Tubulin as a Molecular Component of Coated Vesicles

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ABSTRACT Two proteins of 53,000 and 56,000 mol wt have been found to be associated with coated vesicles (CV) purified from bovine brain and chicken liver. These proteins share molecular weights, isoelectric points, and antigenic determinants with α- and β-tubulins purified from bovine brain. Based on SDS PAGE and electron microscopic analysis of controlled pore glass bead exclusion column fractions, both the tubulins and the major CV polypeptide clathrin were found to chromatograph as components of a single kinetic particle. In addition, tubulin and CV antigens assayed by a sensitive enzyme-linked-immunoadsorbent method eluted from the columns with constant stoichiometry. These data provide evidence that tubulin is a molecular component of coated vesicles.

CV[1] are transient intracellular structures derived from coated endocytic pits (33, 41). Found in virtually all cell types, they are recognized morphologically by the presence of an ordered array of material coating the cytoplasmic surface (9, 57) and biochemically by the presence of clathrin, a 180,000-mol-wt protein (30, 31). The central role of CV in receptor-mediated endocytosis has been well documented (11, 43). In addition, CV also appear to play a role in the intracellular shuttling of proteins from the endoplasmic reticulum to the Golgi and plasma membrane (44).

CV have been isolated from many different tissues and cell types (24, 31, 57). Regardless of the initial source, one of the predominant protein bands observed when isolated CV are analyzed by SDS PAGE is invariably the 180,000-mol-wt protein clathrin. When CV are dissociated with Tris or urea, one prominent structure is seen as a triskelion-shaped complex composed of three 180,000-mol-wt clathrin molecules and three light chains of 32,000 and 36,000 mol wt (17, 54). Other proteins that are generally observed by SDS PAGE include several in the 100,000–120,000-mol-wt range and three in the 50,000–55,000-mol-wt area (15, 16, 57). The role of these accessory proteins is not known although it has been suggested that the 110,000-mol-wt protein may be a membrane-bound clathrin-binding protein that plays a role in the assembly of clathrin subunits to form the cagelike structure (53).

CV have been observed in association with various elements of the cytoskeleton. Recent morphological findings have suggested an association among coated structures, MT (49) and actin filaments (46). Presumably such associations may be illustrative of the mechanism by which CV shuttle from the cell surface to specifically targeted intracellular compartments. In addition to the morphological observations, the similarity of molecular weights between α- and β-tubulins and two of the proteins of 50,000–55,000 mol wt invariably associated with isolated CV led us to investigate the possibility that these two proteins might be tubulin and to question the nature of their association with CV.

We used a variety of purification techniques, in particular the CPG bead chromatographic procedure of Pfeffer and Kelly (35), to explore more fully the association between tubulin and coated structures isolated from either bovine brain or chicken liver. The relative amounts of various CV proteins associated with each of the fractions obtained by CPG chromatography were calculated using a number of very sensitive enzyme-linked immunoassays developed in our laboratory.

MATERIALS AND METHODS

Purification of CV and MT: CV from bovine brain were purified by the method of Nandi et al. (27). CV from chicken liver were obtained using the methods of either Pearse (32) or Nandi et al. (27). Bovine brain MT were purified using the procedure described by Shelanski et al. (47). The protein

[1] *Abbreviations used in this paper:* anti-CV, anti–coated vesicle antibody; anti-LC, anti–light chain antibody; CPG, controlled pore glass; CV, coated vesicles; ELISA, enzyme-linked immunoadsorbent assays; HRP, horseradish peroxidase; MAP-2, microtubule-associated protein-2; MT, microtubules; pI, isoelectric point; S-1000, Sephacryl S-1000.

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concentration of each preparation was determined by the Lowry method (22). The purity of each preparation was determined using SDS PAGE according to Laemmli (21) and by electron microscopy of negatively stained samples.

Two-dimensional Gel Electrophoresis: Two-dimensional electrophoresis of CV and MT samples was accomplished by the Ames and Nikaido (2) modification of the method of O'Farrell (28). Typically, 25–80 μg protein samples were first isoelectric-focused in 4% acrylamide tube gels (12 cm × 3.5 mm). Gels contained 2% Nonidet P-40, 8 M urea (Ultra-pure; Schwarz-Mann, Orangeburg, NY) and 2% carrier ampholytes (LKB Instruments, Gaithersburg, MD). The ampholyte profile used was 3 parts pH 4–6, 2 parts pH 7–9, 1 part pH 5–7, and 1.5 parts pH 3.5–10. Gels were focused for a minimum of 9,000 V-h at 400–500 V and then for an additional hour at 1,000 V. To prevent reaggregation of proteins prior to entering the gels in the first dimension, some CV samples were modified through alkylation using either *N*-ethyl maleimide or iodoacetamide according to the method of Dreesman and Benedict (10).

After isoelectric-focusing, tube gels were equilibrated for 1 h in two changes of two times Laemmli sample buffer. They were then cast in place horizontally across a 10% Laemmli SDS PAGE slab gel with 1% agarose in 0.125 M Tris, pH 6.8, and 0.2% SDS. After electrophoresis, the slab gels were stained with silver using the procedure of Merril et al. (25).

Gels containing proteins of known isoelectric points (pI) (pI markers; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) were cofocused with sample gels in the first dimension to determine the pH distribution. The pI markers were then visualized by Coomassie Blue staining of the gels. Alternatively, gels were cut into 1-cm segments and equilibrated in dH_2O, and the pH of each solution was determined.

Permeation Chromatography: CV samples were further purified by permeation chromatography on a CPG bead column by the method of Pfeffer and Kelly (35). Typically, 100 ml of CPG-2,000 glass beads (mean pore diameter 1,902 Å, 120–200 mesh, lot #10B07; Electro-Nucleonics Inc., Fairfield, NJ) was treated with 0.45 μ-filtered 1% (wt/vol) polyethyl glycol (20,000 mol wt). After being swirled and allowed to settle five times, the mixture was decanted and the beads were rinsed with distilled H_2O at least five times. The beads were suspended in 300 ml column buffer (10 mM Hepes, pH 7.0, 0.2 M sucrose, 0.3 M NaCl, 10 mM EGTA, 0.02% $NaNO_3$) and thoroughly degassed. The beads were packed with vibration into a 0.9 × 58 cm column. A 1 ml sample (1–2 mg/ml) was applied to the column, eluted at a rate of 3 ml/h and 1 ml fractions collected.

Immunoassays: ELISA were carried out essentially as described by Raugi et al. (38). For a typical assay, 100-μl aliquots of the solutions to be tested for the presence of antigen were allowed to adsorb to 96-well vinyl microtiter plates for 1 h at 25°C. The antigen solution was removed, and 200 μl of PBS containing 2 mg/ml BSA (PBS-BSA) (Sigma Chemical Co., St. Louis, MO) were added to the wells and incubated for an additional 60 min to saturate all protein-binding sites. For direct-binding assays, 100 μl of appropriately diluted primary antibody were incubated in the treated wells for 2 h at 37°C. Primary antibody solutions were diluted such that the amount of antibody present was in excess of that needed to bind all the vinyl-bound antigen. After a brief wash with PBS, the wells were incubated with 100 μl of an HRP-conjugated second antibody for 2 h at 37°C. Where mouse antibodies were used as primary probes, rabbit anti-mouse IgG-HRP was used as the second antibody at a final dilution of 1:2,000 in PBS-BSA. Where rabbit antibodies were used as primary probes, goat anti-rabbit IgG-HRP was used at a final dilution of 1:2,000 (Sigma Chemical Co.). The bound HRP concentration, and hence indirectly the concentration of the vinyl-bound antigen, was measured spectrophotometrically by monitoring the HRP-mediated conversion of a clear substrate solution into a colored product. Following incubation with the HRP-conjugated second antibody, the wells were washed with 5 cycles of filling and draining the wells with a wash buffer (PBS containing 2 mM EDTA, 0.1% Triton X-100, and 1.0% normal horse serum) followed by two rinses with PBS. 150 μl of substrate solution containing 0.04% phenylenediamine, 0.012% H_2O_2 in 0.1 M dibasic $NaPO_4$, 0.05 M citrate pH 5.0 were added to each well and incubated for 30 min at 25°C. The reaction was stopped by adding 20 μl of 10% SDS to each well. The amount of product formed was measured by recording the optical density at 490 nm with a Dynatech plate scanner (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, VA).

Competitive ELISA assays were used to characterize our antibody probes. Competitive assays were carried out exactly as the direct assays except that the primary antibody probes were preincubated with aliquots of the test solutions for 2 h at 37°C. The preincubated solutions were then incubated in vinyl plates that previously had been coated with a solution known to contain the antigen being quantitated. The subsequent procedures were carried out exactly as described for the direct assays. It is important to note that in the competition format, the free antigen in the preincubation is competing with the vinyl-bound antigen for antibody binding. Thus, a number of control experiments with each antibody and antigen were performed to insure that all our experiments were carried out under antibody-limiting conditions.

We used several antibodies as primary probes in our ELISA assays. Tubulin was detected with either a rabbit anti–bovine brain tubulin kindly provided by Dr. Thomas Pollard (Johns Hopkins University) or a rabbit anti–chicken brain tubulin purchased from Miles Laboratories, Inc. (Elkhart, IN). These antibodies were used at final dilutions of 1:1,000 and 1:400 respectively. To detect CV proteins we used two different mouse monoclonal antibodies directed against CV determinants. One antibody (anti-LC) was produced by the mouse monoclonal cell line CVC1 obtained from the American Type Culture Collection (Rockville, MD). Anti-LC has been shown by immunoelectroblots to be directed against the 36,000-mol-wt light chain polypeptide of clathrin triskelions isolated from bovine brain (7, 18). In our experiments this antibody showed no cross-reactivity with CV isolated from chicken liver. Two different preparations of anti-LC were used in our experiments. In some experiments anti-LC was prepared by ammonium sulfate precipitation and DEAE-Affigel Blue chromatography (Bio-Rad Laboratories, Richmond, CA) from media obtained after growing CVC1 cells in culture. Alternatively anti-LC from ascites fluid was used without further purification. In the latter case, 10^6 cells of CVC1 were injected intraperitoneally into Pristane-primed mice to establish an ascites tumor. Ascites fluid was collected daily after 7–9 d.

Another monoclonal antibody directed against a CV determinant was prepared in collaboration with Dr. D. Fambrough (Carnegie Institute). This antibody, anti-CV, has been characterized using an ELISA with CV adsorbed to vinyl microtiter plates. In our competitive ELISA assays we found that the following concentrations of other proteins did not compete for antibody binding: 1 mg/ml conalbumin, 1 mg/ml phosvitin, 250 μg/ml rabbit IgG, 125 μg/ml bovine IgG, and 250 μg/ml tubulin. In these experiments half-maximal inhibition of anti-CV binding was achieved at 0.25 μg/ml chicken liver CV protein. Immunofluorescence experiments carried out in Dr. Fambrough's laboratory showed that the anti-CV bound to various chicken-derived cell lines in a pattern of punctate dots previously observed to be characteristic of plasma membrane-associated coated pits (4, 14). Also, preliminary immunoelectroblot experiments suggest that anti-CV is directed against the 180,000-mol-wt clathrin protein band obtained after SDS PAGE separation of CV proteins.

Electrophoretic Transfer and Immunoblot Assay: CV and MT proteins were electrophoretically transferred from a 10% SDS PAGE gel prepared according to the procedure of Laemmli (21) onto nitrocellulose paper according to the method of Towbin et al. (52). Transfers were carried out at 5–10°C at 30–40 V (2–3 A) in a trans-blot apparatus (Bio-Rad Laboratories). The nitrocellulose paper was then cut into strips and incubated in ethanolamine buffer (10% vol/vol ethanolamine, 0.25% gelatin, 10 mM Tris pH 7.4, 0.15 M NaCl) for 2 h at room temperature to saturate additional protein-binding sites. After quenching, the strips were incubated in a 1:100 dilution of either rabbit antitubulin IgG or preimmune rabbit IgG in incubation buffer (50 mM Tris pH 7.5, 0.15 M NaCl, 5 mM EDTA, 0.25% gelatin, 0.1% Triton X-100) for 4 h at room temperature. The strips were then washed with two changes of the same buffer (5 min each) and incubated 2 h longer with ^{125}I-goat anti-rabbit IgG (5 × 10^6 cpm/strip). The strips were then washed for five cycles of 5 min each in incubation buffer to remove unbound ^{125}I-goat anti-rabbit IgG. The strips were dried and placed next to Eastman Kodak X-Omat XAR-2 film at −80°C with a Cronex enhancing screen (E. I. Dupont De Nemours & Co., Wilmington, DE).

S-1000 Chromatography: 300 ml of preswollen S-1000 Superfine (Lot. No. HE24927; Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) were washed three times with distilled H_2O, suspended in 500 ml column buffer (10 mM HEPES, 10 mM EGTA, 0.2 M sucrose, 0.3 M NaCl, 0.02% sodium azide, pH 7.0), degassed thoroughly, and poured into a 60 × 0.9 cm column. The column was washed overnight by elution of an amount of buffer in excess of five times column volume before sample application. Typically, 1 mg of sample was applied (in 1-ml volume), eluted at 3.5 ml/h, and 1.5 ml fractions were collected.

Electron Microscopy: Pellets obtained from pooled CPG column fractions centrifuged at 200,000 *g* for 40 min were fixed with 1% glutaraldehyde and 2 mg/ml tannic acid in CV isolation buffer. After 1 h at room temperature, the pellets were rinsed with isolation buffer and treated with 1% OsO_4 in 0.1 M sodium cacodylate, pH 7.2, for 40 min at room temperature. After several rinses with distilled water, the pellets were stained en bloc with 1% aqueous uranyl acetate for 30 min and then dehydrated over 45 min with ethanol: 50%, 70%, and 95% at 4°C and three changes of absolute at room temperature. The pellets were incubated with two changes of propylene oxide (15 min each) and then infiltrated with a 1:1 mixture of propylene oxide and epoxy resin (Epon 812-Araldite 6009) for 1 h followed by 100% epoxy resin for 1 h. The resin was then replaced with fresh epoxy and cured at 60°C for 2 d. Gold sections were cut using a Sorvall MT-2B ultramicrotome, collected on 200-mesh grids, and stained with uranyl acetate and lead citrate. Micrographs were taken with a Hitachi H-600 electron microscope.

RESULTS

Coelectrophoresis of CV and MT Proteins

Purified CV from a variety of tissues exhibit a number of polypeptides when analyzed by SDS PAGE. Two of these polypeptides have molecular weights between 50,000 and 55,000 (Fig. 1). Because of the apparent similarity of molecular weights, we wished to determine if any of these proteins were α- or β-tubulin.

One criterion for the identification of tubulin among the 50,000–55,000-mol-wt polypeptides is the consistent co-migration of purified bovine brain tubulin with one or more of the polypeptides on SDS PAGE. When these proteins were electrophoresed in adjacent lanes on slab gels, it was apparent that two of the three bands in the 50,000–55,000-mol-wt range found in CV isolated from bovine brain co-migrated with α- (56,000) and β- (53,000) tubulin on 10% acrylamide gels (Fig. 1). Co-migration was also noted at acrylamide concentrations of 7.5 and 12% (data not shown). CV isolated from chicken liver also contained proteins with apparent molecular weights very similar to α- and β-tubulin. However, these proteins were present in a much smaller relative quantity than that found in bovine brain isolated CV and were clearly observable only in overloaded silver-stained gels. Nevertheless, the presence of tubulin associated with chicken liver isolated CV was clearly demonstrated by our ELISA assays described in a following section.

To define the pI of the many CV proteins and, in particular, to determine if the 53,000- and 56,000-mol-wt doublet had pI similar to α- and β-tubulin, we compared the pI values of CV proteins and tubulin by two-dimensional electrophoresis. Purified CV and MT were electrofocused in the first dimension and then separated in the second dimension by SDS PAGE (Fig. 2). The 53,000- and 56,000-mol-wt proteins localized at the same pI and molecular weight as α- and β-tubulin. They had an apparent pI of 5.5 for the "a" band and for α-tubulin and 5.3 for both the "b" band and β-tubulin. Unlike many of the other CV proteins neither of the MT polypeptides had a component which focused at the pI of clathrin.

Examination of CV polypeptides after electrofocusing and electrophoresis in the second dimension revealed at least 18 distinct spots (Fig. 2). Chief among these is a prominent band that migrated in the second dimension and that had the same molecular weight as the clathrin standard. This clathrin band exhibited a distinctly heterologous isoelectric pattern. Although spread from a pI of 6.0 to nearly 7.0, the clathrin was predominantly concentrated in two areas which range from 6.0 to 6.6 and 6.7 to 6.8 and whose peak densities have a pI of 6.4 and 6.7, respectively. Often in the second dimension there was a slightly faster migrating clathrin at both pI; however, this was not always evident at lower protein concentrations.

In addition to the heterogenous pattern produced by clathrin, most of the other polypeptides exhibited two distinct patterns. All had pI distinct from clathrin but, in addition, most also appeared to have a component of the same molecular weight that focuses at the same pI as clathrin (Fig. 2). All of the polypeptides of the 100,000–125,000-mol-wt group electrofocus at pI that are identical to the clathrins; in addition, the 120,000-mol-wt polypeptide also focused at a pI of 6.0 and the 110,000-mol-wt at a pI of 5.8. Polypeptides in the 70,000–90,000-mol-wt range migrated as four well-focused

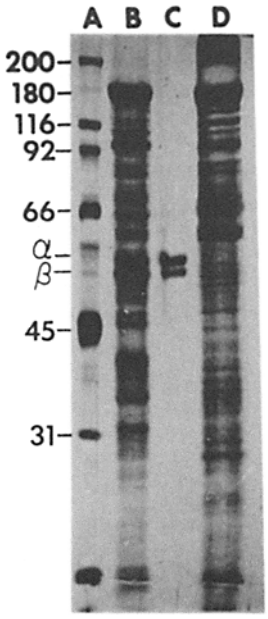

FIGURE 1 SDS PAGE analysis of bovine brain CV (*B*), tubulins (*C*), and chicken liver CV (*D*). Reduced samples (3–5 μg protein) were electrophoresed on 10% slab gels and stained by the silver method of Merrill (25). (*A*) Molecular weight standards.

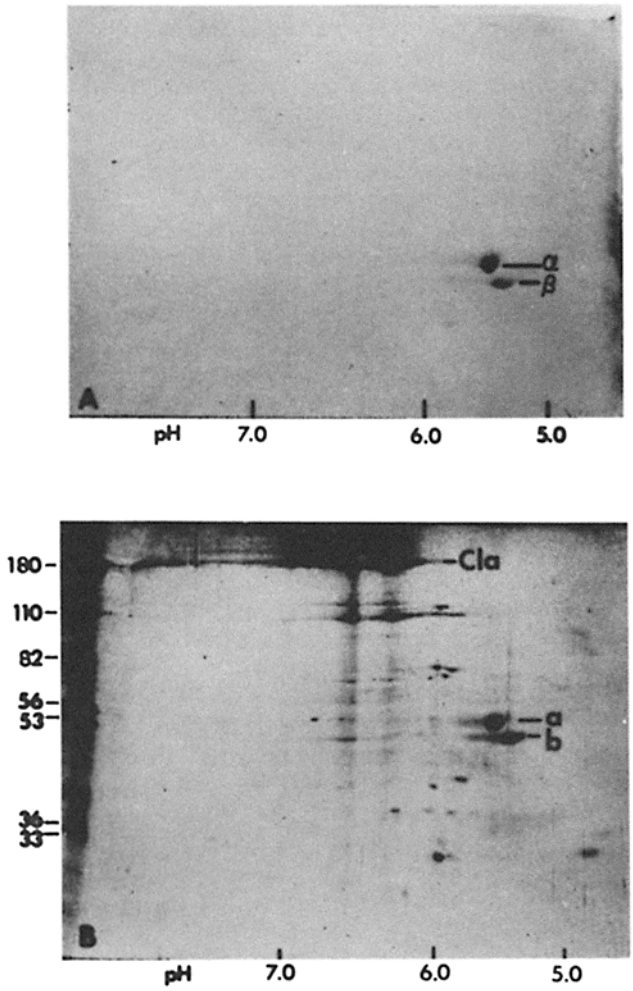

FIGURE 2 Two-dimensional gel electrophoresis of bovine brain MT and bovine brain CV. Samples were prepared as described, isoelectric-focused, and then electrophoresed in the second dimension on 10% slab gels. Gels were stained with silver. Spots *a* and *b* in brain CV (*B*) focused at identical positions as those of α- and β-tubulin (*A*). *Cla*, clathrin.

spots with pI of 6.0 and 5.8 for the 82,000- and 80,000-, 6.1 and 5.9 for the 74,000- and 76,000-mol-wt polypeptides. To a much lesser degree, each of these proteins also had a component that migrated at the same pI as the clathrins. In contrast, the polypeptides in the 40,000–47,000-mol-wt range all focused as discrete spots at a pI of 5.5 for the 48,000-, 5.8 for the 43,000-, 6.1 for the 40,000- and 6.4, 6.1, and 5.9 for the 38,000-mol-wt protein. Only very small amounts of these spots were found migrating at the pI of clathrin. Polypeptides of the 30,000- to 36,000-mol-wt light chain family, two of which are usually purified associated with clathrin, migrated at pI of 4.5, 4.7, and 6.0 for the 36,000-, 34,000-, and 32,000-mol-wt polypeptides respectively.

If the 53,000- and 56,000-mol-wt bands from CV are in fact α- and β-tubulin, they would be expected to share antigenic determinants with the tubulins. To test this hypothesis, MT and CV polypeptides from SDS PAGE were electrophoretically transferred to nitrocellulose, and the polypeptides on the paper were incubated with antibodies specific for tubulin. When autoradiograms were developed, both the α- and β-bands and the 53,000- and 56,000-mol-wt bands from the CV bound antibodies. Preimmune sera did not bind (Fig. 3).

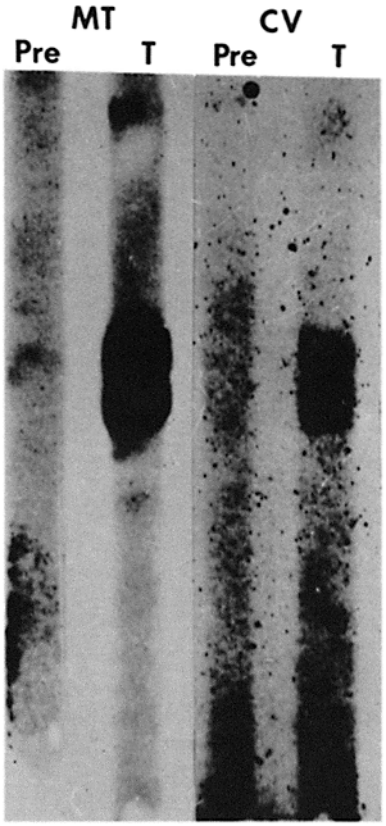

FIGURE 3 Immunoblot analysis of MT and CV proteins with antitubulin antibodies. MT and CV proteins purified from bovine brain were separated by SDS PAGE and then electrophoretically transferred to nitrocellulose paper. Both MT (*MT*) and CV (*CV*) transfers were incubated with either preimmune serum (*Pre*) or antitubulin serum (*T*) followed by a second incubation with ^{125}I-goat anti-rabbit IgG. The strips were then washed and analyzed by autoradiography.

Kinetic Association of Tubulins with CV

Given the finding that α- and β-tubulin co-purify with CV, we wished to determine if the tubulins were associated with CV in a common kinetic particle or if their apparent co-purification was an artifact of the preparative techniques. Isolated CV were therefore chromatographed over a CPG bead exclusion column with a mean pore size of 1,902 Å. Depending on the purity of the particular CV preparation, two distinct elution profiles, as detected by absorbance at 280 nm, were obtained. One typical profile (Fig. 4) had two distinct protein peaks centered at fractions 19 and 25. Fractions 22–27 when analyzed by SDS PAGE (Fig. 4*B*) contained the principle CV polypeptide, the 180,000-mol-wt protein clathrin. It should be noted that the 53,000- and 56,000-mol-wt proteins identified as α- and β-tubulin also eluted in these fractions. Densitometric scans of the silver-stained gels demonstrated that the ratio of tubulin to clathrin remained constant in all of the fractions within this peak (data not shown). Fractions 22–27 were pooled, pelleted at 200,000 × *g* for 40 min, and examined as thin sections in the electron microscope (Fig. 4*C*). In a typical image, 95% of the visible structures were identified as CV that appeared to be of a uniform size. A small number of uncoated membrane structures were also observed in these fractions.

In contrast, fractions 18–21, when examined by a similar procedure in the electron microscope, consisted of large, smooth membranous structures (Fig. 4*D*). When analyzed by SDS PAGE, the predominant protein component of these fractions was tubulin, with very little clathrin and smaller amounts of other proteins. It should be noted that we did not observe in the electron microscope any MT-like structures in either peak with either thin-sectioned or negative-stained samples. Material eluting in fractions 28–35 proved to be nonpelletable at 100,000 *g* for 1 h.

The other typical elution profile obtained with certain CV preparations (Fig. 5*A*, absorbance 280 nm) exhibited no membranous void volume material and consisted of a CV-containing peak (fractions 23–29) that by SDS PAGE analysis was similar to the second peak in Fig. 4 (data not shown). Fractions 30–36 (Fig. 5*A*) eluted with the included volume marker and contained low amounts of clathrin and some proteins of less than 50,000 mol wt. Like the more included material from the preparation in Fig. 4, material in fractions 30–36 (Fig. 5*A*) was nonpelletable upon centrifugation. The large absorbance at 280 nm for the peak fractions 30–36 (Fig. 5*A*) is due to the presence of vitamin B12 that was used as an internal included volume marker in this chromatograph.

Immunoassay of Column Purified CV

We were able to verify the presence of CV and MT proteins in the fractions from CPG bead columns by direct-binding immunoassays of the column fractions. Two monoclonal antibodies were used to monitor the presence of CV. One monoclonal recognizes the 36,000 polypeptide light chain (anti-LC) of bovine brain CV, and the other recognizes a determinant of chicken liver CV (anti-CV). Polyclonal antitubulin antibodies were used to detect α- and β-tubulin.

Although the elution profile from the glass bead column in Fig. 5 exhibited two distinct protein peaks (peak I, fractions 23–29, and peak II, fractions 30–36), it was clear from the antibody-binding data (Fig. 5*A*) that CV- and MT-binding activities were localized in the CV-containing peak (peak I). When the fractions from peak I were pooled, concentrated, and rechromatographed, protein eluted from the CPG bead column as two closely spaced peaks (Fig. 5*C*) with the bulk

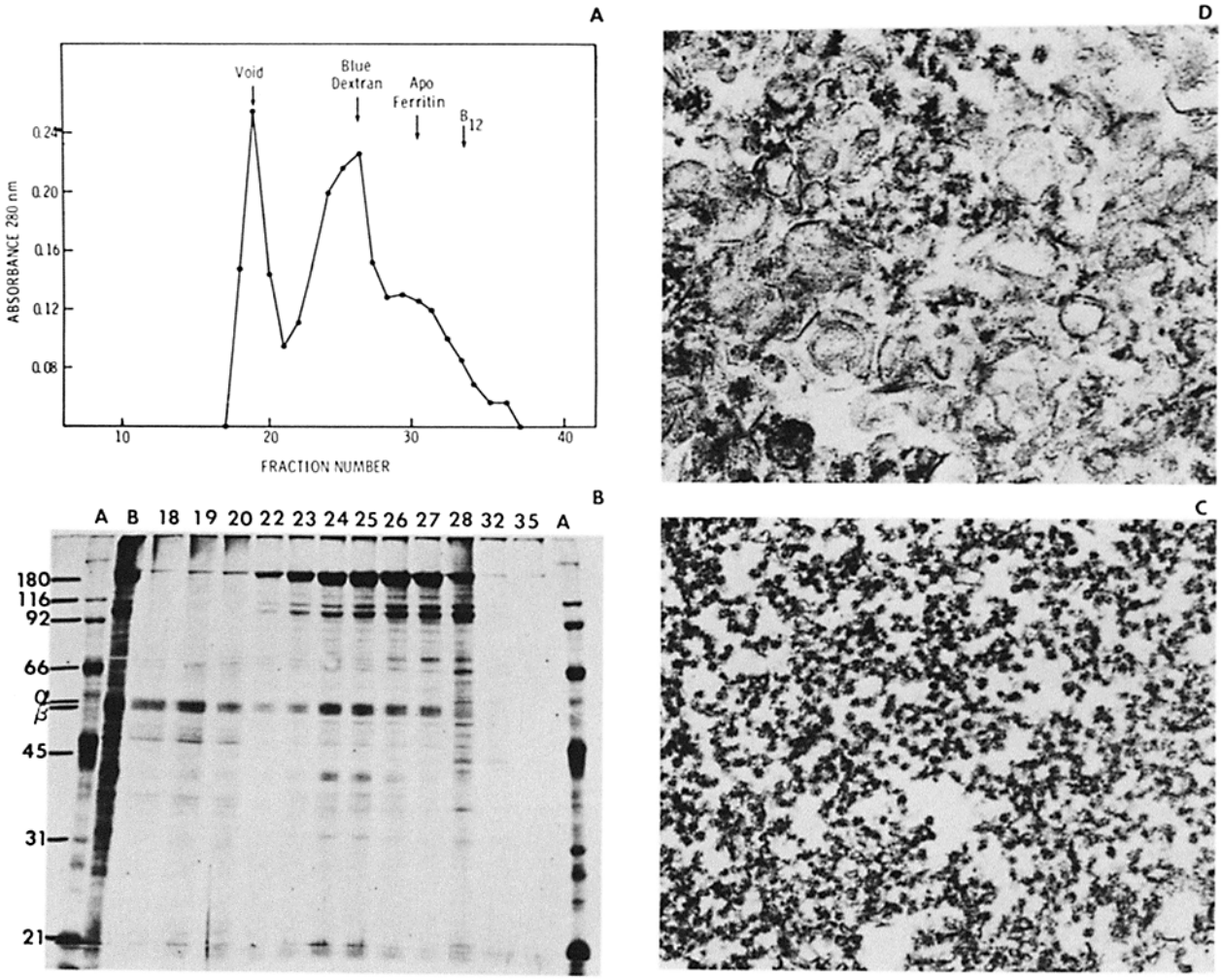

FIGURE 4 Permeation chromatography of bovine brain CV. Brain CV purified by the method of Nandi et al. (27) were chromatographed on a CPG bead column (CPG 1,902 Å), and 1 ml fractions were collected. Fractions containing protein (*A*), as determined by absorbance at 280 nm, were analyzed by SDS PAGE on a 10% gel and stained with silver (*B*). Numbers above lanes in (*B*) correspond to fraction source in (*A*). (*B*, lane *A*) molecular weight standards; (*B*, lane *B*) starting material (bovine brain CV). Protein molecular weight markers for the column were run separately and included blue dextran (2×10^6 mol wt), apoferritin (4.8×10^5 mol wt), and vitamin B12 (1,450 mol wt). The void volume for the CPG bead column corresponds to 1,902 Å diameter for a spherical particle. Fractions 22–27 (*C*) and fractions 18–21 (*D*) were centrifuged at 200,000 *g* for 40 min; pellets were collected and prepared for electron microscopy as described in Materials and Methods. × 90,000.

of the protein eluting at the position of peak I. The antibody-binding profiles for rechromatographed peak I were coincident with the protein profile.

Rechromatography of peak II resulted in the elution of a single peak of protein (Fig. 5*E*) that eluted in approximately the same position as the original peak II. However, most of the antibody-binding activity eluted at the relative position of peak I in fractions containing small amounts of protein, suggesting the presence of contaminating peak I material in the pooled fractions from peak II. Light chain eluted almost exclusively at the peak I position whereas some CV and tubulin activities also eluted at the peak II position.

If the MT and CV polypeptides co-migrate as a single kinetic particle on the CPG bead column, the ratio of the MT and CV should remain constant in fractions containing this particle. All bound antibody ratios remained constant over the fractions comprising peak I but not over the fractions within peak II (Fig. 5*B*, *D*, and *F*). The calculated antibody-binding ratios for rechromatographed peak II remained constant only over a narrow range of fractions (26–29, Fig. 5*F*), presumably due to contaminating peak I material in the pooled fractions. The tubulin/light chain and the CV/light chain ratios were not constant over the peak II region. However, in some cases (Fig. 5*B* and *F*), the tubulin/CV ratios were constant over this region reflecting the presence of coeluting tubulin and CV antigens. From these ratios, light chain appeared to be present only in peak I.

To determine whether the calculated antibody-binding ratios were valid over the range of antigen concentrations obtained from the CPG column, we coated vinyl wells with undiluted and diluted samples of CPG column–eluted material and assayed these samples as described. With our antibody probes we found that for column fractions containing detectable amounts of absorbance 280 nm material, the absorbance at 490 nm obtained by the ELISA procedure (a measure of the amount of antigen in the solution) was linear over a hundredfold range of coating antigen concentrations (data not shown).

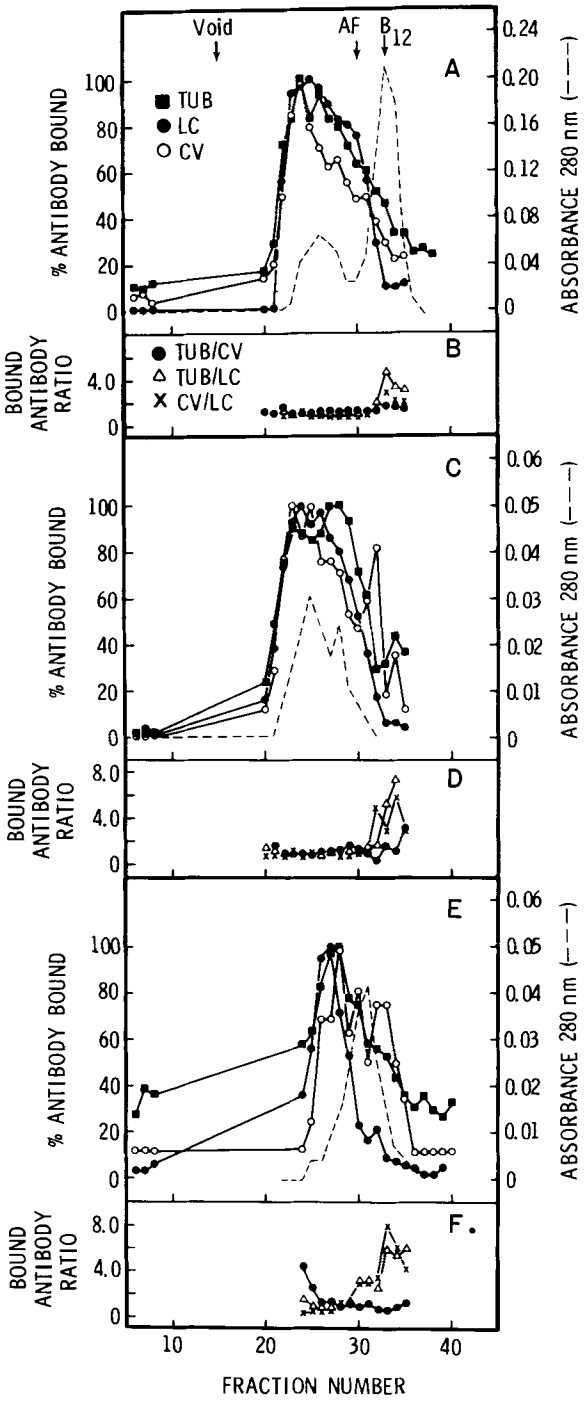

FIGURE 5 Permeation chromatography of bovine brain CV: antibody analysis. (*A*) Density-gradient isolated bovine brain CV (1.5 mg) were applied to a CPG bead column (58 × 0.9 cm). The CV were eluted as in Fig. 4, and 1 ml fractions were assayed with antitubulin (1:1,000 dilution), anti-CV monoclonal (1:10^5), and anti-LC (1:1,000). The relative percent of antibody bound was determined by ELISA. Peak I and peak II fractions were pooled separately, concentrated, and reapplied to the CPG bead column. Fractions from rechromatographed peak I (*C*) and rechromatographed peak II (*E*) were once again assayed for antibody-binding. Relative antigen levels of tubulin (*TUB*), CV (*CV*), and 36-mol-wt light chains (*LC*) were calculated and expressed as ratios of antibody-binding activity for density-gradient isolated CV (*B*), pooled and rechromatographed peak I (*D*), or pooled and rechromatographed peak II (*F*). Molecular weight markers are as in Fig. 4.

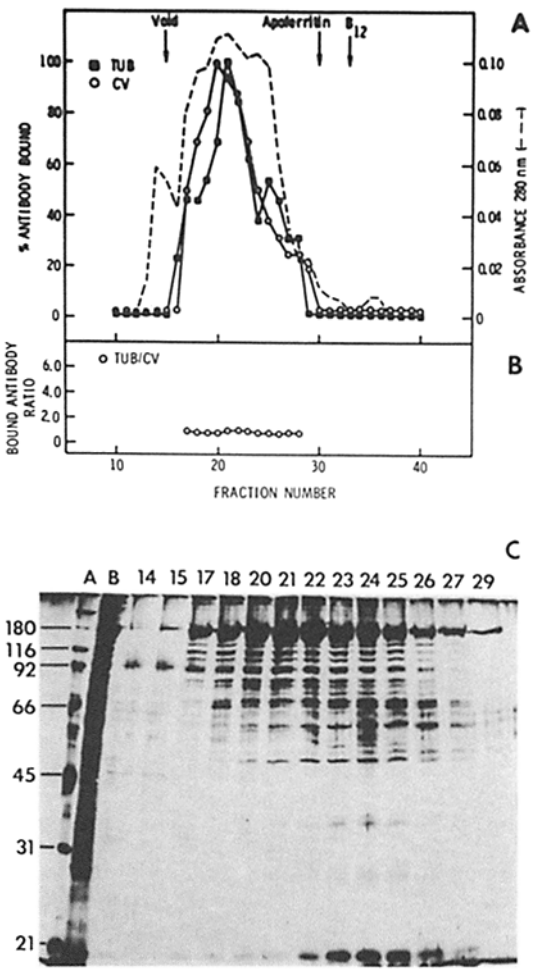

FIGURE 6 Permeation chromatography of chicken liver CV. Chicken liver CV were isolated on D_2O-Ficoll gradients according to Pearse (32). 1.3 mg was applied to a CPG bead column as before (Fig. 4). 1 ml fractions were assayed for protein and antibody-binding activity as before (Fig. 5). Fractions containing protein as determined by absorbance at 280 nm (*A*) were analyzed by SDS PAGE on a 10% gel and stained with silver (*C*).

Chicken liver CV were also applied to the same CPG bead column. The protein eluted with a small amount of material in void volume followed by a broad peak centered around fraction 21 (Fig. 6*A*). Unlike the bovine brain CV elution profile (Fig. 5), the elution profile from this preparation of chicken liver CV did not contain a distinct second peak of material. Tubulin and CV coeluted with the broad protein peak whereas no antibody-binding activity was detected in the void-volume fractions. The ratio of tubulin to CV remained constant, indicating the presence of tubulin in the same kinetic particle as CV (Fig. 6*B*). Because there was no cross-reactivity with chicken liver CV, the anti-bovine brain 36,000-mol-wt light chain monoclonal (anti-LC) could not be used to monitor the presence of chicken liver CV light chain.

Sephacryl-1000 (S-1000) is also sometimes used as a final step in purifying CV (26). We therefore applied the same bovine brain CV as used in the CPG column to this gel matrix. Protein eluted from the S-1000 column as a single peak with a broad shoulder (Fig. 7). There was no separation of material into two distinct peaks as for the CPG bead

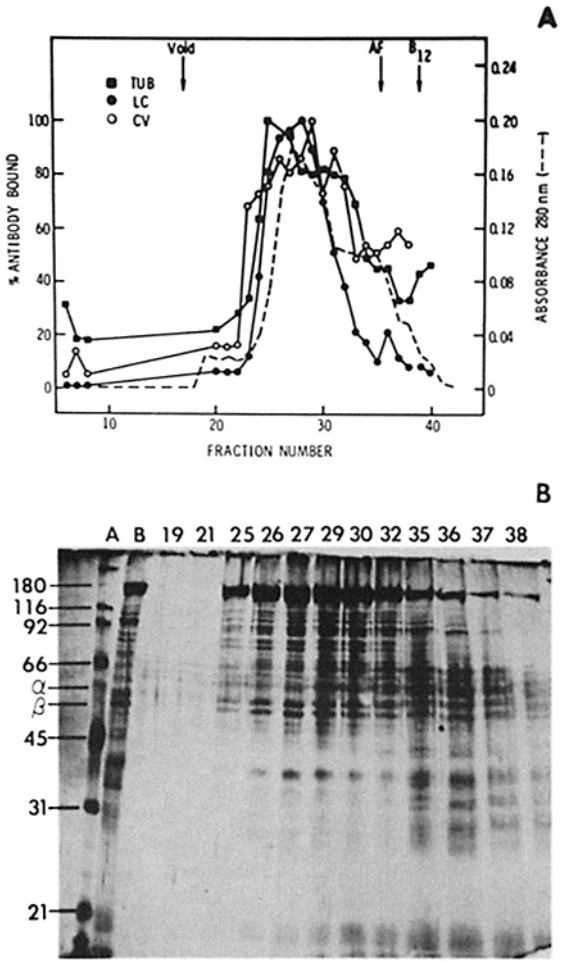

FIGURE 7 Gel filtration of bovine brain CV on sephacryl S-1000. Bovine brain CV were isolated on D_2O-sucrose gradients according to the method of Nandi et al. (27). 1.5 mg of material in 1 ml of column buffer was chromatographed. 1 ml fractions analyzed for protein (absorbance at 280 nm) and antibody-binding as before (Fig. 5). Fractions containing protein as determined by absorbance at 280 nm (*A*) were analyzed by SDS PAGE on a 10% gel stained with silver (*B*).

column even when lesser amounts of protein were applied to the column. Similarly, antibody-binding was detectable in all the fractions of eluted protein, except for light chain activity that was present only in the material nearest the void and not in the peak shoulder fractions. As in the CPG bead column, tubulin and CV appeared to coelute from the S-1000 column (Fig. 7).

DISCUSSION

Our results demonstrate that the 53,000- and 56,000-mol-wt proteins found in highly purified preparations of CV isolated from bovine brain share SDS PAGE mobility, pI, and antigenic determinants with α- and β-tubulin isolated from bovine brain. In addition, we have shown that these two proteins are associated with the same kinetic particle as the 180,000-mol-wt CV protein clathrin and the 33,000 and 36,000 CV light chain components. These latter results are based, in part, on our findings that the 53,000-, 56,000-, 180,000-, 33,000-, and 36,000-mol-wt components co-purify through velocity and equilibrium sedimentation centrifugation followed by exclusion chromatography through CPG bead columns. Depending on the purity of the initial preparation, two CPG elution profiles were obtained. In addition to a CV-containing peak, some preparations contained large membranous vesicles that eluted in the void volume (Fig. 4). Both peaks, however, appeared to contain similar amounts of tubulin. If it is assumed that the tubulin in the void peak is in association with the large vesicles found in these fractions, the large amount of tubulin in the second peak cannot be explained by the small amount of membrane contaminants and therefore must be in association with the CV themselves. The kinetic association of α- and β-tubulin and CV was also shown in experiments in which CPG bead column purified CV were assayed by an ELISA method. These experiments demonstrated that the great majority of the MT protein co-purified with the 180,000- and 36,000-mol-wt CV proteins. This association had a constant stoichiometry since the ratio of MT to CV remained constant in all fractions in the CV-containing peak.

These findings are in basic agreement with those of Rubenstein et al. (45). They showed that when sucrose-density purified CV were electrophoresed through agarose, the original material was resolved into two components, one consisting of CV containing the typical CV polypeptides including the 50,000–55,000-mol-wt proteins, and a second fraction containing smooth membrane vesicles with very little protein. Thus, the findings of these authors support our conclusion that the CV proteins clathrin, the two light chain species, and α- and β-tubulin are components of a single kinetic particle.

We also obtained similar results when we examined the MT components of CV isolated from chicken liver by two different procedures. The D_2O-sucrose method of Nandi et al. (27) and the D_2O-Ficoll method of Pearse (32). Regardless of the isolation method, the CV components, as assayed by our anti-CV monoclonal antibody probe, and the MT proteins chromatographed in a single peak. These results show that, as in bovine brain CV, the MT proteins and the clathrin of chicken liver CV are associated in the same kinetic particle. Unlike those purified from bovine brain, CV isolated from chicken liver consistently have much less associated α- and β-tubulin. This conclusion is based on a comparison of the relative densities of the silver-stained SDS PAGE gels that demonstrate that bovine brain CV contain predominant bands of protein at 53,000 and 56,000 mol wt (Fig. 1*B*), whereas at similar clathrin concentrations chicken liver CV contain barely detectable silver-stained bands in the 50,000–55,000-mol-wt region (Fig. 1*D*).

The difference in the amount of MT protein that co-purifies with CV may reflect the differing roles that coated structures presumably serve in these two very different tissues. We expect that the role served by coated structures in bovine brain involves an association between the numerous MT found in this tissue and CV. However, in the liver, many of the functions served by coated structures may not involve an association between CV and MT. We expect that within any particular cell type there are various subpopulations of CV, some of which are not associated with MT. Our data, then, may suggest that the predominant subpopulation of coated structures in bovine brain is associated with MT, whereas only a minor subpopulation of CV in chicken liver has this capability. Thus, many of the CV isolated from liver would not carry along associated MT protein.

In the course of these experiments we used a number of different procedures to isolate CV. Independent of the method used, the relative amount of tubulin in each preparation remained the same. Depending on the method employed, however, and even with different preparations isolated by identical procedures, the relative amounts of non-CV material varied from one preparation to the next (Figs. 4 and 5). Our results are in basic agreement with those of Pfeffer and Kelly (35), and both of our results make obvious the need for rigorous purification beyond the centrifugation techniques now employed, including those that employ D_2O (27, 32).

S-1000 has been used by some investigators as a final purification step in CV preparations (26). In our experiments, when bovine brain CV were chromatographed on CPG bead columns, they were resolved into two distinct peaks whereas when the same starting material was chromatographed on an S-1000 column, a single broad peak was obtained. Thus our data demonstrate that S-1000 is not as effective as CPG in separating CV from contaminating material.

The role of CV in protein and membrane transport has been well documented (3, 4, 11, 12, 32, 41, 42, 44, 46). The mechanism employed to direct a vesicle to any one of several potential intracellular sites is not, however, understood. The presence of MT proteins in CV suggests that these cytoskeletal elements may play a role in directing the transport of CV-derived vesicles through the cytoplasm.

MT have been implicated in the transport of many endocytosed ligands and the secretion of a variety of cellular products (20, 23, 36, 39). There is also considerable evidence for the role of MT in the intracellular movement of other membrane-bounded organelles and particles (1, 6, 13, 56). In addition, it has been shown that the in vitro association of pancreatic secretory granules with MT is dependent on MT-associated proteins. MAP-2, in particular, can cross-link MT with CV (49) and is also capable of binding to actin and inducing the formation of actin filament bundles (48). It is known that tubulin binds to, and may at least partially insert in, phospholipid vesicles (5, 8, 19, 40). Indeed, tubulin binds preferentially in vitro to rat hepatic membrane fractions that are involved in protein, membrane, and organelle translocation (40). Since MAP-2 is known to cross-link MT (51), and because phospholipid vesicle-bound tubulin appears to retain its MAP-2-binding capability (19), perhaps it is MAP-2 that mediates the interaction of CV, via membrane-bound tubulin, with the cytoskeletal network. The phosphorylation of MAP-2 has been suggested as a possible control mechanism for this system (49), since the addition of cAMP enhances MT-organelle associations (49, 51) and cAMP has been shown to stimulate the phosphorylation of MAP-2 (50).

An alternate mechanism has also been suggested that could control the CV-MT interaction. It has been reported (29) that a cyclic nucleotide-Ca^{2+} independent protein kinase present in highly purified brain CV phosphorylates a 50,000-mol-wt CV component. This 50,000-mol-wt protein has been identified as the tau protein (34), which is a MT-associated protein involved with the regulation of MT formation (55). The exact roles of these MT-associated proteins (tau, MAP-2) in the regulation of transport of cellular organelles remains, at this time, speculative. Further studies are necessary to interpret this data in terms of any in vivo mechanisms.

Although much information is available concerning the specific proteins and structure of CV, the manner in which the ligand-containing vesicle is guided to a particular destination remains unclear. In the past, CV have been observed as a ubiquitous contaminant of MT preparations, suggesting that there may be some functional association. The demonstration in this report that α- and β-tubulin are associated in the same kinetic particle as clathrin and CV light chains provides further evidence of a role for MT in the translocation of cytoplasmic CV.

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