tau factor and the role of tau in microtubule assembly. *J. Mol. Biol.* 116:227–247.
8. Cleveland, D. W., M. A. Lopata, P. Sherline, and M. W. Kirschner. 1981. Unpolymerized tubulin modulates the level of tubulin mRNAs. *Cell.* 25:537–546.
9. Connolly, J. A., V. I. Kalnins, D. W. Cleveland, and M. W. Kirschner. 1977. Immunofluorescent staining of cytoplasmic and spindle microtubules in mouse fibroblasts with antibody to tau protein. *Proc. Natl. Acad. Sci. USA* 74:2437–2440.
10. Crowther, R. A., J. T. Finch, and B. M. F. Pearse. 1976. On the structure of coated vesicles. *J. Mol. Biol.* 103:785–798.
11. Franke, W. W., M. R. Lüder, J. Kartenbeck, H. Zerban, and T. W. Keenan. 1976. Involvement of vesicle coat material in casein secretion and surface regeneration. *J. Cell Biol.* 69:173–195.
12. Goldstein, J. L., R. G. W. Anderson, and M. S. Brown. 1979. Coated pits, coated vesicles and receptor mediated endocytosis. *Nature (Lond.).* 279:679–685.
13. Herzog, W., and K. Weber. 1978. Fractionation of brain microtubule-associated proteins: isolation of two different proteins which stimulate tubulin polymerization in vitro. *Eur. J. Biochem.* 92:1–8.
14. Kadota, K., M. Usami, and A. Takahashi. 1982. A protein kinase and its substrate associated with the outer coat and the inner core of coated vesicles from bovine brain. *Biomed. Res.* 3:575–577.
15. Keen, J. H., M. C. Willingham, and I. Pastan. 1979. Clathrin-coated vesicles: isolation, dissociation and factor dependent reassociation of clathrin baskets. *J. Biol. Chem.* 256:2538–2544.
16. Keen, J. H., M. C. Willingham, and I. H. Pastan. 1979. Clathrin and coated vesicle proteins: immunological characterization. *Cell.* 16:303–312.
17. Kirchhausen, T., and S. C. Harrison. 1981. Protein organization in clathrin trimers. *Cell.* 23:755–761.
18. Reference deleted in proof.
19. Klausner, R. D., N. Kumar, J. N. Weinstein, R. Blumenthal, and M. Flavin. 1981. Interaction of tubulin with membrane vesicles. I. Association with vesicles at the phase transition. *J. Biol. Chem.* 256:5879–5885.
20. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:580–685.
21. Mains, R. E., and B. A. Eipper. 1976. Biosynthesis of adrenocorticoropic hormone in mouse pituitary tumor cells. *J. Biol. Chem.* 251:4115–4120.
22. Mellon, M. G., and L. I. Rebhun. 1976. Sulfhydryls and the in vitro polymerization of tubulin. *J. Cell Biol.* 70:226–238.
23. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
24. Pallas, D., and F. Solomon. 1982. Cytoplasmic microtubule-associated proteins: phosphorylation at novel sites is correlated with their incorporation into assembled microtubules. *Cell.* 30:407–414.
25. Patzelt, C., D. Brown, and B. Jeanrenaud. 1977. Inhibitory effect of colchicine on amylase secretion by rat parotid glands. Possible localization in the Golgi area. *J. Cell Biol.* 73:578–593.
26. Pauloin, A., I. Bernier, and P. Jollès. 1982. Presence of cyclic nucleotide-Ca^{++} independent protein kinase in bovine brain coated vesicles. *Nature (Lond.).* 298:574–576.
27. Pearse, B. M. F. 1975. Coated vesicles from pig brain: purification and biochemical characterization. *J. Mol. Biol.* 97:93–98.
28. Pearse, B. M. F. 1976. Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. *Proc. Natl. Acad. Sci. USA* 73:1255–1259.
29. Pearse, B. M. F. 1978. On the structural and functional components of coated vesicles. *J. Mol. Biol.* 126:803–812.
30. Pearse, B. M. F., and M. S. Bretscher. 1981. Membrane recycling by coated vesicles. *Annu. Rev. Biochem.* 50:85–101.
31. Pfeffer, S. R., and R. B. Kelly. 1981. Identification of minor components of coated vesicles by use of permeation chromatography. *J. Cell Biol.* 91:385–391.
32. Reaven, E. P., and G. M. Reaven. 1980. Evidence that microtubules play a permissive role in hepatocyte very low density lipoprotein secretion. *J. Cell Biol.* 84:28–39.
33. Redman, C. M., D. Banerjee, K. Howell, and G. E. Palade. 1975. Colchicine inhibition of plasma protein release from rat hepatocytes. *J. Cell Biol.* 66:42–59.
34. Rubenstein, J. L. R., R. E. Fine, B. D. Lusky, and J. E. Rothman. 1981. Purification of coated vesicles by agarose gel electrophoresis. *J. Cell Biol.* 89:357–361.
35. Schaffner, W., and C. Weissman. 1973. A rapid, sensitive and specific method for the determination of protein in dilute solution. *Anal. Biochem.* 56:502–514.
36. Shelanski, M. L., F. Gaskin, and C. R. Cantor. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. USA.* 70:765–768.
37. Sherline, P., Y.-C. Lee, and L. S. Jacobs. 1977. Binding of microtubules to pituitary secretory granules and secretory granule membranes. *J. Cell Biol.* 72:380–389.
38. Steinman, R. M., and Z. A. Cohn. 1972. The interaction of soluble horseradish peroxidase with mouse peritoneal macrophages in vitro. *J. Cell Biol.* 55:186–204.
39. Unanue, E. R., E. Ungewickell, and D. Branton. 1981. The binding of clathrin triskelions to membranes from coated vesicles. *Cell.* 26:439–446.
40. Ungewickell, E., and D. Branton. 1981. Assembly units of clathrin coats. *Nature (Lond.).* 289:420–422.
41. Weingarten, M. D., A. H. Lockwood, S. Y. Hwo, and M. W. Kirschner. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA* 72:1858–1862.
42. Woodward, M. P., and T. F. Roth. 1978. Coated vesicles: characterization, selective dissociation and reassembly. *Proc. Natl. Acad. Sci. USA* 75:4394–4398.
43. Zisapel, N., M. Levi, and I. Gozes. 1980. Tubulin: an integral protein of mammalian synaptic vesicle membranes. *J. Neurochem.* 34:26–32.